The experimental model of mesenchymal stem cells growth and differentiation based on application of porous scaffold from bacterial origin poly(3-hydroxybutyrate)-poly(ethylene glycol)

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Abstract. Nowadays, the biotechnologically produced polymers, e.g. poly(3-hydroxyalkanoates) (PHAs), are widely used for biomedical applications (e.g. in tissue engineering), because of biodegradation and high biocompatibility. PHAs are used for development of various medical devices. These biopolymers can be also used for development of experimental models, e.g. 3D cell culture model.

To develop 3D cell culture model, the porous scaffolds based on novel biopolymer poly(3-hydroxybutyrate)-poly(ethylene glycol) (PHB-PEG) were produced. This biopolymer was synthesized by original method with use of an effective producer of PHAs, *Azotobacter chroococcum* 7B. The porous scaffolds were made by gas porous formation technique using ammonium carbonate as porogen. Human mesenchymal stem cells (MSCh) were used as cell culture, which tends to maintain 3D growth in experimental conditions. MSCh attachment, growth and proliferation were estimated with a standard method of viability cells and biocompatibility of polymeric materials, XTT (Biological Industries, Israel). Confocal microscopy was also used to examine cells 3D growth. The obtained data demonstrated that MSCh penetrated in produced PHB-PEG porous scaffolds and maintained in the pores 3D growth. Thus, the developed biopolymer scaffold can be used for application in experimental biology as 3D cell culture model as well as in tissue engineering as medical device.

1 Introduction

Nowadays, polyhydroxyalkanoates (PHAs) have attracted a great deal of interest as a promising material in regenerative medicine. PHAs are a family of biopolymers synthesized by different bacteria as an intracellular carbon and energy storage materials [1-3]. These products possess some unique properties including high biocompatibility and degradation nontoxic components. The biocompatibility, biodegradation and applications of PHAs in tissue engineering were studied by many research groups [4-

9, 27]. Particular attention has been focused on the use of poly(3-hydroxybutyrate) (PHB), mechanical, chemical and biological properties of which have been repeatedly as well as development of various PHB-based medical devices described in the literature [10-13, 26].

Many efforts have been made to change the physics-chemical properties of PHB by copolymerizing and blending PHB with other polymers [14-16, 25]. The most important copolymers of PHB are: the copolymer with hydroxyvaleric acid, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) [17-18]; with poly(ethylene glycol), poly(3-hydroxybutyrate)-poly(ethylene glycol)(PHB-PEG) [19]; and with the both, the terpolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-poly(ethylene glycol) (PHB-HV-PEG), which was lately described [20].

We used copolymer PHB-PEG produced by Azotobacter chroococcum 7B, one of the most efficient PHB producers. Compared to PHB, PHB-PEG displays advantages due to their water related properties, protein and significant improvement in biocompatibility [19]. That is why this copolymer can be used for development medical devices, such as drug delivery systems and drug sustained release system [21], flat and porous scaffolds.

In this study, we attempted to construct 3D cell culture model using PHB-PEG as a porous matrix and mesenchymal stem cells (MSCs) as a widly used cell culture to investigate tissue engineering devices.

2 Materials and methods.

2.1 Materials.

Poly(ethylene glycol) 300 g/mol (PEG 300), sodium salt, components of growth media: $K_2HPO_4 \times 3H_2O$, $MgSO_4 \times 7H_2O$, NaCl, $Na_2MoO_4 \times 2H_2O$, $CaCO_3$, $FeSO_4 \times 7H_2O$, sodium citrate, $CaCl_2$, KH_2PO_4, sucrose, agar, phosphate-buffer saline (PBS). (Sigma-Aldrich), $(NH_4)_2CO_3$ (Chimmed, Russia) were used.

