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Cultivation and differentiation of multipotent mesenchymal bone marrow cells on polyhydroxyalkanoate scaffolds

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***Abstract*—A study of mesenchymal stem cell viability and differentiation on the different scaffolds prepared from polyhydroxyalkanoates is presented.**

Keywords—electrospinnig, polyhydroxyalkanoates, PHAs, biocompatibility, mesenchymal stem cells, MSCs

I. Introduction

Electrospinning method attracts considerable attention now because variation of the process parameters allows producing fibers with different characteristics, such as mechanical properties, structure, and fiber orientation. Scaffolds with optimal characteristics can be used for the specific tasks of tissue engineering. Ultrafine fibers promote better cell adhesion because of the greater available surface area compared to the matrix in the form of films. For some polymers, such as polylactide, polycaprolactone, collagen, optimal parameters are known for a number of cells, including osteoblasts, fibroblasts, neurocytes [1-3]. Not many works are available regarding PHAs. Despite the great potential of application of ultrafine fibers, there is not enough knowledge about the influence of these parameters of PHAs ultrafine fibers on cell behavior. Previously we have investigated the influence of different ultrafine fiber properties as well as the PHA composition, orientation, and fiber diameter on NIH 3T3 fibroblast line [4]. However, mesenchymal stem cells and their differentiation on ultrafine fibers are more interesting. The aim of this study was to evaluate MSC adhesion, proliferation and differentiation to osteoblasts on different scaffolds prepared from polyhydroxyalkanoates.

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II. Materials and Methods

A. *Materials*

Well purified samples of the copolymers 3- and 4-hydroxybutyric acid (P3HB/4HB), the most promising members of PHAs, were used for construction of scaffolds. The polymer samples were synthesized in the Institute of Biophysics SB RAS using strain *Cupriavidus eutrophus* B-10646 by patented technology [5]. 2D scaffolds in the form of films were prepared from polymer/chloroform solution poured onto teflon mold. Nonwoven scaffolds formed by ultrafine fibers were prepared by electrospinning method with automatic device Nanon 01A (MECC Inc., Japan).

For 3D porous scaffolds were used two techniques: the technique of salt leaching and freeze-drying of polymer/chloroform solution.

B. *Biocompatibility and differentiation assay*

Mesenchymal stem cells were obtained from the femurs of Wistar rats euthanased by overdose of ether anesthesia. Bone marrow was isolated aseptically after removing neck bone; the open medullary canal was rinsed by syringe injection of 1 ml of α-MEM supplemented with 20% calf serum and 50 IU heparin, bone marrow was washed out carefully and suspended, then centrifuged and washed twice with nutrient medium. The cell suspension was transferred to a Petri dish and placed in a CO_2 incubator. After 24 hours, the medium was changed for removing of the non adherent cells to obtain a population of mesenchymal cells with a purity of 90%. Cells after 3-4 passages were used for further studies. For differentiation of MSCs to osteoblasts DMEM supplemented with 10% fetal calf serum, antibiotic solution, 0.15 mM ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate was used. Cultivation was carried out for 21 days with media replacement on every third day.

Morphology and distribution of cells on scaffolds were studied by electron microscopy using raster electron microscope HITACHI TM-3000 with microanalysis system BRUKER XFlash 430 H (Japan). Cells were pre-fixed with 4% formalin solution and gold plated (10 mA, 40 sec) using a vacuum coaters Emitech K575X (Quorum Technologies Ltd., UK), the images were taken at a voltage of 5 and 15 kV. Cell viability was assessed by the ability of cell mitochondria to

