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Collagen-chondroitin sulfate-hydroxyapatite porous composites: preparation, characterization and *in vitro* biocompatibility testing

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LUCİA MOLDOVAN[1], OANA CRACİUNESCU[1], ELENA IULİA OPRİTA[1], MİRELA BALAN[1], OTİLİA ZARNESCU[2]

[1]National Institute of Research and Development for Biological Sciences, Department of Cellular and Molecular Biology, 296 Splaiul Independentei, sector 6, 060031, Bucharest, Romania

[2]University of Bucharest, Faculty of Biology, 91-95, Splaiul Independentei, sector 5, Bucharest, Romania

Corresponding author: Lucia Moldovan, 296, Splaiul Independentei, Bucharest, Romania, tel/fax: +40212200882, e-mail: luci@dbio.ro

Abstract

The aim of this study was to develop two new variants of porous composites based on collagen-chondroitin sulfate-hydroxyapatite (COL-CS-HA) mixtures. Electron microscopy examination showed that the composites had a microporous structure, with HA deposits non-homogeneously disposed on COL fibrous bundles. Their porosity was higher than 90 % and the biodegradation in the presence of collagenase was reduced after UV treatment of the scaffolds. Composite biocompatibility was evaluated by MTT assay and light microscopy after cultivation in a human osteoblast primary culture. All COL-based materials showed a good in vitro biocompatibility, but COL-CS-HA composites presented better cell proliferation and adhesion than COL material. These porous composites demonstrate potential for future application as cell scaffolds in bone tissue engineering.

Keywords: hydroxyapatite, collagen, chondroitin sulfate, porous composite, microstructure, scaffold, biocompatibility

Introduction

In recent years, porous composites have proved to be effective scaffolds for bone regeneration. These scaffolds, built from synthetic or natural materials, serve as temporary substitutes for the native extracellular matrix and guide the cell proliferation and adhesion *in vitro* and *in vivo* [1-3]. It has been well established that the specific interaction of cells with their surrounding extracellular matrix is responsible for promoting and regulating the regeneration processes of lost or damaged tissues [4]. Current strategies for bone regeneration focus on the extension of cell-matrix basic principles for the development of implantable matrices that mimic natural tissue. The physico-chemical and biological properties of the biomaterials used in bone regeneration are the key factors in determining their functional performance [5].

The synthetic calcium phosphate ceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP) are biocompatible and osteoconductive, as they are made from a material similar to the inorganic component of bone [6]. However, the ceramic is brittle and does not have alone the properties of cortical bone [7].

Collagen (COL)- and glycosaminoglycan (GAG)-based materials are the most widely used natural polymers for tissue regeneration. Type I COL, the major organic component of the bone matrix, has excellent biocompatible properties. It is easily degraded and resorbed by the body and allows good attachment to bone cells [8], but its mechanical properties are relatively low in comparison to bone [9]. Similarly, decorin and biglycan proteoglycans are natural constituents of bone matrix and are predominantly composed of chondroitin sulfate (CS) chains. Furthermore, they have the physical and biological properties needed for tissue grafting biomaterials.

It was reported that the combination of COL fibers and GAG polymers provides a chemical composition and mechanical and biological properties closer to native bone tissue [10]. On the other hand, a series of COL-HA composite matrix have been previously developed and used as temporary bone substitutes [11, 12]. The addition of COL to a ceramic material was shown to provide many advantages for medical applications: shape control, spatial adaptation, increased HA particle adhesion to the scaffold's wall and ability for clot formation [13].

In this study we have developed and characterized two new variants of porous composites based on COL-CS-HA mixtures. In order to use them for bone regeneration, the biocompatibility and cell behavior were evaluated after cultivation in a human osteoblast primary culture.