2.2 Growth conditions

A PHA producer A. chroococcum strain 7B ,was used [26-29] and maintained on Ashby's medium, containing 0.2 g/l $K_2HPO_4 \times 3H_2O$, 0.2 g/l $MgSO_4 \times 7H_2O$, 0.2 g/l NaCl, 0.006 g/l $Na_2MoO_4 \times 2H_2O$, 5.0 g/l $CaCO_3$, 20 g/l sucrose, and 20 g/l agar. PEG 300 was added initially in the medium at a concentration of 150 mM. The polymer isolation and purification from A. chroococcum for biocompatibility study comprised the following stages: (1) polymer extraction with; (2) separation of polymer solutions from cell debris by filtration; (3) polymer precipitation from chloroform solution with isopropanol; (4) subsequent repeated cycles of dissolution in chloroform and precipitation with isopropanol, and (5) drying at 60°C. Molecular weight (Mw) of PHB-PEG were determined by viscosimetry: the viscosity of the PHB solution in chloroform was measured at 30°C on an RT RheoTec viscometer (RheoTec, Germany); the molecular mass was calculated using the Mark-Kuhn-Houwink equation according to.

2.3 Fabrication of PHB-PEG scaffolds and films

The PHB-PEG porous structures were prepared by porous formation method with gas using ammonium carbonate as porogen. The ammonium carbonate powder was mixed with 3% (w/v) PHB-PEG solution in chloroform. The mixed solution was poured in Petry dish and after the chloroform evaporation the polymer matrix was placed in hot water to decompose ammonium carbonate and to create pores. The obtained scaffolds were washed 5 times in distilled water and dried about 24 hours in 37°C. The morphology of PHB-PEG substrate was observed by SEM (JSM-6380LA; JEOL, Japan) at an accelerating voltage of 15 kV. Before the observation, the samples were coated with gold using a sputter coater. The dimension of the ES substrates was measured from the SEM photographs (n =10). All substrates were sterilized by autoclaving at 120°C for 20 min

To produce polymer films the copolymer was first dissolved at a concentration of 5 wt % in chloroform. The resulting solution was then cast onto the glass coverslip. After solvent evaporation, the polymer-coated coverslip (hereafter referred to as polymer film) was placed in a vacuum dryer overnight to remove any remaining solvent.

2.4 Cell culture

Mesenchymal stem cells (MSCs) (Biolot, Russia) were used for biocompatibility testing of polymer as the main cell culture applied in regenerative medicine. The cells were cultivated in MEM (Biolot, Russia) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin solutions (Biolot, Russia). Cells were incubated at 37ºC in a humidified 5% CO2 atmosphere and the medium was changed every three days. Cells were released before confluence with trypsin-versen solution (0.05% (v/w) trypsin and 0. 02% (v/w) EDTA in PBS) (PanEco,Russia) and counted with a hemocytometer(MiniMed, Russia).

2.5 Cell viability test

Cell proliferation and viability were measured by the cell proliferation test based on the cleavage of the tetrazolium salt to soluble formazan salt by mitochondrial activity of viable cells (XTT Cell Proliferation Kit, Biological Industries, Israel). The specimens with a dimension of 5x5 mm were introduced in 96-well plate and incubated with growth media during 24 hours for protein absorption for better cell attachment. Then the cell suspension was added on the top of every sample in a mount 3000 cells per well. At the end of the experimental time (4 and 7 days), polymer structures with attached cells were gently and quickly transferred from wells of incubated tissue culture plate to respective wells of new plate with preliminarily added 100 µl fresh medium. Then 50 µl XTT reagent solution was added to the cell monolayers on polymer films in each well, and the multi-well plates were incubated at 37ºC for a 4 h . Polymer films were removed and samples were quantified spectrophotometrically at 450 nm with reference wavelength at 640 nm. Viable cell numbers on matrix were then determined from the standard curve based on their XTT

absorbance. The data were obtained as optical density (OD). The data on polymer scaffolds and films were calculated as percentage of control cell growth on 2D culture plate.

2.6 Confocal microscopy

Also the cell growth on PHB-PEG matrix was examined using confocal microscopy.

3D virtual model of MSC staining with Calcein AM distribution in the polymer scaffold was constructed using a LSM710 laser scanning confocal microscope (Carl Zeiss, Germany), and the Zeiss LSM Image Browser 4.2.0 software.

3 Results and discussion

In this study 3D cells growth model consisted of biodegradable polymer PHB-PEG and the MSCs was developed. The porous scaffold from PHB-PEG was obtained by porous formation method by gas with 50x50 mm and 3-4 mm thickness (Fig 1a). Pore size was estimated on SEM assay and average size was 90-110 μm (Fig. 1b). It was shown [28] that MSC cells prefer a surface with pore size about 100-150 μm to 250-300 μm, that is why the suitable morphology of scaffolds is a very important feature for cells growth. Fig.1c shows the polymer surface with high magnification: the inner surface of scaffold pores had increased roughness that can also improve matrix adhesive properties for cells.

