Bacterial Cellulose-polyhydroxyalkanoates Composites

Synthesis, physico-chemical characterization and biological evaluation for tissue engineering

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The paper focuses on the development of composite materials based on bacterial cellulose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV). Natural composites were obtained from bacterial cellulose membranes and chloroform copolyester solutions (various mass ratios). The composite membranes were characterized by FT-IR spectroscopy, X-ray diffraction, SEM morphology, contact angle measurements and thermogravimetric analysis. Cellulose nanofibrils coating and/or precipitation with PHBHV particles was observed. The biocompatibility of composite materials was assessed by using L929 mouse fibroblast cells monolayers. Possible applications of these composite materials are focused on blood vessel engineering and/or wound dressing management.

Keywords: bacterial cellulose, polyhydroxyalkanoates, composites, tissue engineering

Recent developments in tissue engineering approaches involve the use of three-dimensional scaffolds to function as the template for cellular activities to repair, rebuild and regenerate damaged or lost tissues. It is generally agreed that a biomaterial to be used in tissue engineering needs to possess certain material characteristics such as biocompatibility, suitable surface chemistry, desired mechanical properties and biodegradability [1].

Composite materials based on biodegradable natural macromolecules are very important constructs for tissue engineering applications. Research efforts are being made in developing fully biodegradable composite biomaterials [1].

In this respect cellulose and polyhydroxyalkanoates (PHAs) based composites could be certainly important in this field due to their intrinsic biodegradability and biocompatibility.

Cellulose and its derivatives are the most abundant natural polysaccharides on earth, with an annual production of over 100 billion tons for cellulose. Cellulose is biodegradable in nature by microbial or fungal enzymes that can break down the glucosidic linkages, but in humans, biodegradation is relatively limited or absent due to the lack of these enzymes.

The two main sources of cellulose are trees and cotton. In addition to these sources, microorganisms can synthesize cellulose. *Acetobacter xylinum* is the only species known to be capable of producing cellulose in commercial quantities [2-4].

Cellulose has traditionally been sourced from plants. However, refining of plant cellulose typically involves harsh, aggressive processing to remove non-cellulose materials such as lignin and hemi-cellulose. Fortunately, an alternative source of cellulose where no chemical or mechanical refining is necessary is available. Bacterial cellulose (BC) has been developed as an alternative to plant cellulose. Due to its high water-holding capacity, high crystallinity, high tensile strength and fine web-like network structure, which means that it can be formed into any size or shape, BC could be used as a promising biomaterial [5-9].

Bacterial cellulose (BC) belongs to the products of primary metabolism and it has a protective role whereas plant cellulose plays a structural role. In terms of chemical structure, bacterial cellulose is identical to that produced by plants. However, bacterial cellulose membranes possess excellent mechanical strength and high surface area when compared to plant derived cellulose due to the highly crystalline structure and reduced fibre diameter. These properties make it an interesting biomaterial for applications as nutritional component, artificial skin, composite reinforcement, nerve regeneration etc. [4-9].

PHAs are a class of natural thermoplastic polymers. Due to their properties, similar to those of conventional plastics and to their biodegradability, they have attracted much interest as alternatives to synthetic polymers, the more so as they can be produced from renewable resources and processed with the aid of equipment used for polyolefins or other synthetic materials. As these biopolymers are biodegradable and biocompatible, they are suitable for many biomedical applications such as surgical sutures, long term carriers of drugs and tissue engineering [10-14]. Among the PHA biopolymers family, poly-3-hydroxybutyrate (PHB) is produced by *Ralstonia eutropha*. PHB is a biocompatible, biodegradable and thermoplastic polymer, with the thermoplastic-like properties offering the possibility for potential replacement of non-degradable polymers currently used such as polyethylene and polypropylene [15]. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) is a bacterially derived co-polymer which is produced by fermentation [16].

Belonging to the family of poly(hydroxyalkanoates), this natural polymer has been the focus of several studies investigating its thermal, physical and mechanical properties and its use in scaffolds for tissue engineering.

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The development of composites from PHAs and bacterial cellulose is an interesting approach of biomaterials taking benefits of PHAs and pure cellulose properties. While plants mostly produce cellulose as a complex with lignin, hemicelluloses, pectin, biogenetic products, this polymer is produced in a pure form by some kinds of microbial cells such as: *Acetobacter, Agrobacteria, Rhibozia and Sarcina* strains. The diameter of BC fibrils is one thousand of plant cellulose fibrils; thus, BC has a large specific surface area, higher water retention value and high tensile strength compared with plant cellulose.

