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Resorbable scaffolds modified with collagen type I or hydroxyapatite: *in vitro* studies on human mesenchymal stem cells

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Poly(L-lactide-co-glycolide) (PLGA) scaffolds of pore size within the range of 250–320 μm were produced by solvent casting/porogen leaching method. Afterwards, they were modified through adsorption of collagen type I and incubation in simulated body fluid (SBF) to allow deposition of hydroxyapatite (HAp). The wettability of the scaffolds was measured by sessile drop test. Scanning electron microscopy (SEM) evaluation and energy dispersive X-ray analysis (EDX) were also performed. SEM evaluation and EDX analysis depicted the presence of HAp deposits and a collagen layer on the pore walls on the surface and in the bulk of the scaffolds. Wettability and water droplets penetration time within the scaffolds decreased considerably after applying modifications. Human mesenchymal stem cells (hMSC) were cultured on the scaffolds for 28 days and cell morphology, proliferation and differentiation as well as calcium deposition were evaluated. Lactate dehydrogenase (LDH) activity results revealed that cells cultured on tissue culture polystyrene (TCPS) exhibited high proliferation capacity. Cell growth on the scaffolds was slower in comparison to TCPS and did not depend on modification applied. On the other hand, osteogenic differentiation of hMSC as confirmed by alkaline phosphatase (ALP) activity and mineralization results was enhanced on the scaffolds modified with hydroxyapatite and collagen.

Key words: *collagen type I, human mesenchymal stem cells, hydroxyapatite, poly(L-lactide-co-glycolide), scaffolds*

1. Introduction

Progressive ageing of societies in more developed countries causes an increase in public demand for advanced medical care. As a consequence, the rapid development of such branches of bioscience as tissue engineering and regenerative medicine has occurred. One of the biggest old age problems is osteoporosis and fragility of the bones which results in frequent bone fractures and general worse health condition [1], [2].

Tissue engineering of bone involves a series of strategies using biologically based mechanisms to repair and restore damaged and diseased tissues [3]. The key elements include a specific living cell type, a material scaffold that forms supporting structure for culturing the cells *in vitro* and surgical delivery *in vivo*, and growth stimuli [4]. Among materials for scaffolds, aliphatic polyesters have come under careful scrutiny for their biocompatibility, good mechanical properties and resorbability [5]. Moreover, to enhance the biological profile, biocompatible layers from natural components of bone extracellular matrix (ECM), like hydroxyapatite or collagen, have been formed [6], [7].

The goal of this study was to manufacture composite biodegradable scaffolds with defined properties such as porosity, microstructure, surface properties and presence of biologically active molecules, and to investigate their performance *in vitro* in contact with

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human mesenchymal stem cells, with the aim to use them in the future for bone tissue engineering.

2. Materials and methods

2.1. Preparation of scaffolds

The scaffolds were made from poly(L-lactide-co-glycolide) (PLGA) (molar ratio of L-lactide to glycolide comonomers 85:15, number-average molecular weight M_n = 100 kDa, polydispersion d = 2.1, polymerization initiator – Zr(acac)$_4$; produced at the Center of Polymer and Carbon Materials, Polish Academy of Sciences, Zabrze) [8]. The scaffolds were prepared by the solvent casting/porogen leaching method. As porogen sodium chloride particles (POCh, Gliwice, Poland) with a size within the range of 250–320 µm were utilized (previously sieved to obtain desired fraction with the use of Multiserw, Wadowice, Poland). To produce the scaffolds a mixture of porogen (85 vol.%) and copolymer dissolved in dichloromethylene (POCh, Gliwice, Poland) was tightly packed into polypropylene syringes, dried in air for 24 h and in vacuum oven under reduced pressure for 48 h. Afterwards, each syringe was cut into 2 mm thick slices, and the samples were placed in ultra-high quality water (UHQ-water, Purelab, Elga, UK) to remove salt particles. The water was changed repeatedly until the conductivity level was below 2 µS/cm (conductometer CC-315, Elmetron, Poland) and then samples were dried for 48 h at 37 °C.

