NEW COMPOSITES BASED ON BACTERIAL CELLULOSE AND PHAS FOR TISSUE ENGINEERING APPLICATIONS

P.O. Stanescu¹, C. Zaharia¹, V. Fratila¹, B. Galateanu², E. Vasile¹
¹ University Politehnica of Bucharest, Advanced Polymer Materials Groups, Bucharest, Romania, ² University of Bucharest, Biochemistry and Molecular Biology, Bucharest, Romania
* zaharia.catalin@gmail.com

Keywords: bacterial cellulose, polyesters, composites, tissue engineering

Introduction

Composite materials based on biodegradable natural biopolymers are very important constructs for tissue engineering applications. Research efforts are being harnessed in developing fully biodegradable composite biomaterials. In this respect cellulose and polyhydroxyalkanoates (PHAs) based composites could be certainly important in this field due to their intrinsic biodegradability and biocompatibility. Cellulose is the most abundant biopolymer on earth, with an annual production of over 100 billion tons. 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 [1-3].

Cellulose has traditionally been sourced from plants. However, refining of plant cellulose typically involves harsh, aggressive processing to remove noncellulose 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 [4-8].

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 fiber diameter. These properties make it an interesting biomaterial for applications as nutritional component, artificial skin, composite reinforcement, nerve regeneration etc [3-8].

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 [9-13]. 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 [14]. Poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) is a bacterially derived co-polymer which is produced by fermentation [15]. Belonging to the family of poly(hydroxyalkanoates), this biopolymer has been the focus of several studies investigating its thermal, physical and mechanical properties and its use in scaffolds for tissue engineering.

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, biogenicetic 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 focuses on the synthesis and characterization of composite materials based on
bacterial cellulose and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) as biodegradable scaffolds for tissue engineering applications.

**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 xylinum DSMZ (ICCF 398)*.

Hydrated (99% water, fig.1) BC membranes (5 mm thick) were carefully dried at 30 °C for 48 h and used for composites preparation. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate2%) (PHBH) copolymer was dissolved in boiling chloroform in closed round bottom flask and under stirring for 24 h (3, 5 and 9 wt. %).

Dried bacterial cellulose membranes were immersed in polyester solutions for 24 h and the composite materials obtained were left to dry at room temperature for 12 h. Composite membranes were prepared with the following compositions (BC/PHBH gravimetric ratio): 1/1, 1/2, 1/5. For comparison PHBH films were prepared by casting from chloroform solutions on Petri 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 of 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% CO2. 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 discussion**

Macroscopically relatively homogenous membranes were obtained for all PHBHV/BC composites. Pure BC and composite membranes appear transparent milky and white/brownish respectively (fig.1-2).

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 (CH2), 1371 cm⁻¹ (CH), 1158 cm⁻¹ (C=O-C), 1105 cm⁻¹ (C-O-C), 1052 cm⁻¹ (C-O), figure 3. 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 4).

XRD analysis was performed on cellulose membranes, PHBHV films and BC/PHB/V composite 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° (figure 5). Characteristic diffraction peaks for PHBHV copolymers are shown at 2θ diffraction angles of 13.54°, 16.97°, 21.62°, 22.56° respectively 25.59° (figure 6). Diffraction patterns obtained for the composite membranes show mainly the cellulose peaks at 1/1 ratio between BC/PHBHV (figure 7). 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 to unmodified bacterial cellulose membrane. Crystallite dimension of 2.68 nm characteristic for cellulose membranes increases for composites due to the 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 8-12 shows 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 8 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 (figure 9) reveals the ultrafine network structure composed of a random assembly of cellulose nanofibrils less than 100 nm wide. Figure 10-12 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 13 shows TGA results with DTG curves also (figure 14). The curve obtained for the pure BC membrane shows two significant events of weight loss. The first gradual one involving approximately 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 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 that a second important event is observed starting at around 260 °C 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 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 28° in good agreement with the hydrophilic nature of the material. On the other hand the value of the polyester 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 (73°). The computation of the free surface energies relies on Young-Dupre 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 ethyleneglycol.

\[ W_A = \gamma_L (1 + \cos \theta) \]  
\[ W_A = \gamma_S + \gamma_D - \gamma_{SL} \]  
\[ \gamma_{SL} = \gamma_S + \gamma_L - 2\sqrt{\gamma_S \gamma_L + \gamma_P \gamma_D} \]

The values obtained for these materials (1/1 ratio 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 results showed that the cytotoxicity of the BC/PHBHV composite membranes was assessed on L929 murine cell line. Figure 15 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 16. Biological investigations showed that most of the cells retained their typical morphology with extensions proving that the composite materials did not exhibit cytotoxic effect.

Conclusions

New composite materials based on PHBHV/BC were prepared by immersion of the cellulose membranes in chloroform polyester solutions (different PHBHV/BC mass ratios). Development of biodegradable PHAs/BC composites for tissue engineering (blood vessel engineering) is a challenging concept to develop. A composite material based on PHAs/BC composites will ensure the required mechanical integrity of the scaffold and controllable biodegradability.

Acknowledgments

This work was supported by UEFISCDI, project PNII-Partnerships in Priority Areas, number 158/2012.

References


NEW COMPOSITES BASED ON BACTERIAL CELLULOSE AND PHAS FOR TISSUE ENGINEERING APPLICATIONS

Tables and figures

Fig.1. Swollen bacterial cellulose obtained from Acetobacter xylinium DSMZ

Fig.2. Dried bacterial cellulose membranes obtained from Acetobacter xylinium DSMZ

Fig.3. FTIR spectrum for pure bacterial cellulose membrane

Fig.4. FTIR spectrum for PHBHV/BC composite

Fig.5. XRD diffractogram for bacterial cellulose membrane

Fig.6. XRD diffractogram for pure PHBHV polyester
Fig. 6. XRD diffractogram for BC/PHBHV composite 1/1 ratio

Fig. 8. SEM microphotograph showing the pure PHBHV polyester film

Fig. 9. SEM microphotograph showing the structure of pure bacterial cellulose

Fig. 10. SEM microphotograph showing the structure of 1/1 BC/PHBHV composite
NEW COMPOSITES BASED ON BACTERIAL CELLULOSE AND PHAS FOR TISSUE ENGINEERING APPLICATIONS

Fig. 11. SEM microphotograph showing the structure of 1/2 BC/PHBHV composite

Fig. 12. SEM microphotograph showing the structure of 1/5 BC/PHBHV composite

Fig. 13. TGA results for bacterial cellulose membrane, PHBHV powder and film, 1/1, 1/2 and 1/5 BC.PHBHV composites

Fig. 14. DTG curves for bacterial cellulose membrane, PHBHV powder and film, 1/1, 1/2 and 1/5 BC.PHBHV composites
Table 1. Contact angle measurements and free surface energies for PHBHV, BC and composite materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Contact angle, °</th>
<th>Free surface energy* mN/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>91.35</td>
<td>21.24</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>PHBHV 2% film</td>
<td>28.86</td>
<td>71</td>
</tr>
<tr>
<td>Pure BC membrane</td>
<td>73</td>
<td>21</td>
</tr>
<tr>
<td>Composite BC/PHBHV, 1/1</td>
<td>73, 50, 15</td>
<td>21</td>
</tr>
</tbody>
</table>

* based on equation Young-Dupre and Fawkes

Fig. 15. 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. 16. Optical microphotographs OM cells cultured grown on: a – blank test; b - BC/PHBHV 1/1 composites.