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New Scaffold Structure Based on Collagen. Fabrication and Biocompatibility Evaluation

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Development of bioactive material template for in vitro and in vivo synthesis of osteoinductive and biodegradable bone material was intensevely studied over the last decade and the research in the field of partial substitution of bone tissue, use a very large range of natural and synthetic polymers, inorganic components and their composites. Despite of composites collagen hydroxiapatite with a mimetic osseous composition until now was not defined a scaffold model suitable to biofunctionality of native osseous structure. The goal of the article is fabrication of a new scaffold structure, based on collagen fibrils with length 1–1.5 cm, thikness 0.1–0.3 and having a shroud structure. Collagen crosslinking was performed with aldehides in such way that aminic groups became bloked and carboxylic groups remain free in order to involve hydroxiapatite and biocompatible synthetic polymer (polyvinil alchol, polilactide) coupling. Crosslinking temperature for collagen fibrils is 70°C being a suitable temperature for resistance to "in vivo" resorbtion. Infrared spectra was performed and the amount of the hidroxyl bonds was correlated with hydrophilic [2] balance estimated from contact angle measurements. The morphology and the surface composition were determined with an Environmental Scanning Electron Microscope FEI/ Phillips **XL30 ESEM** *and all physical chemical properties especially surface features were used as basic factors in future cell growth and proliferation process. The main aim of biocompatibility tests is to multiply and to differentiate cells in vitro in osteoblasts from marrow. The environment of culture was supplemented with specific media containing Na β glicerofosfat and the cell was differentiated in osteoblasts. As arguments for differentiation were proposed the evidence of specific markers: osteonectine, sialoproteines and osteocalcine. Osteoprogenitors cells culture were tested on various samples of scaffold. Cell cultures were tested for alkaline phosphatase*

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at a week after culture. The technique uses p-nitrophenole which is going to be change by alkaline phosphatase in dinitro-phenole.

Keywords: biocompatibility; collagen; new scaffold

INTRODUCTION

Development of bioactive material template for *in vitro* and *in vivo* synthesis of osteoinductive and biodegradable bone material was intensively studied over the last decade [1]. The research in the field of partial substitution of bone tissue, use a very large range of natural and synthetic polymers, inorganic components and their composites [2]. Collagen being one of the most abundant natural polymer in human body, to biomaterials based on collagen were devoted many studies in last decade [2,3]. On the other hand natural and synthetic hybrid materials have the advantages of good mechanical features from synthetic part and good biocompatibility from the natural component [4]. Especially, composites as synergetic biomaterials are found suitable for biomedical applications, being representative for their remarkable ratio quality/price. Despite of composites collagen hydroxiapatite with a mimetic osseous composition until now was not defined a scaffold model suited to biofunctionality of native osseous structure.

Aliphatic polyesters such as polyglycolic acid (PGA), polylactic acid (PLLA), their copolymers (e.g. PLGA) and polycaprolactone (PCL) are the most commonly used polymers for tissue engineering scaffold applications [5–7]. The degradation products of these polymers (glycolic acid and lactic acid) are present in the human body and are removed by natural metabolic pathways. Biodegradable synthetic poly(hydroxy acids), such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymer of poly(DL-lactic-co-glycolic acid) (PLGA), and collagen have been widely used for tissue engineering as temporary scaffolds to accommodate transplanted cell masses, and as materials for assisting surgeries in clinical applications [8–13]. The synthetic polymer meshes demonstrate good biocompatibility, good mechanical properties, and are easy to handle. Their rate of degradation can also be controlled to meet the rate of new tissue formation in tissue repair. However, these synthetic polymers are relatively hydrophobic. These hydrophobic properties, together with the large mesh interstices, hinder smooth cell seeding. In contrast, collagen offers the advantage of specific cell interactions and hydrophilic properties, but scaffolds constructed entirely of collagen have poor mechanical strength.

Therefore, synthetic biodegradable polymers and collagen have been hybridized to combine their advantages [14–20].

The goal of the article is the fabrication and evaluation of the biocompatibility, of a new scaffold structure, based on collagen and biocompatible polymers. Collagen crosslinking was performed with aldehides in such a way that amine groups became blocked and carboxylic groups remained free in order to involve synthetic polymer (polyvinyl alcohol, polylactide) coupling.