This paper discusses the synthesis, physico-chemical characterization and biological evaluation of natural composite materials based on bacterial cellulose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) as biodegradable scaffolds for tissue engineering applications.

Experimental part

Materials and Methods

Bacterial cellulose membranes were kindly provided by National Institute for Chemical Pharmaceutical Research and Development (ICCF Bucharest, Romania). The microorganism used in all the experiments for obtaining BC was *Acetobacter xylinium DSMZ* (ICCF 398).

Hydrated (99% water) BC membranes (5 mm thick) were carefully dried at 40°C for 48 h and used for composites preparation. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate 2%) (PHBHV) copolymer was dissolved in boiling chloroform in closed round bottom flask and under stirring for 24 h (5 wt. %).

Dried bacterial cellulose membranes were immersed in copolyester solutions for 24 h and the composite materials obtained were left to dry at room temperature for 12 h followed by vacuum drying. Composite membranes were prepared with the following compositions (BC/PHBHV gravimetric ratio): 1/1, 1/2, 1/5. For comparison PHBHV films were prepared by casting from chloroform solutions on Petri glass plates.

FT-IR spectra were taken on a Jasco 4200 spectrometer equipped with a Specac Golden Gate attenuated total reflectance (ATR) accessory, using a resolution of 4 cm^{-1} and an accumulation of 60 spectra, in the 4000–600 cm^{-1} wavenumber region. XRD patterns were obtained using a RIGAKU miniflex II diffractometer with CuKα radiation. Morphological information including internal structure was obtained through the scanning electron microscopy analysis of the gold-coated specimens. The analysis has been performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG) with a resolution of 1.2 nm and with an X-ray energy dispersive spectrometer (EDS)

Thermogravimetric measurements of each sample were performed at 10°C/min, in nitrogen atmosphere, from ambient temperature up to 600°C, using TGA Q500 equipment (TA Instruments). The samples weight was 2.2 ± 0.1 mg.

KSV CAM 200 apparatus was used for static contact angle measurements performed on dried films. Ultrapure water droplets were used with a drop volume of 20 μL. The measurement of each contact angle was made within 10 s after each drop to ensure that the droplet did not soak into the compact. The contact angles reported were the mean of 10 determinations. Surface free energies were also calculated for polyester films, cellulose membranes and composite materials.

For biological tests macrophage cell line was employed. The macrophage is considered to be an important cell in the initial non-specific host response against biomaterials [24-25]. Macrophages are responsible for the elimination of foreign bodies in the organism.

The biocompatibility of composite materials was assessed by using L929 mouse fibroblast cells monolayers grown on polymer films covering approximately 90% of the well surfaces in 24-well cell culture plates. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) at 37°C in a humidified incubator with 5% CO_2. After 24 h incubation, polymer films were removed and the number of cells grown both on their surface and adjacent well bottom were measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based cell viability test. This assay is based on the ability of dehydrogenase enzymes from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form insoluble dark blue formazan crystals. Cell lysis and crystals solubilization was performed and the absorbance at 540 nm is directly proportional to the number of viable cells. Finally, the cells were microscopically examined for detecting cytotoxicity visible signs, cellular lysis or cellular components dimensions and conformation (optical microscopy, ZEISS - Axiovert 135 Microscope). A cellular viability test (MTT) was also employed and the number of cells grown on polymer films was calculated relative to an equivalent area of cells culture plastic.

Results and discussions

Macroscopically relatively homogenous membranes were obtained for all BC/PHBHV composites. Pure BC and composite membranes appear transparent milky and white/brownish respectively (fig.1).

