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Properties and modification of porous 3-D collagen/hydroxyapatite composites

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ABSTRACT

A freeze drying technique was used to form porous three-dimensional collagen matrixes modified by the addition of a variable amount of nano-hydroxyapatite. For chemical cross-linking EDC/NHS were used. Physical cross-linking was achieved by dehydrothermal treatment. Mechanical properties, morphology, dissolution, porosity, density, enzymatic degradation and swelling properties of materials have been studied after cross-linking. The density of scaffolds and its compressive modulus increased with an increasing amount of hydroxyapatite and collagen concentration in the composite scaffold, while the swelling ratio and porosity decreased. The studied scaffolds dissolved slowly in PBS solution. DHT cross-linked collagen matrices showed a much faster degradation rate after exposure to collagenase than the EDC cross-linked samples.

1. Introduction

Biomaterials play a very important role in tissue engineering. Numerous scaffolds produced from a variety of biomaterials have been used in biomedical field in attempts to regenerate different tissue and organs in the body [1]. An ideal scaffold should have the following characteristics: biocompatibility, non toxicity, suitable mechanical properties and a biodegradation rate that matches the rate of tissue regeneration. In addition, the biodegraded product should not have negative effects on the surrounding tissues and organs [1–3]. The architecture of scaffolds used for tissue engineering is of critical importance. Scaffolds should have high porosity and an interconnected porous structure to provide adequate space for the cell's seeding, growth and proliferation [1,2,4].

Many methods have been developed to prepare porous three-dimensional biodegradable scaffolds for tissue engineering, including freeze-drying, gas-forming foam, three-dimensional printing, thermal-induced phase separation, electrospinning [5,6], laser treatment [7] and precipitation of crystals [8,9].

Collagen is an especially abundant protein in animals. It is the main protein of connective tissue and the main component of the skin. As an extracellular matrix protein it is widely used as a biomaterial for tissue regeneration and implantation. Bone and teeth are both made of collagen with the addition of mineral crystals, mainly hydroxyapatite. Collagen-based materials are widely used in reconstructive medicine. Various applications of collagen in tissue regeneration may include: artificial skin, bone graft substitutes, dental implants, implants for incontinence, artificial tendons and blood vessels, corneal implants, nerve regeneration, regeneration of cartilage, regeneration of skin and organs. However, soluble collagen can be extracted only from very young tissue. This fact makes soluble collagen very expensive and rather rare material [10]. Scientific reports indicate that they are several research groups working on preparation of appropriate materials for ideal scaffold. In particular the collagen based materials with sufficient mechanical parameters which could be used as artificial bone are of the huge interest [2–10].

The disadvantage of using collagen as a biomaterial for tissue repair is its high degradation rate, which leads rapidly to a loss of mechanical properties [2]. Many attempts have been made to overcome this problem through the means of mixing collagen with either natural (e.g. elastin [11,12], chitosan [10,12], glycoaminoglycans – GAGs [13,14]) or synthetic polymers (e.g. poly(vinyl alcohol) – PVA [15], polycaprolactone – PCL [6,16], polylactic acid – PLLA [6], polyglycolic acid – PGA [6,17]) or by adding mineral crystals [18,19]. Likewise, the physical properties of collagen biomaterials are improved by applying different cross-linking method. Different chemical and physical cross-linking methods are used for crosslinking of protein materials. The physical cross-linking agent such as gamma radiation, UV-irradiation, heat and dehydrothermal treatment can be used [6,20]. However, the energy of UV and γ-radiation can destroy the native structure of the protein. In DHT, cross-linking is induced by heating dry collagen under vacuum to ~100 °C. The DHT technique induces an increase in tensile strength and some fragmentation in the collagen's molecular structure [20]. Chemical cross-linking can produce highly cross-linked material in a very short time. There are several chemical compounds capable of cross-linking

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proteins, including glutaraldehyde, formaldehyde, polyepoxy compounds, acyl azide, carbodiimides and hexamethylene-diisicyanate [6,20,21]. Anyway, these treatments are not sufficiently cytocompatible due to the potential toxicity of some of the cross-linking agents utilized. Covalent cross-linking using EDC/NHS {N-(3-dimethylamino propyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS)} is a widely used method in biomaterials preparations [20,22–24].