Materials and methods

Preparation of composite scaffolds

COL type I was extracted in our lab from bovine tendons by pepsin treatment [14], purified by precipitation at a salt concentration of 2.4 M NaCl and dialysed against distilled water. The COL solution had a concentration of 11.2% hydroxyproline, 0.8% hexosamine and a molecular weight of 320kDa. CS was isolated and purified from bovine tracheal cartilage by alkaline treatment and ethanol precipitation [15]. The yellowish powder had a content of 28.7% uronic acid and a molecular weight of 27 kDa. In order to fabricate the composite scaffolds, a solution of COL type I (0.8%, w/w) was mixed with a solution of chondroitin sulfate (1%, w/w) and hydroxyapatite powder (Merck), in weight ratios of 1:0.5:1 (variant I) and 1:0.5:2 (variant II). The mixtures were frozen at -40^{0}C and freeze-dried for 24h. Scaffolds obtained from type I collagen alone were used as controls. The freeze-dried materials were exposed to UV radiation, for 8h, in an UV sterilization cabinet (Scie-Plas, England). The microstructure of the scaffolds was then observed in the low vacuum mode using a scanning electron microscope (ESEM, Quanta 400, FEI, Philips, Holland).

Density and porosity measurement

The density (d) and porosity (ε) of COL and composite scaffolds were measured by water displacement method [16]. Briefly, a sample with a known weight (w) was immersed in a graded test tube holding a known volume of water ($v1$). The sample was kept in water for 30 min and pressed to force air from the scaffold and allow the water to penetrate and fill the pores. The total volume of water plus the water-impregnated sponge was recorded as $v2$. The water-impregnated scaffold was removed from the test tube and the residual water volume was recorded as $v3$. The following equations were used:

$d = w/(v2-v3)$ (1) and

$\varepsilon = (v1- v3)/(v2- v3)$ x 100 % (2)

Three measurements were taken for each average value.

In vitro degradation test

This test was performed using bacterial collagenase [17]. Briefly, UV-treated and untreated scaffolds of about 5mg dry weight were incubated in 0.1 M Tris-HCl (pH7.4) containing 2U/ml bacterial collagenase (*Clostridium histolyticum*, EC 3.4.24.3, Sigma Chemical Co.), at 37^0C. After 24h, the reaction was stopped and the extent of scaffold degradation was determined by measuring the amount of protein in the supernatant using the Bradford assay [18]. Biodegradability was calculated by comparison with the control sample, considered to be 100% degraded. The experiments were performed in triplicate.

In vitro biocompatibility test

A primary culture of rat osteoblasts was established from calvaria bones of newborn rats, using the enzymic digestion method, as previously described [19]. Cell viability was quantitatively determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test [20]. Briefly, cells from the first passage were injected into COL and COL-CS-HA composite scaffolds, at a density of 2 x 10^6 cells/cm^2 and cultured in standard culture conditions for 24h, 48h and 72h, respectively. After each period of time, the culture medium was replaced with 500µl of freshly prepared MTT solution (0.25mg/ml MTT) and incubated at 37^0C, for 3h. Water-insoluble dark blue formazan crystals formed in the viable cells were solubilized with 1ml isopropanol and the absorbance of each well was measured at 570nm using an UV-VIS spectrophotometer (Jasco V-650, Japan). Concentration of converted dye directly correlated to the viability of metabolically active cells in culture. Cell viability was calculated by comparison with the control sample (COL scaffold) considered to be 100 % viable cells at each period of time. The experiments were performed in triplicate.

Cell morphology

To analyze osteoblast morphology, cell-seeded COL-CS-HA scaffolds maintained in culture for 7, 14 and 21 days were fixed in Bouin solution, dehydrated in ethanol, cleared in toluene and embedded in paraffin. Paraffin sections (7µm) were stained with a 1 µg/ml 4′, 6-diamidino-2-phenylindole (DAPI) solution. The sections were then examined under the fluorescence microscope (Zeiss Axiostar Plus). The photomicrographs were taken by digital camera (AxioCam MRc 5, Carl Zeiss) driven by software AxioVision 4.6 (Carl Zeiss).

Results and discussion

The bone matrix is a natural composite material which consists of calcium phosphate in the form of HA crystals and COL (mainly type I), proteoglycans, small amounts of lipids and peptides [21]. The predominant bone substitutes are resorbable composite materials that mimic tissue composition and structure, containing COL type I fibrils and calcium phosphate crystals [22]. It was shown that both COL type I and HA enhanced osteoblast differentiation [23], but combined together they accelerated osteogenesis and behaved mechanically in a superior way to the individual components [12]. In this study, we have prepared two bioresorbable porous composites consisting of COL type I, HA and the glycosaminoglycan CS which occur in natural bone composition..