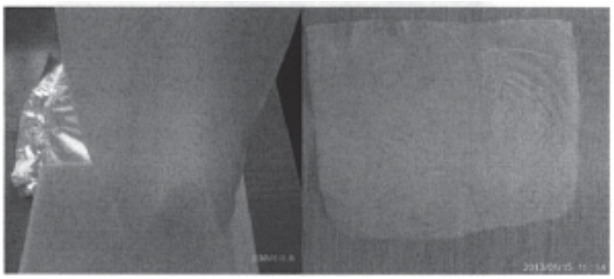

Fig.1. Swollen and dried bacterial cellulose membranes obtained from Acetobacter xylinium DSMZ

FTIR-ATR analysis was performed on BC membranes, PHBHV films and newly obtained composite materials. The BC spectrum revealed the presence of characteristic peaks for cellulose at 3338 cm^{-1} (OH), 2970 and 2895 cm^{-1} (CH_2), 1371 cm^{-1} (CH), 1158 cm^{-1} (C-O-C), 1105 cm^{-1} (C-C), 1052 cm^{-1} (C-O), figure 2. In the spectrum of composite an intense peak at 1725 cm^{-1} appears and it corresponds to specific vibration of C=O from polyester (PHBHV, figure 3).

XRD analysis was performed on cellulose membranes, PHBHV films and BC/PHBVcomposite membranes. The high degree of crystallinity of cellulose is evidenced by 3 diffraction peaks for bacterial cellulose at 2θ diffraction angles of 14.5, 17 and 22.76° (fig. 4).

Characteristic diffraction peaks for PHBHV copolymers are shown at 2θ diffraction angles of 13.54, 16.97, 21.62, 22.56° respectiv 25.59. Diffraction patterns obtained for the composite membranes show mainly the cellulose peaks at 1/1 ratio between BC/PHBHV. When the ratio is in favour of polyester the peaks characteristic for PHBHV could be seen on the diffractograms. The analysis of crystallite dimensions based on Scherrer equation shows a modification of dimension for composites as compared

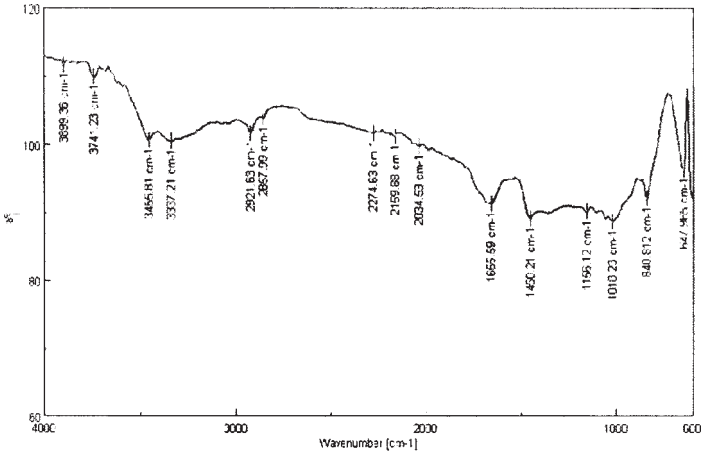

Fig.2. FTIR spectrum for pure bacterial cellulose membrane

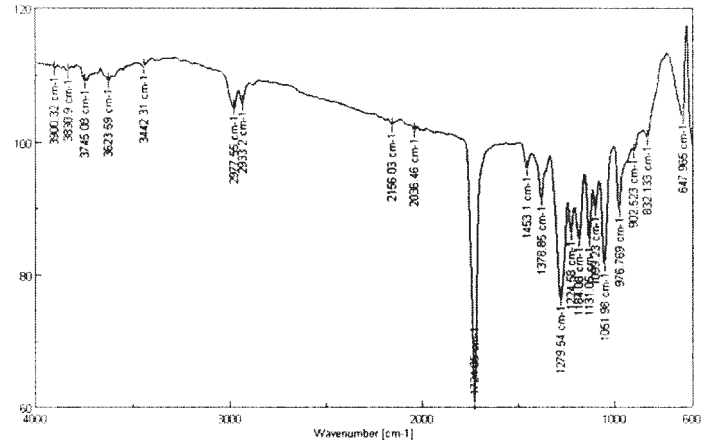

Fig.3. FTIR spectrum for BC/PHBHV composite

to unmodified bacterial cellulose membrane. Crystallite dimension of 2.68 nm characteristic for cellulose membranes increases for composites due to te presence of PHBHV copolymer (8.86 nm for pure polyester). 1/1 BC/PHBHV has a crystallite dimension of 3.23 nm, 1/2 BC/PHBHV 3.45 nm (slight increase with the polyester concentration) reaching for 1/5 ratio a dimension of 5.04 nm. In this way it is clear that crystallinity of bacterial cellulose is modified by the presence of copolyester.