Cell cytotoxicity testing was used to estimate the cells attachment to 3D-matrix from PHB-PEG and MSCs growth on the scaffold. The 3D-matrix and the film made from the same polymer were compared. As indicated in Figure 3 it was a distinct difference between the cell attachment on the PHB-PEG three dimensional scaffold and on the flat film.

On the 4th day of incubation, there was observed the similar indicators in the amount of attached cells in both cases. When compared the same results on the 7th day of incubation, the fall of the number of cells in the thin film and a clear increase of number of cells in the case of the test matrix were observed. This is an indication that the surface area of the porous matrix is greater and the cells continue to divide and grow, while on the flat film these processes are slowed down or stopped.

Obtained data from confocal microscopy supports this assumption. Figure 3 demonstrates a 3D virtual model of live cells (staining with Calcein AM) distribution in the porous polymer scaffold. The MSCs demonstrated a relatively uniform distribution in the volume of PHB-PEG scaffold.

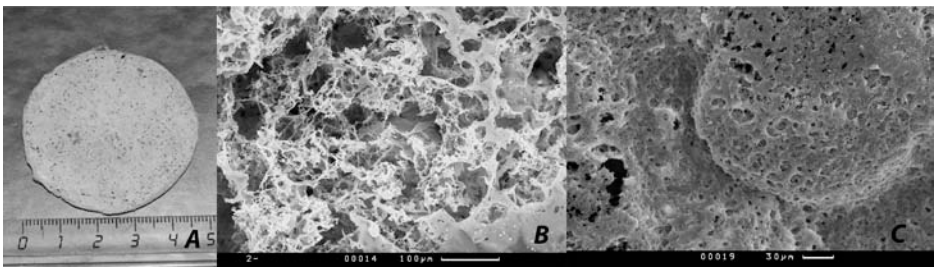

Fig. 1. The PGB-PEG Porous scaffold: the appearance (a) and scanning electron microscopy (SEM) micrographs(b, c).

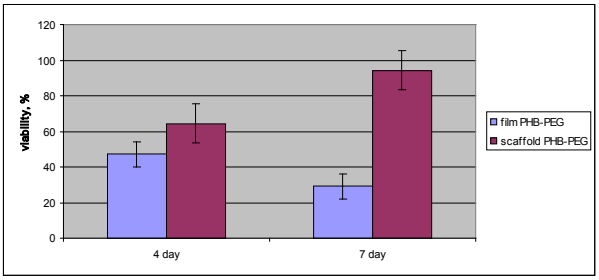

Fig. 2. Adhesion and cell proliferation on tested biopolymer scaffolds evaluated by XTT test

4 Conclusions.

Thus, the 3D cell growth model based on poly(3-hydroxybutyrate)-poly(ethylene glycol) was developed. It was shown that the polymeric matrix has suitable characteristics such as pore size for cells attachment, growth and proliferation. It was confirmed by viability test and scanning electronic and confocal microscopy. Moreover, the obtained data can be used for development of experimental models in cell biology as well as medical devices in tissue engineering.

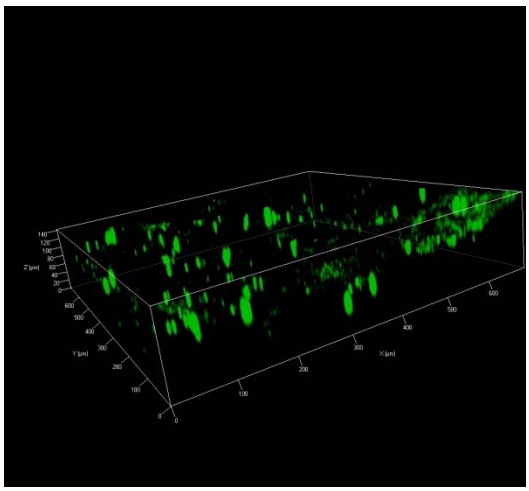

Fig. 3. MSC cell culture spreading in the porous flat scaffold (confocal microscopy, Calcein AM dye).

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