Evaluation of the composite scaffolds

A scaffold used for bone tissue engineering requires a porous structure with a porosity no less than 70% and interconnected pores which allow cell growth and proliferation [24]. The COL-CS-HA composites prepared in this work had highly three-dimensional porous structures (Figure 1). The pore structure of these scaffolds was formed as a consequence of the freeze-drying technique used in their fabrication. Previous studies showed that the

morphology of the pores is dependent upon the freezing temperature of the mixture before lyophilization [25]. In the present study, microporous structures were obtained when COL solution and COL-CS-HA mixtures were frozen at -40^{0}C. Our SEM images showed that all scaffolds had a honeycomb structure with pores of several microns in diameter. HA deposits were observed non-homogeneously disposed on COL fibrous bundles. The amount of HA deposits on the COL surface was higher in variant II than in variant I composites (Figure 1B, C).

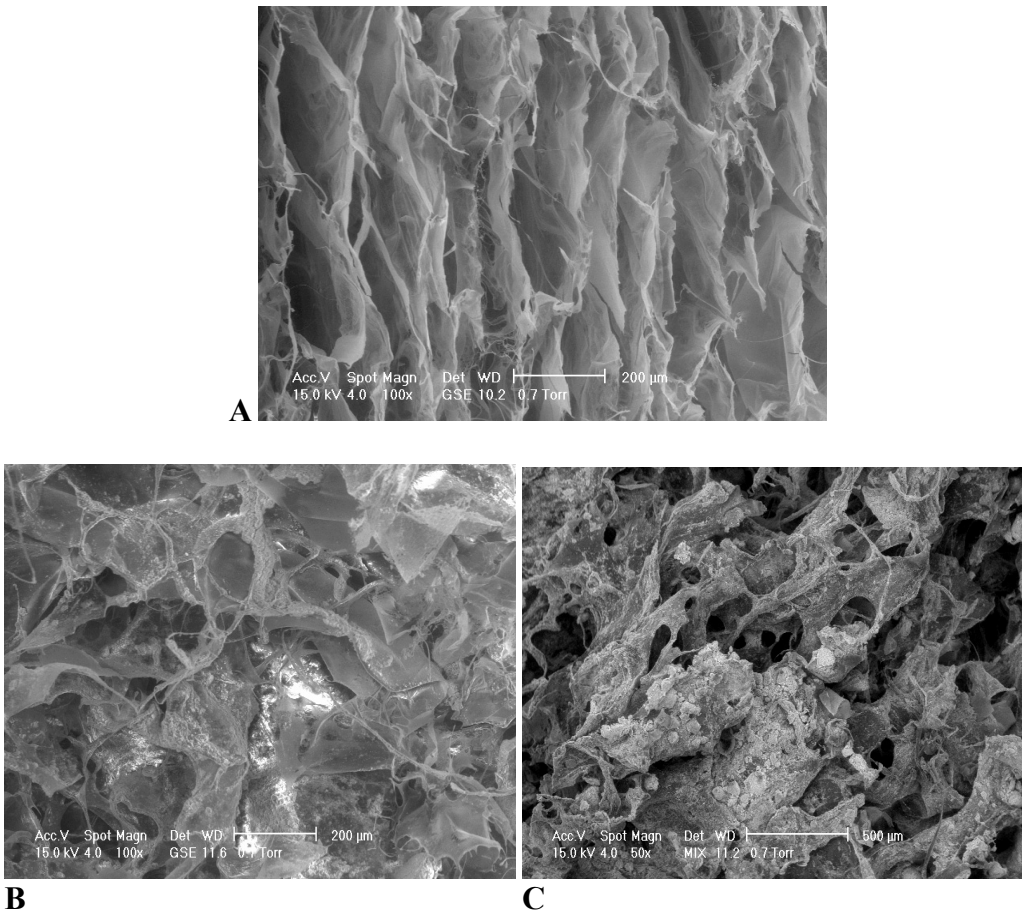

Figure 1. ESEM images presenting the cross-section morphologies of COL scaffold (A) and COL-CS-HA composite variants I (B) and II (C).

The porosity and density calculated for COL-CS-HA composite scaffolds are presented in Table 1.