MATERIALS AND METHODS

The collagen/polymer scaffolds was the obtaining of biocompatible porous structures by compounding synthetic polymers – (poly(L-lactic acid) (PLLA), poly(D,L-lactic-co-glycolic acid) (PLGA) and poly (caprolactone) (PCL) with natural polymer – collagen, the main protein of extracelullar matrix. Type I collagen was extracted from bovine derma as fibrillar gel following a known technological process [21]. The biocomposites were prepared in a polymer:collagen ratio of 2:1, 1:2 and 1:1 by added synthetic polymer solution, solved in chloroform, into aqueous fibril collagen gel. The obtaining of biocomposite matrices was achieved by lyophilizing of synthetic polymer/collagen blends after dialyse against 1 M KCl. Both uncrosslinked and crosslinked samples were prepared. As standard sample the uncrosslinked and crosslinked collagen matrices were chosen. These several types of matrices were obtained: collagen/PLLA (1:2), crosslinked collagen/PLLA (1:2), collagen/PLGA (1:2), crosslinked collagen/PLGA (1:2), collagen/PVA (1:2), crosslinked colagen/PVA (1:2), collagen/PCL (1:2), crosslinked collagen/PCL (1:2), collagen/PCL (1:1), crosslinked collagen/PCL (1:1).

Structural characterization was performed by infrared spectroscopy with a Shimazu-620 equipment. Chemicals used for cell culture and for cell staining were obtained from Sigma (Germany). Tissue culture flasks were from Costar (Cambridge, MA, USA).

RESULTS AND DISCUSSIONS

In Figures 1 and 2 are presented the infrared spectra for collagen and two scaffolds (matrices) of collagen/PLGA and collagen/PVA. It can be seen that there are almost no differences when the ratio between collagen and polymer is changed. In the case of collagen/PVA matrices it can observe that the peaks at 1734 and 1716 cm^{-1} (corresponding to the COOH group) are shifted. Moreover the peak at 1734 cm^{-1} (free acid) is smaller than that at 1716 cm^{-1} (bonded acid) in the collagen/polymer matrices.

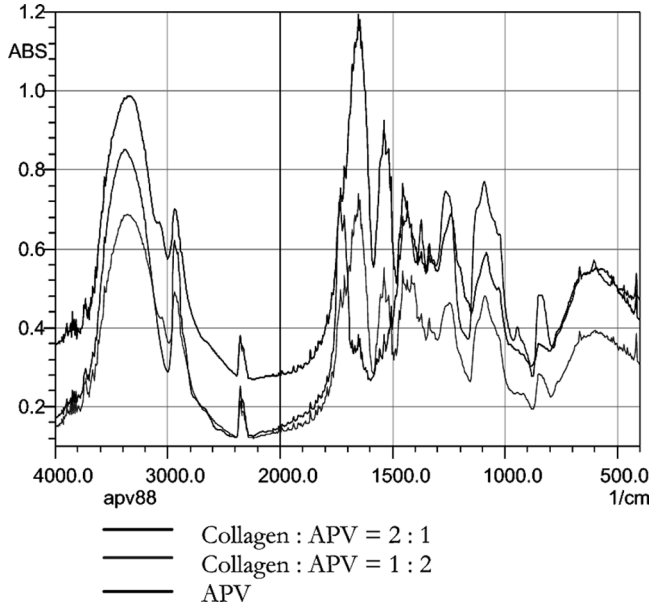

FIGURE 1 Collagen/PLGA matrices.

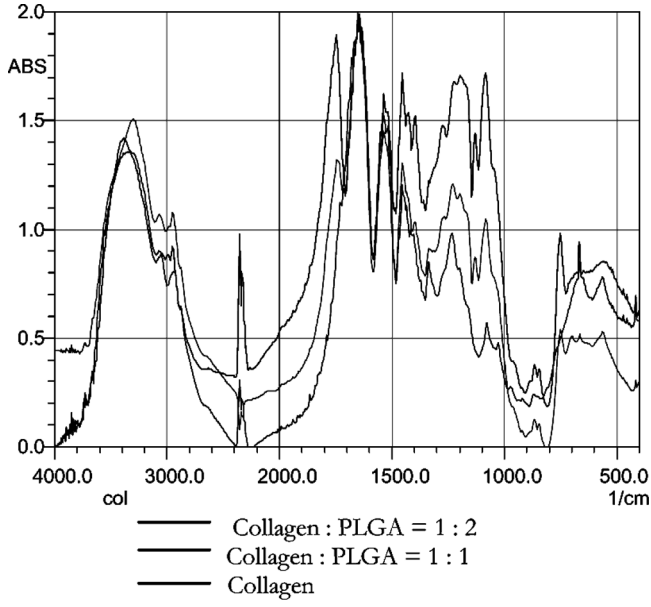

FIGURE 2 Collagen/PVA matrices.