2.2. Scaffold modifications

Hydroxyapatite deposition was proceeded by immersing scaffolds in simulated body fluid (SBF). SBF was prepared by dissolving $NaHCO_3$, Na_2SO_4, KCl, K_2HPO_4, $MgCl_2·6H_2O$, NaCl, $CaCl_2$ (all chemicals from POCh, Gliwice, Poland) in UHQ-water; the pH was adjusted by HCl and TRIS to 7.4 at 37 °C. The inorganic ion concentrations were the following: 426 mM Na^+, 15mM K^+, 7.5 mM Ca^{2+}, 4.5 Mg^{2+}, 446.4 Cl^-, 12.6 HCO_3^-, 3 mM HPO_4^{2-}, 1.5 mM SO_4^{2-}, and were three times those of human blood plasma [9]. The scaffolds were immersed in SBF in 20-mL syringes and then the vacuum was created by blocking them and pulling back the plunger to enable penetration of the pores by SBF solution. Afterwards, the scaffolds were incubated in SBF at 37 °C for 12 days, SBF was changed every two days. In the end, the scaffolds were rinsed in tap water.

The scaffolds were also modified through collagen adsorption. 0.1% collagen solution in 0.1 M acetic acid (for cell culture, from calf skin, catalogue no. C 8919, Sigma, Germany) was diluted to the concentration of 40 µg/ml in phosphate buffer saline with the following composition: 137 mM NaCl, 6.44 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4.

Before immersing the scaffolds in collagen solution they underwent the same procedure of removing air from the pores as in the case of hydroxyapatite modification. Afterwards, the scaffolds were incubated in PBS at 37 °C for 2 h. Next, they were rinsed in UHQ-water to remove remnants of the buffer salts and unbound collagen.

In both cases after incubation in modification solutions the samples were placed in the oven at 37 °C for 72 h, packed in sterilization bags (Tyvdec^R Roll, ASP, Johnson & Johnson) and sterilized by the hydrogen peroxide plasma (Sterrad 120, ASP, Johnson & Johnson).

2.3. Physico-chemical evaluation of scaffolds

Wettability of the scaffolds was determined by sessile drop method (DSA 10 Mk2, Kruss) using UHQ-water as a measuring liquid. Measurements were performed at room temperature and ambient humidity.

The sample microstructure was examined by SEM at 15 kV by Nova 200 NanoSEM (FEI, USA). Elemental composition of the samples was studied by energy dispersive X-ray spectroscopy (EDX, Link AN 10000, UK; Noran Vantage EDS system, USA).

2.4. Cell seeding and cultivation on scaffolds

Human mesenchymal stem cells (hMSCs) were obtained from the University Hospital Carl Gustav Carus Dresden from patient 315 (caucasian male, age 37) and used for cell culture experiments in passage 4. The scaffolds were pre-wetted with PBS with the use of the syringes (in a similar way as described in the procedure of hydroxyapatite/collagen deposition), placed for a few seconds on the sterile paper filter, transferred into 24-well plates (Nunclon) and cells suspension (50 000 cells in 60 µl) was dropped on the top of each scaffold. Then 1 mL Dulbecco's Modified Essential Medium (DMEM, F0415), with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2 mM of L-Glutamine (all from Biochrom) was poured. HMSCs were cultured up to 28 days and the medium was exchanged twice a week.

2.5. Methods for studying cell morphology, viability, proliferation and differentiation

The morphology of the cells was examined at 1 kV and 15 kV by Gemini DSM 982 Scanning Electron Microscope (Carl Zeiss, Oberkochen, Germany). The scaffolds with cultured cells were fixed in 2% glutaraldehyde for 10 min and dehydrated in graded ethanol series (10%, 30%, 50%, 70%, 80%, 90% and 100% ethanol for 20 min each). After that, scaffolds underwent critical point drying procedure (BALTEC CPD 030, Witten, Germany), and were sputter-coated with a thin carbon layer in order to enhance their conductivity.

Cell proliferation was measured by activity of lactate dehydrogenase (LDH). Before LDH analysis the lysis buffer (1% Triton X-100, Sigma Aldrich, Germany) solution in PBS and working solution (INT/Na-lactate with catalyst(A) Diaporase/NAD+ solutions (both Cytotoxicity Detection Kit, TaKaRa Bio, Inc., Japan) in 1:46 proportion) were prepared. To each scaffold 500 µl of lysis buffer was added, and they were shaken and sonicated for 1 h. Then 50 µL of lysate and 50 µL of working solution were placed into the wells of 96-well plates and the mixture was shaken for 3 min. The reaction was stopped by adding 50 µL of 0.5 M hydrochloric acid (Merck, Germany). Finally, the absorbance was measured spectrophotometrically at 492 nm.

