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Effects of different crosslinking methods on the properties of collagen–calcium phosphate composite materials

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ABSTRACT

The purpose of this study is the preparation and characterization of porous collagen/calcium phosphates (Col/CaP) composites. Collagen scaffolds with high porosity were prepared by freeze–drying technique. Col/CaP scaffold were created by new method – by deposition of calcium phosphate within collagen matrix in two steps using freeze–drying process before immersing samples in calcium solution. To find the optimal preparative method, we prepared diverse Col/CaP scaffolds using different collagen concentration and various crosslinking method: crosslinking with carbodiimide (EDC/NHS) and dehydrothermal treatment (DHT).

This study explores the effect of the different crosslinking method on the properties of scaffolds, such as: microstructure (porosity and density), dissolution, water uptake, mechanical properties and collagenase degradation. The results obtained showed that crosslinking the scaffolds by either EDC/NHS or DHT have good mechanical and morphological properties compatible with their potential application in bone regeneration. The results demonstrated that properties of Col/CaP scaffolds changed significantly with different crosslinking method. However, while EDC/NHS increased the scaffolds' resistance to dissolution and degradation by collagenase, DHT decreased the swelling ratio and resistance to dissolution in PBS solution.

Based on our study, 2% collagen concentration and EDC/NHS as crosslinking reagent are recommended to design the scaffold for use in bone engineering.

1. Introduction

Human bone is a highly ordered and complex structure consisting mainly of inorganic apatite crystals (natural ceramics) and organic collagen fibers (Col) [1].

Bone defects and losses, which can arise from several causes such as tumors, trauma, bone diseases (e.g. osteoporosis) are very common in our society [2]. Recent research advances in bone regeneration in tissue engineering have focussed on the development of three dimensional (3D) porous scaffolds, which should have the following characteristics: biocompatibility, non toxicity, suitable mechanical properties, high porosity, interconnected porous structure and a biodegradation rate that matches the rate of tissue regeneration [3–5]. Scaffold play a very important role in tissue engineering – that can serve as a support, reinforce and in some cases organize the tissue regeneration or replacement in a natural way [6]. Bone tissue engineering strategies are emerging as attractive alternatives to autografts and allografts in bone tissue reconstruction [7].

Calcium phosphate (CaP) ceramics are widely used in bone regeneration (excellent biocompatibility, bioactivity, biodegradability, osteoinductivity and osteoconductivity) [8]. However, due to the slow degradability ceramic materials do not appear to be very attractive for purposes of tissue engineering. Wherefore, increasing interest has been shown in ceramic–polymer composites as potential materials to regeneration of bone losses [9]. The addition of biodegradable polymers can improve the degradability of the composite materials and change their properties, such as mechanical properties [10]. Therefore, for the regeneration of bone, the ceramic materials with addition of biodegradable polymers, both synthetic and natural, such as collagen, chitosan, silk [11,12], poly(D,L-lactide) [13] or poly-lactic-glycolic acid [14] have been investigated.

However, due to the fact that the natural bones contain mainly collagen and hydroxyapatite, many researchers try to obtain collagen/hydroxyapatite composites for bone regeneration [15]. Collagen is one of the most abundant protein obtained from animal and fish tissues [16–18]. Collagen can be extractable in soluble

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or insoluble form from different animal tissues [15]. Calcium phosphates are available commercially (with different crystal size), as hydroxyapatite extracted from bones or they can be produced wet by the direct precipitation of calcium and phosphate ions [15]. Porous collagen or gelatine–CaP composites were formulated by different authors, especially scaffolds prepared by freeze–drying method [19–22].

To improve a physical properties of a collagen biomaterials, especially resistance of degradation and mechanical properties, crosslinking is used. A variety of crosslinking methods have been described, including e.g. UV irradiation [23], heat [24], glutaraldehyde [25], carbodiimides [26], transglutaminase [27]. Our previous studies have shown, that resistance to degradation, microstructure, mechanical and swelling properties of the composite scaffold can be modified both by the crosslinking method and by changing the concentration of component contained in the composite [28].

This paper focuses on the development of a novel Col/CaP composites using a new method. Firstly a collagen type 1 (from rat tail tendon) matrices were produced, and secondly, Col/CaP composites were obtained by soaking collagen scaffold into a phosphate solution, freeze–drying of samples and soaking scaffolds in calcium solution. The first aim of this study was to prepare and characterize a porous collagen/CaP matrices by precipitation of calcium phosphate within a collagen matrix and the second aim was to modification of obtained materials by the chemical and physical crosslinking. The effect of crosslinking was examined using measurements: water uptake ability, dissolution, enzymatic degradation, porosity, density and mechanical properties.