The aim of this study was to determine the optimal content of HAp in porous collagen matrices and to study the chemical and physical cross-linking of composite samples with different collagen–hydroxyapatite ratios. The samples were treated using EDC/NHS. Collagen–hydroxyapatite samples have been also crosslinked using temperature to compare chemical cross-linking with physical cross-linking. Such materials can be applied as a scaffold for bone tissue restoration. To clarify the effects of EDC/NHS and DHT cross-linking the following properties were measured: 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 (Fig. 1). Hydroxyapatite (HAp) (nanopowder, <200 nm particle size), 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–hydroxyapatite suspension using a freeze-drying technique. First, collagen solutions with concentrations of 1% and 2% (w/w) were prepared from lyophilized collagen in deionized water using an IKA disintegrator. The starting materials for the final Col/HAp composites were mixed and the final weight ratio was: 80/20, 50/50 and 30/70. The homogenized solutions were put into a polystyrene container and were then placed in a freezer at −80 °C. The completely frozen mixtures were lyophilized at −55 °C and 5 Pa for 48 h (ALPHA 1–2 LD plus, CHRIST, Germany).

2.3. Scaffold cross-linking

The lyophilized porous samples were stabilized using different treatment methods: cross-linking with EDC/NHS and dehydrothermal treatment (DHT). The scheme of obtaining and modification of the studied samples is shown in Fig. 1.

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

Carboimide cross-linking was achieved by immersing the collagen scaffolds in a cross-linking 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 above.

2.4. Scaffold characterization

2.4.1. Scanning electron microscopy (SEM)

The morphology of porous samples was studied using Scanning Electron Microscopy (SEM) (LEO Electron Microscopy Ltd, England). Scaffolds were cut with a razor scalpel after being frozen in liquid nitrogen for 3 min and were sputter-coated with a layer of gold for observation.

2.4.2. Porosity and density measurement

The density and porosity of the produced 3D scaffolds were measured by liquid displacement. 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 isporopanol (V_1) for 5 min. The total volume of isopropanol in the cylinder and isporopanol-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:

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

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

2.4.3. 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 37 °C. At each time point, the scaffolds were taken out from 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 the 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 in the pores and then weighed (W_{wm}). In this way the ability of the scaffold material itself to absorb water was assessed.

Each value was averaged from three parallel measurements. The swelling ratio of the scaffold was defined as the ratio of the weight increase ($W_w - W_d$) to the initial weight (W_d) according to following equation:

$$\text{Swelling ratio}\,(\%) = \left[\frac{W_w - W_d}{W_d}\right] \times 100$$

where W_w represents W_{ws} or W_{wm}.

2.4.4. Dissolution of scaffolds

Water-insoluble collagen and Col/HAp scaffolds were weighed prior to the dissolution study (W_b). The samples were immersed in 5 ml PBS (pH = 7.4) at 37 °C for 1, 2, 3, 7, 21, 42 and 77 days. At each time point, they were removed from the PBS buffer, rinsed with deionized water three times, lyophilized and weighed (W_a). The percentage weight loss was calculated from dry weight before and after being immersed in PBS.

$$\text{Weight loss}\,(\%) = \left[\frac{W_b - W_a}{W_b}\right] \times 100$$

The experiment was carried out for three samples and the average value was recorded.

2.4.5. Collagenase degradation

Collagen and collagen–hydroxyapatite lyophilized scaffolds of ~5 mg were weighed and immersed in 1 ml of 0.1 M Tris–HCl (pH = 7.4) containing 50 mM $CaCl_2$ and incubated at 37 °C for 0.5 h. Then, 1 ml of 0.1 M Tris–HCl containing 50 units of Clostridial Bacterial Type I Collagenase (Sigma–Aldrich) was added to the solution.