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test. MTT (Sigma, Germany) was dissolved at a concentration of 5 mg/mL in PBS and sterile filtered. Then, 100 µL of the MTT solution was added to each well containing scaffold, and thoroughly pipetted. Afterwards, the wells were incubated for 2 h at 37 °C at 7% CO_2. After that time, the scaffolds were washed in PBS 3 times and their pictures were taken by a digital camera.

Osteogenic differentiation of hMSCs was assessed by alkaline phosphatase (ALP) activity and van Kossa staining. ALP activity was measured using a biochemical assay, based on conversion of *p*-nitrophenyl to *p*-nitrophenol (Sigma Aldrich N2765, Germany). To obtain the working solution, the tablet of *p*-nitrophenol was diluted in 20 ml of cold ALP buffer. Then, 25 µL of the lysate (prepared as described in LDH procedure) was mixed with 125 µL of working solution in Eppendorf vials and incubated for 30 min at 37 °C. The reaction was stopped by adding 63 µL of 1 M NaOH (Sigma Aldrich, Germany). Subsequently, the samples were centrifuged for 10 min in 14 000 rpm. Later, 170 µL of final solution was transferred into 96-well plate and the absorbance was measured at 405 nm.

For total calcium content, samples were extracted in 0.5 mL of 0.25M hydrochloric acid. Calcium content was determined by a colorimetric assay using o-Cresoplhthalein complexone (Fluitest® Ca-CPC, Analyticon, Germany) according to the instructions provided by the manufacturer. The absorbance of calcium complex was measured at 590 nm.

Before van Kossa staining the scaffolds were fixed in 3.7% formaldehyde (Sigma, Germany) for 20 min, then they were washed in double distilled water (ddH_2O, Ultra-Pure Water System Arium® 611VF, Sartorius Stedim Biotech, Germany). Later 1 mL of 5% of silver nitrate (Fluka Analytical, Germany) was added, samples were incubated for 15 min under UV and then again washed in ddH_2O. Subsequently, 1 mL of 5% sodium thiosulfate (Fluka Chemika, Germany) was added and incubated for 2 min. In the end, the samples were washed in ddH_2O and pictures were taken with a digital camera.

3. Results

The wettability test showed that non-modified PLGA scaffolds were very hydrophobic. Their water contact angle was of 127° ± 9°. The observation of the shape of the drop as a function of time showed that the drop was not penetrating the material and disappeared after 10 min only because of evaporation (data not presented). Modification with collagen resulted in lower water contact angle (θ = 113° ± 1°) and a penetration of water droplet into the scaffold within about 10 s. For the PLGA scaffold after incubation in SBF it was impossible to measure the water contact angle because of very fast penetration of water droplet, i.e., <1 second. The results show that scaffolds modified with hydroxyapatite had the best wettability, but the wettability of collagen-modified scaffold was also improved.