Figures 5-9 show representative SEM images of a polyester PHBHV film obtained by casting from the chloroform solution, a pure BC membrane, and the 1/1, 1/2 and 1/5 mass ratio BC/PHBHV composite.

Figure 5 shows the surface image of the pure polyester film. Microparticles are observed on a compact, smooth and uniform structure. Images obtained for pure BC membrane (fig. 6) reveals the ultrafine network structure composed of a random assembly of cellulose nanofibrils less than 100 nm wide. Figure 7-9 shows images obtained for of 1/1, 1/2 and 1/5 BC/PHBHV composite ratio. PHBHV particles are somehow embedded within the cellulose nanostructure or they cover the surface of the cellulose nanofibrils.

Figure 10 shows TGA results with DTG curves also (fig. 11).

The curve obtained for the pure BC membrane shows two significant events of weight loss. The first gradual one involving aproximatively 5% mass loss occurs from room temperature up to 220 °C and can be associated with loss of surface residual water; the second one, observed around 320°C, could be attributed to BC pyrolysis. A residue of 20% of carbonaceous materials is noticed. TGA curve for PHBHV copolymer powder shows only one weight loss starting at around 270 °C up to 300 °C due the thermal decomposition of the material. Almost no residue is observed. For polyester film another mass loss is initially observed up to 200°C due to residual solvent from the casting technique for film

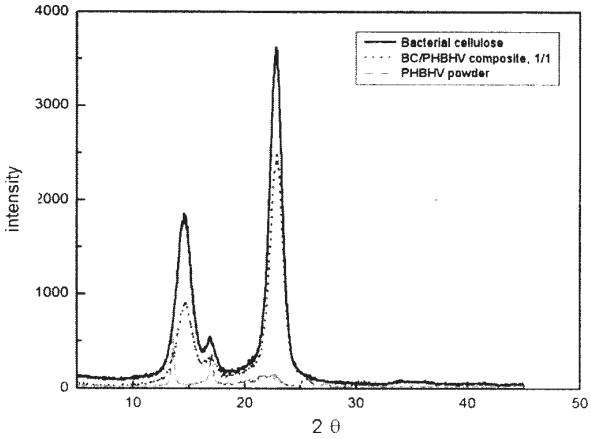

Fig.4. XRD diffractograms for BC membrane, PHBHB powder and BC/PHBHV 1/1 composite

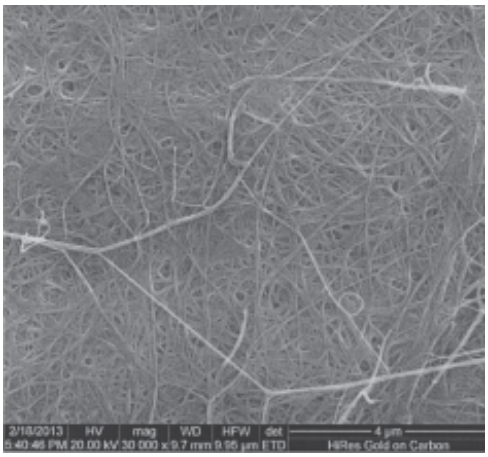

Fig.5. SEM microphotograph showing the structure of pure bacterial cellulose

obtaining. TGA curves obtained for BC/PHBHV composites have a similar behaviour observed for the pure composite components. A continuous mass loss of around 5% is observed from room temperature up to around 250 °C. After