Variant	Porosity (%)	Density(g/cm^3)
COL	97.33	0.0213
COL-CS-HA (I)	96.13	0.0682
COL-CS-HA (II)	92.85	0.1031

Table 1. Physical parameters of the scaffold variants

The highest porosity value (96.13 %) was registered for variant I. Porosity decreased proportional with the increase of the HA quantity, but was higher than 92 % in all variants. This highly porous structure is in favour of cell growth and proliferation. In turn, density was higher for variant II than for variant I and COL alone.

The small variation in porosity values of composites could prove that HA particles are tightly bond to the skeleton of the COL fibril network.

Ishaug et al. (1997) suggested that scaffold materials used for bone formation should provide an appropriate environment for cell proliferation and function and, at the same time, should be biodegradable [26]. Collagenase digestion can represent an *in vitro* measure of degradation rate for a biological implant. Untreated and UV-treated COL and composite scaffolds were analyzed by collagenase digestion. The degraded collagen quantity was lesser for all UV-treated material variants than for the untreated samples (Figure 2). On the other hand, it was observed that HA content did not influenced the biodegradability of UV-treated composites.

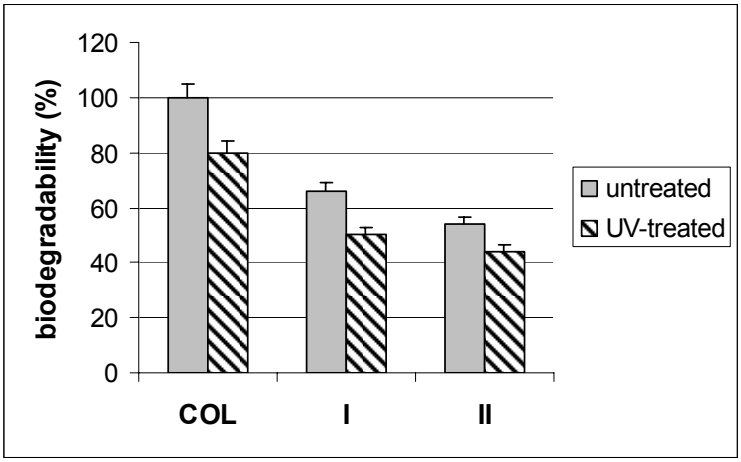

Figure 2. Degradation of composite variants in buffer containing collagenase.
Results are mean of three determinations ± S.D.

Our studies demonstrated that material exposure to UV increased the resistance of COL and composite scaffolds to enzymatic digestion. This result is supported by studies which show that COL fiber can be cross-linked by UV radiation [27]. UV exposure produces radicals from the nuclei of aromatic residues, such as those in tyrosine and phenylalanine and the binding of these radicals results in the observed cross-linking [28]. These cross-links may inhibit the action of collagenase upon COL-based composites and reduce their solubility.

In vitro biocompatibility of composite scaffolds

The biocompatibility of COL-CS-HA composite scaffolds was evaluated *in vitro* by measuring the mitochondrial dehydrogenase activity and observing the behavior of osteoblasts in close contact with these samples.

Biological tests for evaluating material cytotoxicity indicated that COL-CS-HA composites were non-toxic to testing cells. The viability of osteoblasts cultivated in the presence of COL-CS-HA composites was higher than that of cells cultivated into the COL scaffold (control) (Figure 3). Both composite scaffold variants have the cell viability percentage over 103%.

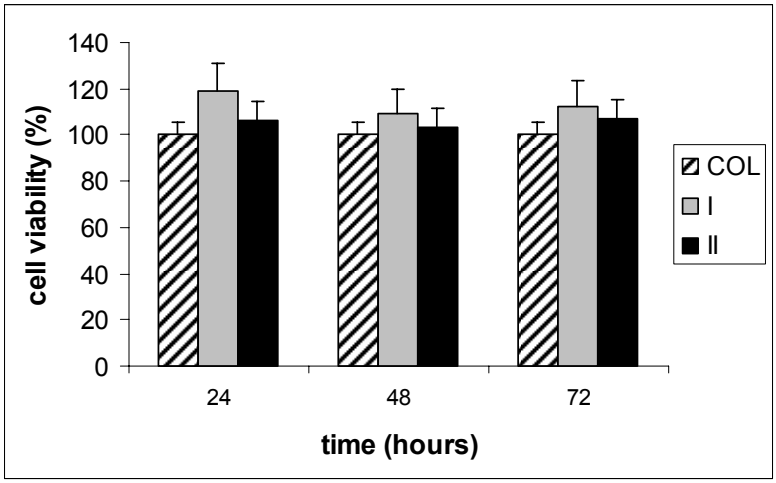

Figure 3. Effect of COL scaffold (control) and COL-CS-HA scaffolds (I and II) on rat osteoblast viability after 24, 48 and 72 hours of cultivation evaluated by MTT assay.
Measurements represent mean of three determinations ± S.D.