Cells Colonization on Collagen/Polymer Matrices

We used the endothelial cell line EA hy 926 (human umbilical endothelial cells) and human osteoprogenitor cells grown in DMEM supplemented with 4,5‰ glucose medium, 10% fetal bovine serum, and sodium selenite 20 μg/L, 30 mg/L ascorbic acid, and antibiotics (100 U/L penicillin, 100 U/L streptomycin, 50 U/L neomycin) for *in vitro* colonization. Human osteoprogenitor cells were obtained by puncture of the postero-superior spine of the iliac crest or by spongy bone biopsies and separated on a Percoll gradient. These cells were positive for alkaline phosphatase. Collagen/polymer matrices were sterilized with 70% ethanol for 24 hours. Matrices were conditioned in the same culture medium for 24 hours and then inoculated with EA hy 926 endothelial cells (50.000 cells/mL) or with human osteoprogenitor cells (75.0000 cells/ml). Cells were maintained in culture at 37°C in incubators with 5% CO_2 in air (v/v), and relative humidity over 95%. The culture medium was changed two times a week. All experiments were done after 1 week of culture. Some of the collagen/polymer matrices (especially uncrosslinked matrices) were dissolved in culture medium after 24 hours in this order: collagen, collagen/PLLA, collagen/PVA, collagen/PLGA, collagen/PCL (1:1) and collagen/PCL (1:2).

Hoechst Staining

The cells were cultured on collagen supports for one week, washed in PBS, fixed in 2% paraformaldehyde for one hour, and then cryoprotected. After washing in phosphate tampon (PB: 0,2 M Na_2HPO_4, and 0,2 M NaH_2PO_4) ph 7.2, the specimens were kept in a solution of PB 0.1 M + 5% sucrose over night at 4°C. In the next day, the probes were immersed in PB containing 5%, 10%, 20% and 50% glycerine at 4°C for 15 minutes, 1 hour, 10 hours and 1 hour, respectively. Specimens were frozen in liquid nitrogen and sectioned with a Leica CM 1800 cryotome. The thickens of the sections were 4–6 μm. The cryosections were washed with PBS for 15 minutes, stained with Hoechst 33258 for 15 minutes (a specific DNA staining), washed in distilled water, mounted in glycerol and examined with a Nikon microscope equipped with epi-fluorescence and a filter G1-B; the micrographs were captured with a Sony DSC-S75 Digital Camera.

Osteoprogenitor Cells Colonization on Collagen/Polymer Matrices

Employing Hoechst staining we have found that matrices used as control sustained growth of osteoprogenitor cells (Figs. 3a, b).

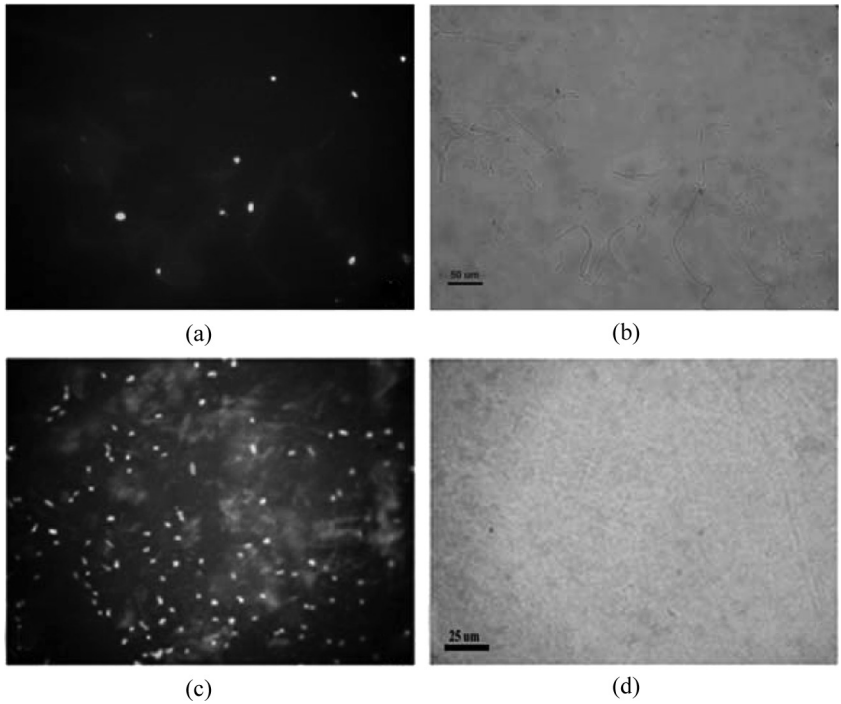

FIGURE 3 Human osteoprogenitor cells growth on collagen supports (control) a, b – human osteoprogenitor cell grown on crosslinked collagen matrices; c, d – human osteoprogenitor cell grown on collagen membrane.