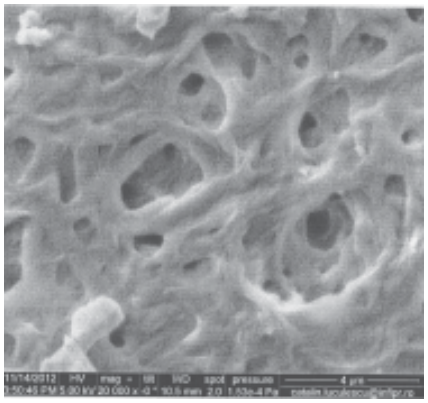

Fig.6. SEM microphotograph showing the pure PHBHV copolyester film

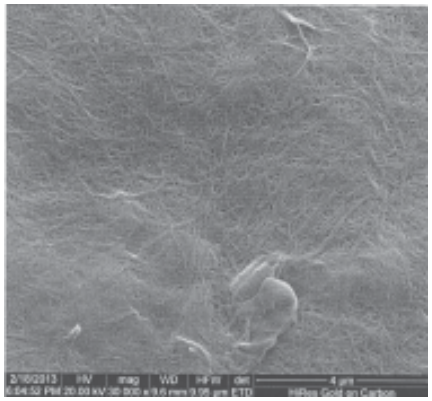

Fig.7. SEM microphotograph showing the structure of 1/1 BC/PHBHV composite

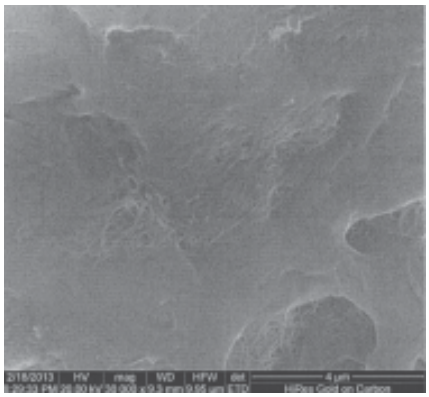

Fig.8. SEM microphotograph showing the structure of 1/2 BC/PHBHV composite

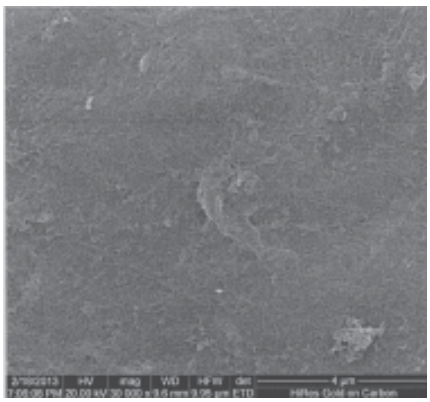

Fig.9. SEM microphotograph showing the structure of 1/5 BC/PHBHV composite

that a second important event is observed starting at around 260 up to 380 °C which is a temperature higher than the temperature observed for pure polyester. The residue mass was observed to depend to a low extent on the PHBHV

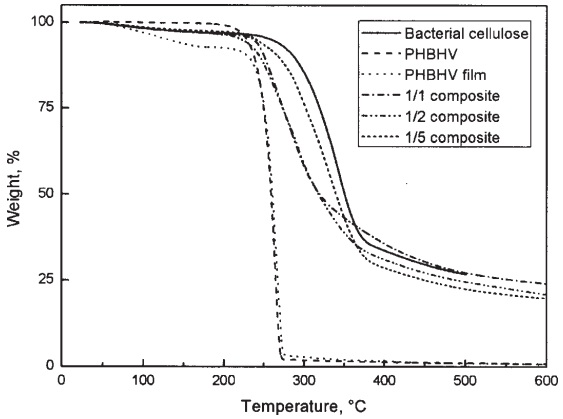

Fig. 10. TGA results for bacterial cellulose membrane, PHBHV powder and film, 1/1, 1/2 and 1/5 BC/PHBHV composites

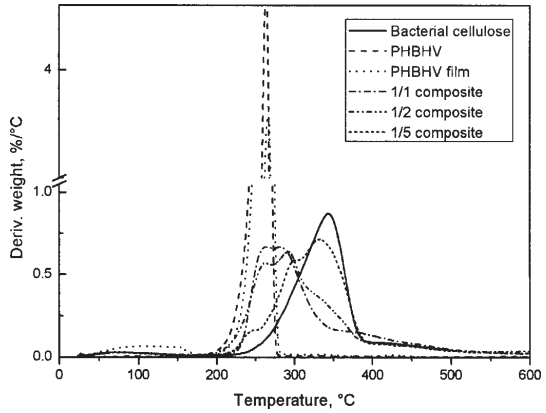

Fig. 11. DTG curves for bacterial cellulose membrane, PHBHV powder and film, 1/1, 1/2 and 1/5 BC.PHBHV composites

relative content. 30% mass residue is observed for most of the composite samples with various BC/PHBHV ratios.