oxidize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT-bromide) to MTT-formazan. Optical density were measured at 540 nm with a microplate photometer 680 Bio-Rad (Bio-Rad LABORATORIES Inc.). The number of cells was estimated from the calibration curve. The following indicators were used to confirm the differentiation of MSCs to osteoblasts: activity of alkaline phosphatase (ALP), osteopontin production and extracellular calcium precipitates [6]. ALP activity of cultured cells was determined using the «Alkaline Phosphatase Detection Kit» (Sigma) according to the technical bulletin. Fluorescence intensity was measured with microplate multimodal reader Mithras LB 940 (BERTHOLD TECHNOLOGIES, Germany) at λ_{ex} 360 nm and λ_{em} 440 nm. Synthesis of osteopontin was detected by immunocytochemical staining using antibodies Anti-Osteopontin IgG labeled FITC, dilution 1/100 (Abcam, USA). Extracellular precipitates represented the salts of calcium and phosphate produced by cells and were analyzed with raster electron microscope HITACHI TM-3000 with microanalysis system BRUKER XFlash 430 H (Japan). The results of analysis were treated using software QUANTAX 70.

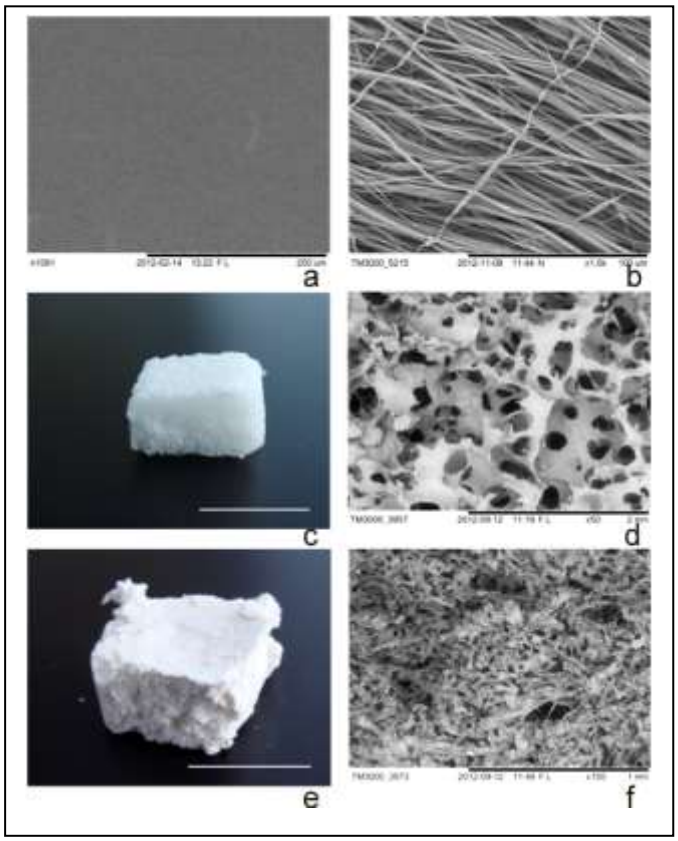

Figure 1 Scaffolds prepared from PHAs: a – films; b – ultrafine fibers; c, d – 3D leached porous scaffolds; e, f – 3D freeze-dried scaffolds (left side – macrophotography, marker 1 cm; right side – SEM images)

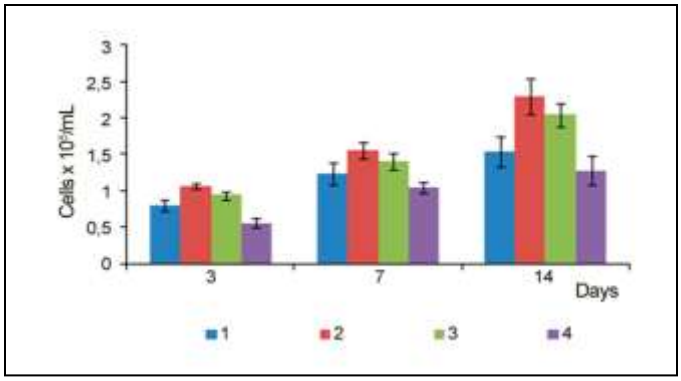

Figure 2 The number of viable cells PHAs scaffolds: 1 – films; 2 – ultrafine fibers; 3 – 3D leached porous scaffolds; 4 – 3D freeze-dried scaffolds

III. Results

The designed scaffolds are shown at Fig. 1. There are a smooth films (water contact angle 65°, roughness 113 nm), ultrafine fiber with diameter of 1.5 μm, 3D leached porous scaffolds (porosity 88%) with a pore size 100-120 μm and lyophilized scaffolds (porosity 82%) with a pore size of 40-60 μm.