Some biomaterials have had a cytotoxic effect for osteoprogenitor cells. This fact was observed by the death cells around material (Fig. 4b). The cells grown around crosslinked collagen/PVA and crosslinked collagen/PLLA show a bit different aspect than control cells (Fig. 4c).

The cells grown around collagen/PLLA had the same morphology compared with the cells grown on borosilicate glass and the cells grown near the crosslinked collagen (Fig. 5).

Although cells around crosslinked collagen/PVA and collagen/PLLA matrices were undergone a normal development, a population of those materials with osteoprogenitor cells had not registered. This fact was observed by cryosection and dyeing of obtained sections with Hoechst 33251.

Endothelial Cells Colonization on Collagen/Polymer Matrices

It is known that a success in a transplant achievement is graft endothelization, a foregone process of implant vascularisation. Besides

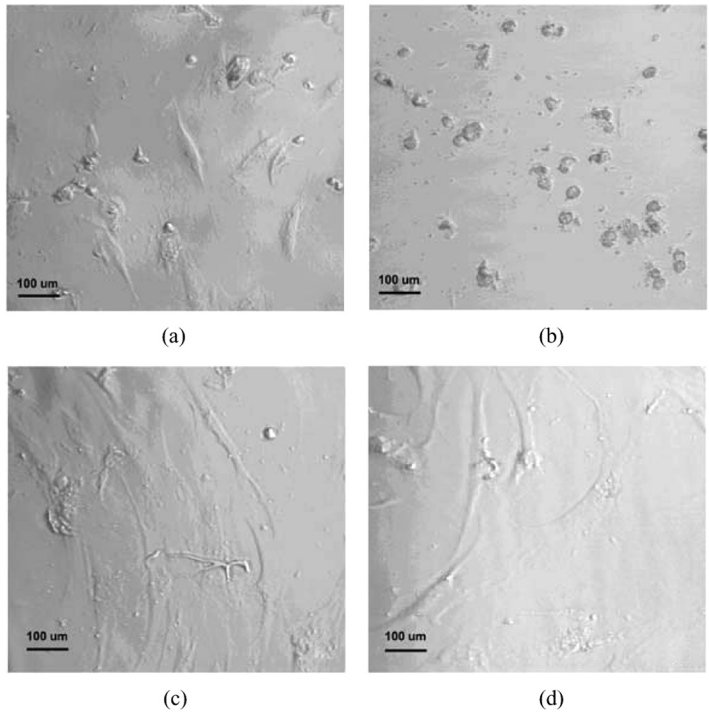

FIGURE 4 Human osteoprogenitor cells – 1 week after seeding. The picture represents cells grown near the matrix scaffolds. a – crosslinked collagen (control); b – crosslinked collagen/PLGA; c – crosslinked collagen/PVA; d – crosslinked collagen/PLLA.

osteoblasts proliferation and maturation and extracellular matrix mineralization, vascularisation has an important role in bone regeneration. Endothelial cells are involved in bone development and remodeling and also can influence bony cells conscription as well as their forming and activation. Thus, microvascularization became the basis of bone regeneration, dependent of interaction between osteoblasts and endothelial cells [22]. For this reason we tested population of collagen/polymers matrices with human endothelial cells. In this case a colonisation of biomaterials which contain collagen and polymers was registred.

The endothelial cells colonized polymeric biomaterials and crosslinked collagen matrices (Figs. 6, 7), but the rate of colonization was very low. As for osteoprogenitor crosslinked collagen/PCL (1:1) and crosslinked collagen/PCL (1:2) proved to be cytotoxic.