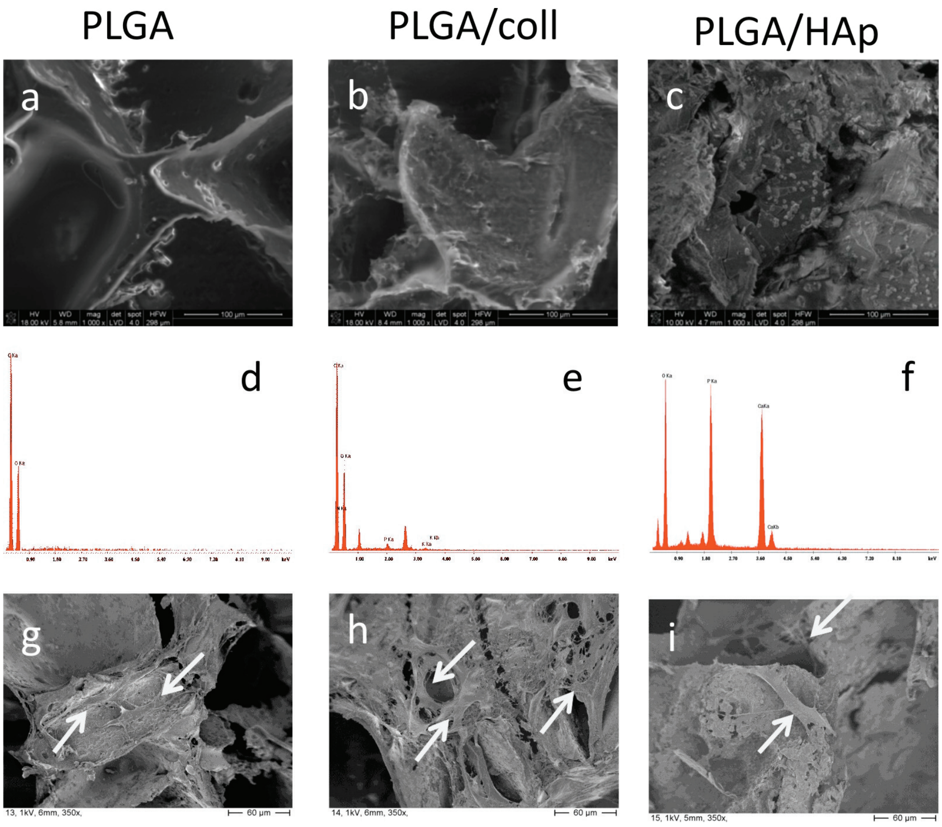

Fig. 1. SEM microphotographs (a), (b), (c) and EDX spectra (d), (e), (f) of PLGA scaffolds before and after culturing hMSC for 28 days (g), (h), (i). Arrows show cells adhering to the pore walls

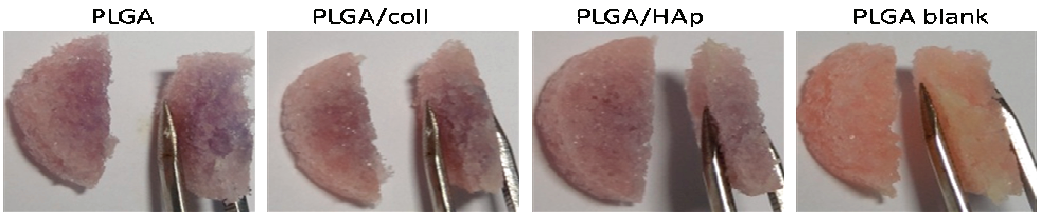

Fig. 2. Results of MTT staining after hMSC cultivation for 48 h

SEM microphotographs (Fig. 1a–c) show that PLGA scaffolds consisted of oval pores of about 250–320 μm which were richly interconnected. The surface of the walls in PLGA scaffolds was smooth (Fig. 1a) while on those soaked in collagen solution a thin adsorbed collagen layer was observed (Fig. 1b). The surface of PLGA scaffolds soaked in SBF was coated with characteristic cauliflower-like structures typical of hydroxyapatite (Fig. 1c).

The results of EDX analysis (Fig. 1d–f) show that as expected, unmodified PLGA scaffolds consisted of carbon and oxygen (Fig. 1d) (hydrogen is not detected by EDX). Modification with collagen resulted in the presence of nitrogen (around 2.4% of atomic ratio) (Fig. 1e). In the case of the scaffolds soaked in SBF calcium and phosphorus were detected; atomic ratio of calcium to phosphorus amounted to 1.6, which is close to that of HAp.

The results of MTT staining after 48 h of cells cultivation show that there was light purple colour originating from cells on all the scaffolds. Moreover, the cells were distributed quite uniformly as cross-sections show, while the blanks remained pink, due to medium absorption (Fig. 2).

Figure 1g–i shows SEM pictures of hMSC cultured on the scaffolds for 28 days. The cells were well spread, with fine developed filopodia and covered the pore walls on the surface and inside the scaffolds.

LDH assay results show that at the later time points cell proliferation was significantly enhanced on TCPS – there were almost 350 000 cells in one well after 28 days of culture (Fig. 3a). The tendency of higher proliferation was visible on pure PLGA scaffold – especially after 28 days, but those results were not statistically significant. On the other hand, osteogenic differentiation of hMSC measured by ALP activity (Fig. 3b) was enhanced on the scaffolds as compared with TCPS after 7 and 21 days.