A. Biocompatibility

All scaffolds were seeded with MSCs in the medium with osteogenic differentiation factors. According to the results MTT assay (Fig. 2) at 3^d, 7^{th} and 14^{th} days, the greatest number of cells at day 14 were recorded for ultrafine fibers and 3D leached porous scaffolds (2.0-2.3 × 10^5 cells/ml); significantly fewer cells were on 3D freeze-dried scaffolds and on the films and were about 1.3-1.6 × 10^5 cells/ml. It is known most suitable pore size for osteoblasts are 100–300 μm. These values correlated to this study: ultrafine fibers and 3D leached scaffolds have large pores, while 3D freeze-dried scaffolds have a smaller pore size (40-60 μm) that affected to adhesion and further development of cells and explained the decrease of cell number detected by the results of MTT assay.

B. Cell differentiation

The results of determination of ALP activity (a marker of osteoblast differentiation) are shown in Figure 3 and correlate with the results of the MTT assay. Greatest ALP activity was on ultrafine fibers and 3D leached porous scaffolds: 4.023 and 3.908 mmol/min × 10^5 cells and significantly lower on 3D freeze-dried scaffolds – 3.0 mmol/min×10^5 cells. Staining with anti-osteopontin antybodies at day 21 of culture confirmed the synthesis of the osteopontin in all the investigated scaffolds at day 21 of experiment (Fig. 4).

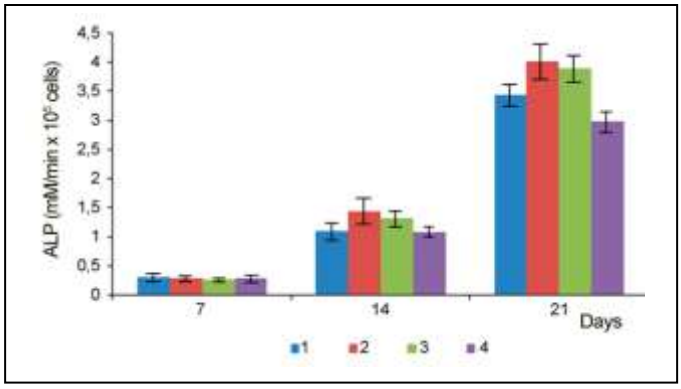

Figure 3 ALP activity of osteoblasts on PHA scaffolds: 1 –films; 2 – ultrafine fibers; 3 – 3D leached porous scaffolds; 4 – 3D freeze-dried scaffolds

Electron microscopy showed that the cells had round shape on the films. Cells located along the electrospun ultrafine fibers had prolate form. There were visually less cells on 3D scaffolds likely due to penetration of cells through pores into the scaffolds. In appropriate conditions adhesion and proliferation of osteogenic cells is accompanied by the formation of bone tissue. Osteoblasts synthesize and excrete proteins out forming organic extracellular matrix of bone that is mineralized by calcium salts. Electron microscopy images showed that the cells on the surface of the scaffolds produced extracellular precipitates salts (Fig. 5). It was noticed that the most active process of salt precipitation was on ultrafine fibers and 3D leached scaffolds with large pores; very low content of mineral crystals characteristic for 3D freeze-dried scaffolds as the total number of cells on this type of scaffolds.

The results of quantitative assay of the mineral salts produced by osteoblasts shown at Fig. 6 and in the Table I.