2. Experimental

2.1. Materials

Collagen (Col) was obtained in our laboratory from the tail tendons of young rats [29]. Ammonium sodium phosphate dibasic tetrahydrate ($NaNH_4HPO_4 \times 4H_2O$), Trizma base (Tris (hydroxymethyl) aminomethane), calcium chloride dihydrate ($CaCl_2 \times 2H_2O$), N-(3-dimethylamino propyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were supplied by the company Sigma–Aldrich (Poland).

2.2. Scaffold preparation

The high porous scaffolds were produced from a collagen suspension using a freeze–drying technique. First, collagen suspension with concentrations of 1% (1Col) and 2% (2Col) (w/w) were prepared from lyophilized collagen in deionized water using an IKA disintegrator. The homogenized suspensions were put into a polystyrene container and were then placed in a freezer at −80 °C. The completely frozen samples were lyophilized at −55 °C and 5 Pa for 48 h (ALPHA 1–2 LD plus, CHRIST, Germany).

2.3. Calcium phosphate (CaP) deposition

Calcium phosphate formation in collagen scaffold was achieved (1Col/CaP and 2Col/CaP). Collagen scaffolds were immersed in a Tris buffer (0.1 M $NaNH_4HPO_4 \times 4H_2O$, 0.05 M Trizma base, pH 7.4) for 3 h. After freezing at −80 °C scaffolds were lyophilized. The dried scaffolds were immersed into a calcium solution in Tris buffer (0.1 M $CaCl_2 \times 2H_2O$, 0.05 M Trizma base, pH 7.4) for 3 h. The next step was freezing and lyophilizing scaffolds. After drying scaffolds were briefly washed in deionized water and freeze–dried.

2.4. Scaffold crosslinking

The lyophilized porous samples were stabilized using different treatment methods: crosslinking with EDC/NHS and dehydrothermal treatment.

For DHT crosslinking, freeze–dried scaffolds were placed under a vacuum at a temperature of 110 °C for 24 h.

Carbodiimide crosslinking was achieved by immersing the collagen scaffolds in a crosslinking solution consisting of 50 mM EDC and 25 mM NHS in 98% ethanol. After reaction for 4 h at room temperature, scaffolds were washed in 0.1 M Na_2HPO_4 twice for 1 h, followed by washing with deionized water for another 2 h, changing the water every 30 min. Samples were frozen again, then lyophilized as described.

2.5. Scaffold characterization

2.5.1. Attenuated total reflection infrared spectroscopy (ATR-IR)

A structure of samples was evaluated by attenuated total reflection infrared spectroscopy using a Genesis II FTIR spectrophotometer (Mattson, USA) equipped in ATR device (MIRacle™ PIKE Technologies) with zinc selenide (ZnSe) crystal. All spectra were recorded in absorption mode at 4 cm^{-1} intervals and 64 scans.

2.5.2. Scanning electron microscopy (SEM) with X-ray microanalysis (EDX)

The morphology of porous samples was studied using scanning electron microscopy (SEM, LEO Electron Microscopy Ltd, England) and energy dispersive X-ray spectroscopy (EDX, Quantax 200, XFlash 4010, Bruker AXS, Germany). Scaffolds were cut with a razor scalpel after being frozen in liquid nitrogen for 3 min and cross-section of the sample was observed.

2.5.3. Porosity and density measurement

The density and porosity of the prepared 3D scaffolds were measured by liquid displacement [30]. The liquid used in this study was isopropanol. A sample with a known weight (W) was immersed in a graduated cylinder in a known volume of isoporopanol (V_1) for 5 min. The total volume of isopropanol in the cylinder and isoporopanol-impregnated scaffold was V_2. The isopropanol-impregnated scaffold was removed from the cylinder and the residual isopropanol volume was recorded (V_3). Each sample was measured in triplicate. The density of the porous samples (d) and the porosity of the scaffolds (ϵ) are expressed as follows:

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

$$\epsilon = \frac{(V_1 - V_3)}{(V_2 - V_3)}$$

2.5.4. Swelling tests

The piece of each dried porous scaffold was weighed and then immersed in 5 ml of phosphate buffer saline (PBS, pH 7.4) for 2, 24, 48 and 72 h. At each time point, the scaffolds were taken out of the solution. The water uptake of the scaffolds was assessed using two different methods.

The first measurement was carried out after being removed from PBS, without pressing soaked samples. After removal from the water or PBS solution, the samples were hung over a table for 1 min until no dripping was observed and then weighed (W_{ws}). In this case we assessed the swelling ability of the scaffold structure with its pore system.

In the second measurement the same swollen samples were pressed between filter paper to remove the excess water remaining

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