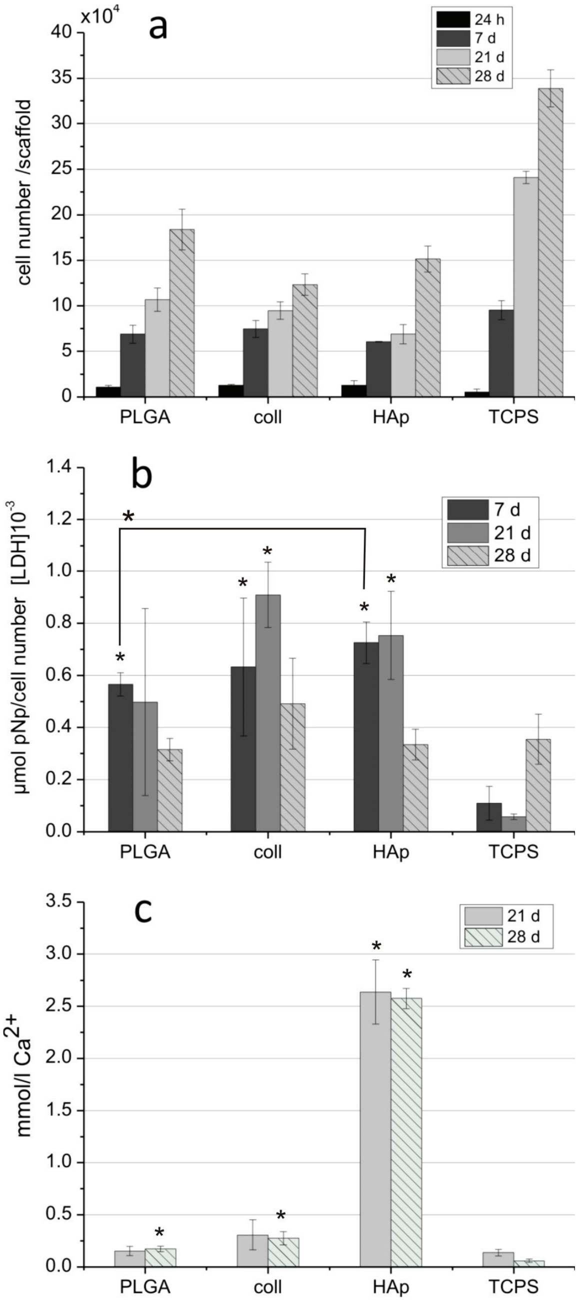

Fig. 3. Results of LDH activity (a), ALP activity (b) and calcium concentration (c) of hMSC cultivation after 24 h, 7 days, 21 days, 28 days. Asterisks indicate a statistical significance from the control TCPS group at certain time point: *$p < 0.05$ according to *t*-test

The measurements of calcium concentration allowed us to evaluate deposition of calcium by cells as well as calcium originating from the scaffold material (Fig. 3c). The results show that calcium concentration on SBF-modified scaffolds was 10-fold higher than that of TCPS and non-modified scaffolds. This is a proof of the presence of hydroxyapatite on the scaffolds. However, it is also worth noting that calcium deposition on PLGA and collagen coated scaffolds was significantly higher in comparison to cells cultured on TCPS. High level of calcium in the case of hydroxyapatite modified scaffolds was the consequence of the coating itself.

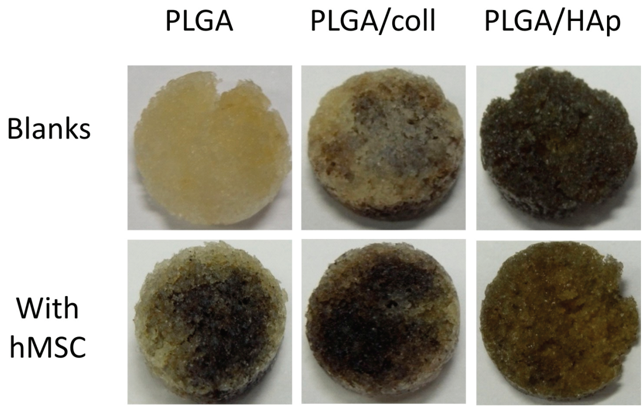

Fig. 4. Von Kossa test on different scaffolds after hMSC cultivation for 21 days

To visualise the presence of calcium ions after soaking with medium without cells (blank) and after hMSC cultivation for 21 days the scaffolds were stained with von Kossa method (Fig. 4). The results show that blank PLGA scaffolds were not stained, while collagen- and HAp-modified scaffolds became brown. Culturing hMSC on all the scaffolds resulted in staining of all types of the scaffolds, but the intensity of staining was the highest for HAp-modified scaffolds.