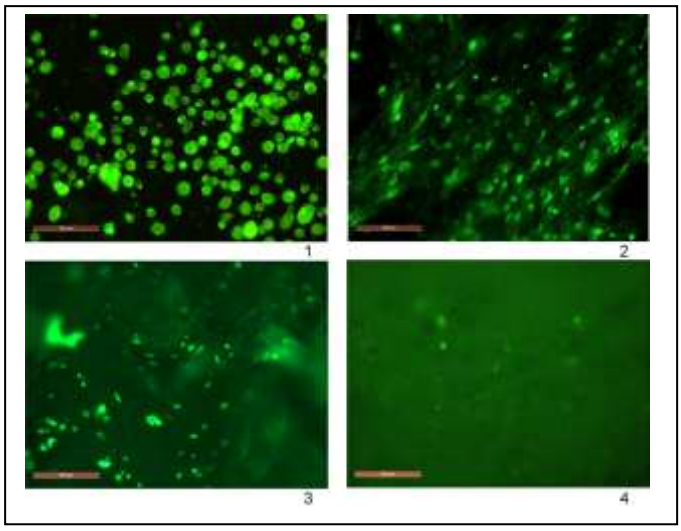

Figure 4 Osteoblasts on different types of PHA scaffolds. Staining with Anti-Osteopontin-antibodies. Marker 100 μm

Spectral analysis showed the presence of calcium-phosphate deposits on cells on all types of PHA scaffolds. The highest values of Ca and P were registered on ultrafine fibers and 3D leached scaffolds and were in 2.0-2.5 times higher than that on the films; and the lowest values was on 3D freeze-dried scaffolds.

Ca/P ratio also was different and was 1.88 on 3D freeze-dried scaffolds, 2.46 on ultrafine fibers, 2.78 on films and 3.12 on 3D leached scaffolds.

So, it is shown that all types of scaffolds made of copolymer of 3- and 4-hydroxybutyric acids, are suitable for MSC cultivations and provide their differentiation into osteoblastic cell. Most suitable scaffolds for osteoblast differentiation are defined ultrafine fibers and 3D leached scaffolds.