Contact angle measurements showed a value of BC membrane contact angle of 26° in good agreement with the hydrophilic nature of the material. On the other hand the value of the copolyester contact angle is approximatively 91° corresponding to the hydrophobic nature of PHBHV film. The composites obtained by the immersion of the BC membranes in polyester solutions in chloroform should have a more hydrophobic surface. This fact is proved by the value of the contact angle (75°, 85°). The computation of the free surface energies relies on Young-Dupré equation (1-2) and Fawkes equation (3) and take into account the values for the contact angles and the superficial tensions of two solvents: water and ethylene glycol.

$$W_A = \gamma_L(1+\cos\theta) \quad (1)$$

$$W_A = \gamma_{S^0} + \gamma_{L^0} - \gamma_{SL} \quad (2)$$

$$\gamma_{SL} = \gamma_S + \gamma_L - 2\left(\sqrt{\gamma_S^D \gamma_L^D} + \sqrt{\gamma_S^P \gamma_L^P}\right) \quad (3)$$

The values obtained for these materials (1/1and 1/5 ratios between BC/PHBHV) are in good agreement with the above presented data and the surface nature of the new composite material (table 1).

Biocompatibility test regarding the cytotoxicity of the BC/PHBHV composite membranes was assessed on L929 murine cell line. Figure 12 shows the adherence of the cells onto the composite membranes with 1/1 BC/PHBHV ratio and control sample (polystyrene - cells culture plastic) and the image of cells growth on a blank test, respectively on composite membranes are presented in figure 13.

Biological investigations showed that most of the cells retained their typical morphology with extensions proving

Material	Solvent		Free surface energy based on Young-Dupre and Fawkes* equations, mN/m
	Water	Ethylene glycol	
	Contact angle, °	Contact angle, °	
PHBHV 2% film	91	71	22
Pure bacterial cellulose membrane	26	24	71
Composite BC/PHBHV, 1/1	75	58	56
Composite BC/PHBHV, 1/5	85	65	46

Table 1
CONTACT ANGLE MEASUREMENTS AND FREE SURFACE ENERGIES FOR PHBHV, BC AND COMPOSITE MATERIALS

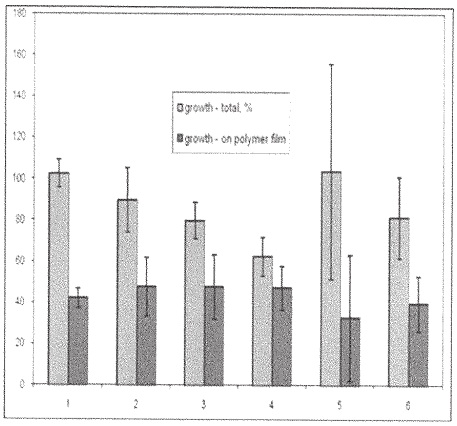

Fig. 12. Cells growth on BC, PHBHV and composite materials membranes: 1 – initial swollen BC; 2 – purified BC membrane; 3- PHBHV film; 4 - BC/PHBHV composite membrane 1/1; 5 – BC/PHBHV composite membrane 1/2; 6 – BC/PHBHV composite membrane 1/5

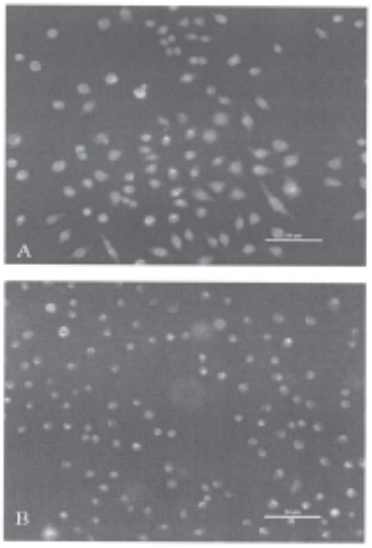

Fig. 13. Optical microphotographs OM cells cultured grown on: a – blank test; b - BC/PHBHV 1/1 composites

that the composite materials did not exhibit cytotoxic effect.

Conclusions

Naturally derived composites based on bacterial cellulose and poly(3-hydroxybutyrate-co-3-hydroxy-valerate2%) were prepared by immersion of cellulose membranes in chloroform copolyester solutions (various BC/PHBHV gravimetric ratios). Development of biodegradable BC/PHAs composites for tissue engineering (blood vessel engineering and/or wound dressing management) is a challenging concept to develop. Such a composite material will ensure the required mechanical integrity of the scaffold and controllable biodegradability.

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