4. Discussion

Morphology of the pores is one of the most important factors, influencing cellular growth and tissue regeneration in the case of tissue engineering scaffolds. If the pores are too small, the cells cannot penetrate the scaffold, as well as nutrients and metabolites. Therefore, the pore size, shape and interconnectivity are vital in designing manufacturing procedure of the appropriate scaffolds. An ideal scaffold for tissue engineering should have such a porosity that would allow effective seeding of the cells, as well as their growth and migration into the material. It should also be permeable for the nutrients and metabolic products [10]. In this study, it was shown by the MTT analysis of scaffolds cross-sections that the cells were uniformly distributed within the whole material volume (Fig. 2). The cell morphology, as observed by SEM (Fig. 1) confirmed that the scaffolds promote cell adhesion, spreading and proliferation.

The scaffolds used in this study were composed of highly interconnected, oval (sometimes unsymmetrical) pores with tortuous structure. The obtained size of pores in the scaffolds was within the range of 250–320 μm. Porosity was controlled by the content and particle size of porogen. The results obtained in this study correspond to those of the previous experiments conducted in our group [11]. The SEM microphotographs revealed also the presence of surface modifications of the scaffolds – collagen type I and hydroxyapatite. Our findings were supported by the elemental EDX analysis, which showed the presence of nitrogen on collagen-modified scaffolds and calcium and phosphorus with atomic ratio Ca/P of 1.6 on SBF-soaked scaffolds (Fig. 1). It was shown in the literature that hydroxyapatite and collagen coatings enhance roughness of the pore walls, and improve attachment, differentiation and proliferation of the bone forming cells [10], [12], [13].

Biochemical tests of LDH show that the cells proliferated best in empty well (TCPS) (Fig. 3a). This is not surprising because tissue culture polystyrene (TCPS) surface is prepared by oxygen plasma modification to enhance adsorption of adhesive proteins from serum and thus support cell adhesion and proliferation [14]. On the contrary, the cells differentiated better on the scaffolds as compared to TCPS (Fig. 3b). Their osteogenic differentiation as measured by ALP, calcium concentration and von Kossa staining was significantly improved. Interestingly ALP activity showed the tendency to be the highest after 21 days of cultivation and to have a substantial drop after 28 days of cultivation. In the other study, a continuous increase of this biochemical osteogenic marker for up to 4–5 weeks followed by a sudden decrease in 3-D culture was observed [15].

The results of calcium concentration assay and von Kossa staining showed enhanced deposition of calcium after 21 days, which indicates the maturation stage of the osteogenic cells. The results are analogous to those of the previous studies reported by Kim et al. [16]. Van Kossa test revealed also the autocalcification of the collagen-coated scaffolds – the material attracts the calcium ions from the medium. High calcium concentration levels in hydroxyapatite-modified scaffolds resulted from the coating itself and were over 10 times higher than in other types of scaffolds and in empty wells. It can be stated that the scaffolds were successfully modified with calcium phosphate, which was additionally supported by SEM images of cauliflower-like structures on the scaffolds' surface and EDX analysis results. Similar results were obtained by other authors, who obtained higher mineralization rate by using biomimetic coatings [10].

5. Conclusions

In this study, highly porous resorbable polymeric scaffolds with the pore size of 250–320 μm and porosity of 85% were obtained; that microstructure supports the cell seeding and infiltration of whole volume of the material. The scaffolds were successfully modified with collagen type I and hydroxyapatite, i.e., main components of natural bone extracellular matrix. The modifications applied improved wettability of the scaffolds, thus enhancing cell infiltration efficiency as well as penetration of nutrients/wastes.

In vitro culturing of hMSCs on the scaffolds resulted in their lower proliferation as compared to TCPS wells. On the contrary, differentiation rate of hMSCs was activated on the scaffolds in comparison to culturing on TCPS. Moreover, enhanced mineralization was observed for the scaffolds enriched with hydroxyapatite and collagen. To sum up, the PLGA scaffolds, especially those modified with hydroxyapatite, are promising materials for osteogenic differentiation of hMSC and regeneration of bone tissue.

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