Effect of COL-CS-HA variants I and II on cell morphology was investigated in a primary culture of rat osteoblasts. Nuclear staining with DAPI showed that many cells appeared healthy, were firmly attached, round or spindle-shaped, with morphology similar to that seen in normal control dishes. Moreover, osteoblasts spread and proliferated well inside the porous scaffolds and retained their normal morphology even after 21 days in culture (Figure 4).

Rat osteoblast cell adhesion did not occur preferentially to HA crystals or to COL fibrils and did not seem to be dependent on the texture or roughness of the biomaterial surface as reported by some authors [29, 30]. It is known that the COL-binding molecules, such as fibronectin, contain RGD sequences that mediate interaction with cell membrane integrins and promote cell attachment through focal contacts and adhesion plaques [31]. Previous studies showed that utilization of COL-based composites organized as three-dimensional scaffolds enhanced the contact guidance process of osteoblasts inoculated onto the biocomposite surface [32]. Our results demonstrated that the association of COL fibers with GAG polymers and HA particles was more effective than COL alone in stimulating osteoblast proliferation and adhesion.

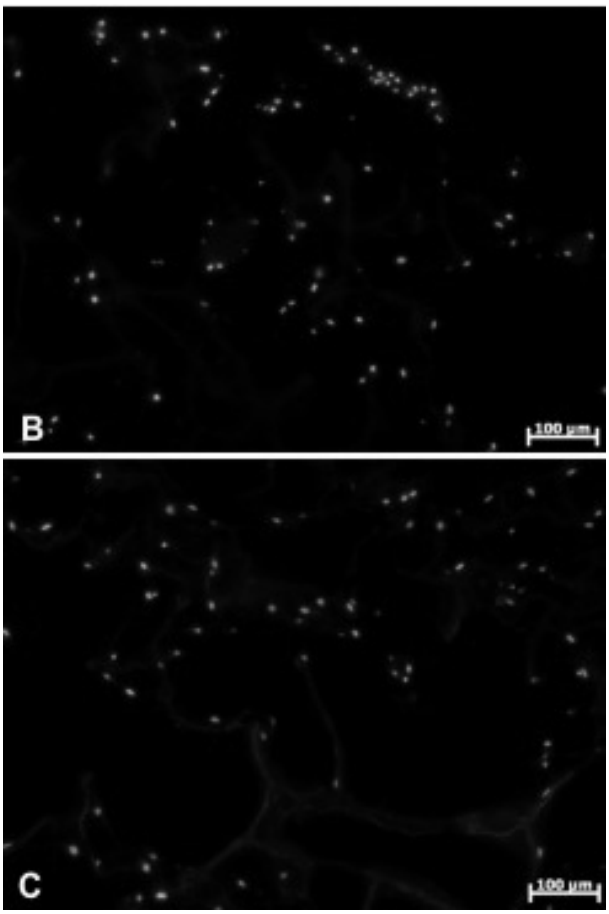

Figure 4. DAPI staining of osteoblast cultured into COL scaffold (A) and COL-CS-HA composite variants I (B) and II (C) for 21 days.

Conclusions

We have prepared two new variants of COL-based biodegradable composites by integrating HA powder in a gel mixture of COL-CS. These composites were constructed as three-dimensional scaffolds with microporous structure and a porosity higher than 90%. Scaffold biodegradation in the presence of collagenase was reduced after their exposure to UV radiation. The COL-based biomaterials were evaluated in a rat osteoblast culture and showed a good biocompatibility. The composite materials presented better cell proliferation and adhesion than the material containing only COL. Both porous composites demonstrate potential for future application as cell scaffolds in bone tissue engineering.

Acknowledgements

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