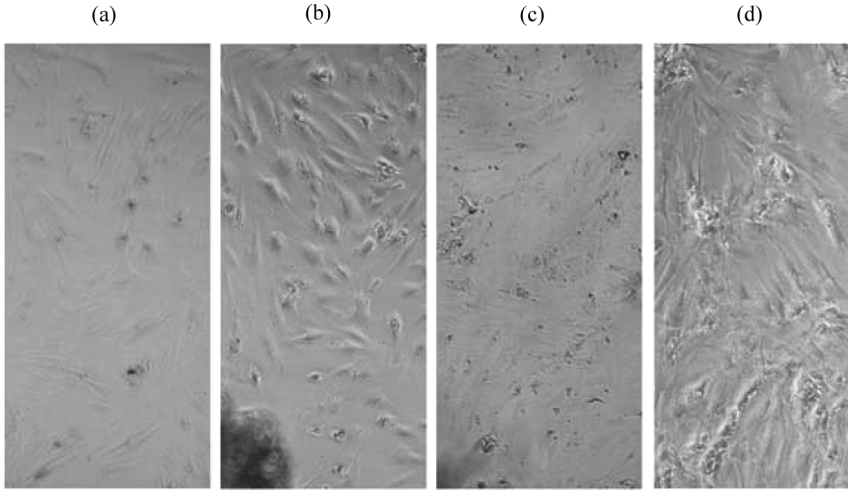

FIGURE 5 Human osteoprogenitor cells – 1 week after seeding. The picture represents cells grown near the matrix scaffolds. a – crosslinked collagen (control); b – collagen/PLLA; c – crosslinked collagen/PLLA; d – cells grown on borosilicate glass (control cells).

CONCLUSIONS

Introduction of synthetic polymer reduced the rate of cell colonisation with endothelial cells hybrid matrices was different for distinct composition of samples. Hereby the highest population with endothelial cells was achieved by crosslinked collagen/PLLA matrices. The structure of those matrices was induced higher hydrophilic ability due to the presence of several acid groups (see IR spectra). Even if the polymer

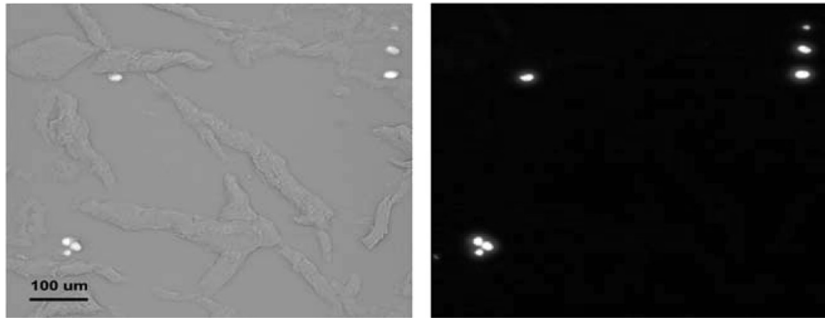

FIGURE 6 Endothelial cells grown on crosslinked collagen.

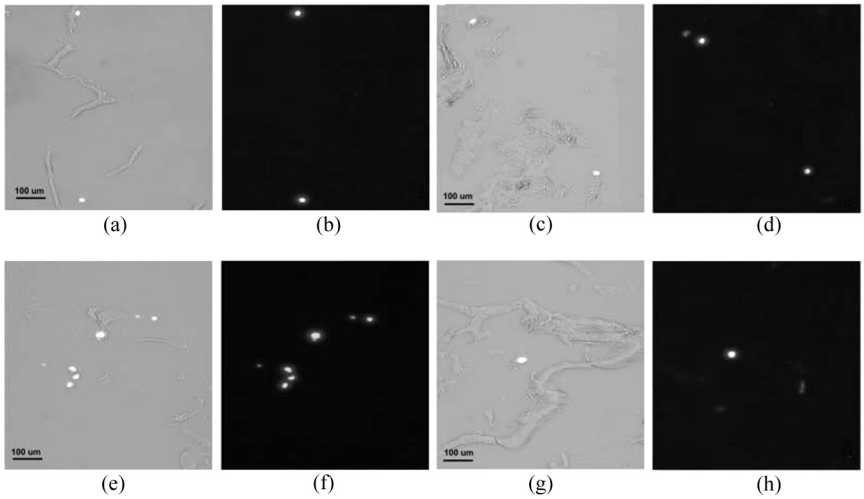

FIGURE 7 Endothelial cells grown on polymeric supports. a, b – collagen/PLLA; c, d – crosslinked collagen/PLLA; e, f – crosslinked collagen/PCL (1:1); g, h – crosslinked collagen/PCL (1:2).

matrices/PLLA, PVA had not cytotoxic effect, the osteoprogenitor cells did not adhere on these supports. Maybe the presence of negative charges on the biomaterial surfaces inhibited the adhesion process because the surface of cells is also negative due to glycocalix.; syalo acids negative radicals.

The higher colonization with human osteoprogenitor cells was found on collagen membranes (unpublished results). A possible explanation of this finding is that during the free drying procedure at 25°C, the collagen fibrils from gel have arranged in a similar way as *in situ* collagen fibrils. Cells development was only on surface of collagen membrane due to 2D structure, which differs from 3D structure of matrices with macro, micro and nanopores.

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