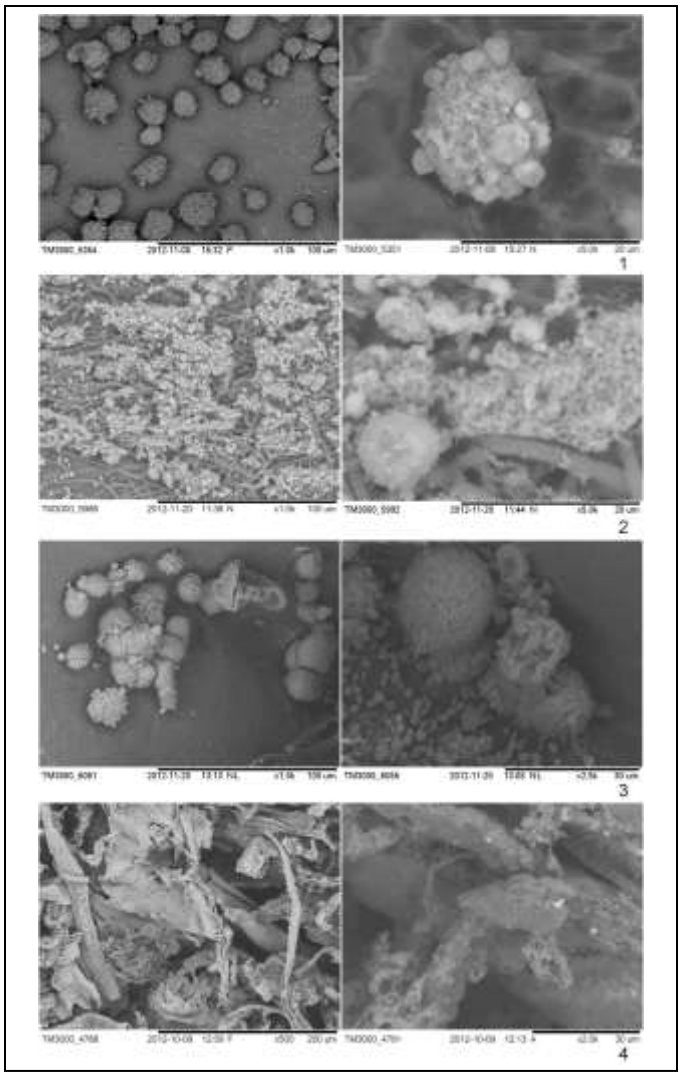

Figure 5 Electron microscopy images of osteoblast cultivated on different types of PHA scaffolds: 1 –films; 2 – ultrafine fibers; 3 – 3D leached porous scaffolds; 4 – 3D freeze-dried scaffolds

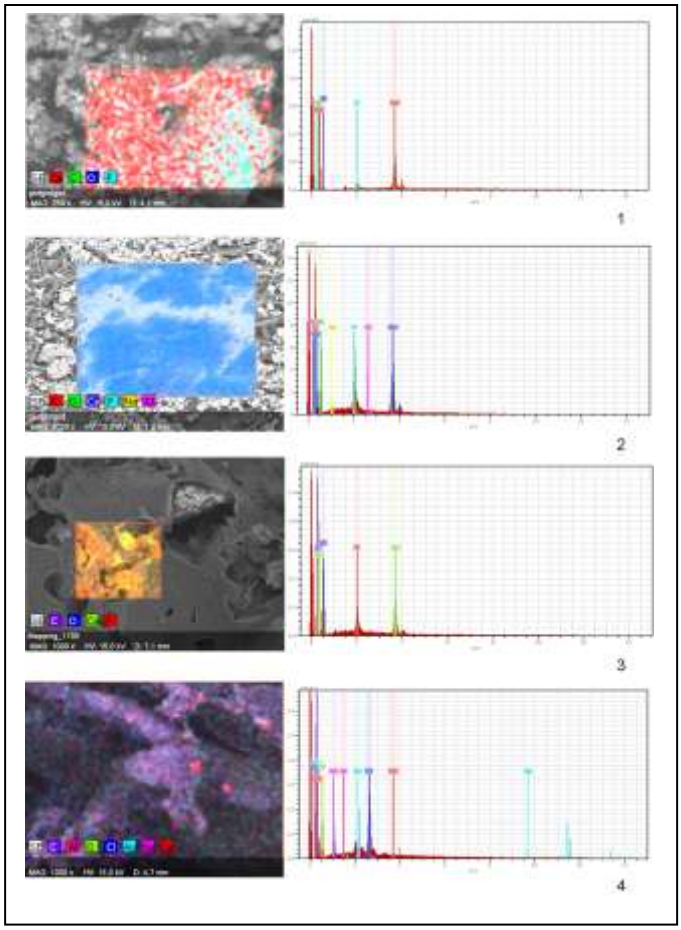

Figure 6 The elemental composition of mineral precipitates produced by osteoblast on different types of scaggolds: 1 –films; 2 – ultrafine fibers; 3 – 3D leached porous scaffolds; 4 – 3D freeze-dried scaffolds

TABLE I. SPECTRAL ANALYSIS OF MINERAL PRECIPITATES PRODUCED BY OSTEOBLASTS OBTAINED FROM MSCS OF BONE MARROW ON PHAS SCAFFOLDS

Type of scaffolds	Value of element, % by weight			
	C	***O***	***Ca***	***P***
Films	50,5	37,4	8,9	3,2
Ultrafine fibers	34,1	37,4	20,2	8,2
3D leached porous scaffolds	50	36,46	10,3	3,3
3D freeze-dried scaffolds	55,7	39,4	3,2	1,7

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