Biocompatibility of PHAs Biocomposites Obtained by Melt Processing

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The present work reports on the biocompatibility of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) loaded with bacterial cellulose and microcrystalline cellulose via melt processing. Biocompatibility was tested by physico-chemical and in vitro methods. Physico-chemical tests of biocomposites, such as reducing substances, acidity, alkalinity, absorbance by UV/VIS, residue on evaporation were performed on aqueous extract. The cell viability was evaluated by MTT assay. Cell morphology evaluation of cell culture treated with composites was visualized by light microscopy. Also, thermal properties of biocomposites were investigated by DSC analysis. The obtained results have shown good biocompatibility of all biocomposites.

Keywords: biocompatibility, physico-chemical tests, cytotoxicity, melt processing

Commercial medical plastics medical plastics exhibit good mechanical properties like flexibility, hardness and low price. These materials are manufactured from fossil fuels, are non-renewable and, furthermore, are not biodegradable. Further, they are designed for single use applications. Due to environmental concerns, there is an increasing interest in the development of biodegradable and biocompatible materials. This implies to protect non-renewable sources, as well as to avoid pollution problems related to the final disposition of non-degradable materials.

The polyhydroxyalkanoates (PHAs) offer the possibility both for potential replacement of non-degradable polymers currently used as polyethylene and polypropylene and to provide a new-type of biopolymers [1-5]. The most common type of PHAs is poly (3-hydroxybutyrate) (PHB). PHB is a biocompatible, environmental friendly and thermoplastic polymer. However, PHB presents many disadvantages that have been preventing its extensive use, such as high cost, thermal degradation and brittleness [6]. In order to overcome these drawbacks, PHB modification has been extensively studied, mainly by three approaches: i) Synthesis of copolymers, like poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV); ii) Introduction of additives, like plasticizers and nucleating agents; and iii) Formulations of composites and blends based on PHB with environmentally degradable polymers [7- 9].

Development of biocomposites based on PHAs and natural fibres has been extensively studied for both medical and packaging applications [3, 8, 10-19].

Cellulose is the most abundant natural biopolymer on Earth, a linear polymer of glucopyranose sugar molecules that is synthesized both by plants and bacteria [20-22]. Bacterial cellulose (BC) differs from plant cellulose with respect to its high crystallinity, ultra-fine network structure, high hydrophilicity, high mechanical properties and biocompatibility [23-26]. Also, it is pure and difficult to make on a large scale as it requires special bacterial cultures. Microbial cellulose has proven to be a remarkably versatile biomaterial and can be used in wide variety of applied scientific endeavors, such as paper products, electronics, acoustics, and biomedical devices [20, 27].

Most of the papers have reported preparation of PHAs-based biocomposites via casting method. For example, bacterial cellulose (BC)/poly (3-hydroxybutyrate) (PHB) composite membranes were prepared from chloroform-swollen BC membranes and PHB chloroform solutions. Relative contents were varied from 25 to 90 wt. % of PHB [28].

The present work reports on the biocompatibility of poly (3-hydroxybuthyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) loaded with bacterial cellulose (BC) and microcrystalline cellulose (MC) via melt processing.

It is a fundamental requirement for a biomaterial to display adequate biocompatibility, which means the ability to remain in contact with living tissue without causing any toxic or allergic side effects [23]. A number of clinical studies will be necessary to prove its usefulness and functionality. Biocompatibility does not only refer to the quality of not having toxic effects on biological systems, but also to the need of having an appropriate host response to ensure satisfactory performance on a specific application [27]. Biocompatibility has been evaluated by physico-chemical, *in vitro* and *in vivo* tests. Physico-chemical procedures are specified by European Pharmacopoeia and envisage tests such as: reducing substances, acidity/alkalinity, absorbance, etc. The purpose of these tests is to check the quality of medical devices made of plastics that come into contact with human tissues or substances and solutions that are introduced into the human body by another route than the oral route.

From our best knowledge there are no papers reporting biocompatibility of materials by physico-chemical methods. Many reports have focused on using PHB, PHBV as biomaterials for *in vitro* and *in vivo* studies [5]. Therefore, blending of PHB with ethyl-cellulose was readily achieved to reduce PHB crystallinity and promote its degradation

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under physiological conditions without influence on biocompatibility [29]. In other study biocompatibility of biocomposites prepared by impregnation of bacterial cellulose into the poly(3-hydroxybutyrate) (PHB) was preliminarily evaluated by cell-adhesion studies using fibroblast cells [30]. The result of study demonstrated high biocompatibility of nanocomposites much better than pure PHB. In other paper, bio-composite scaffolds by freeze-drying using poly(3-hydroxubutyrate-co-4-hydroxy-butyrate) and bacterial cellulose as raw materials and trifluoroacetic acid as co-solvent were prepared [31]. The study revealed the cells incubated with composite scaffold for 48h were capable of forming cell. In recently study, [poly(3-hydroxybutyrate-co-3-hydroxyvalerate 2%)]/ bacterial cellulose composite membranes successfully assessed the cytotoxicity on L929 murine cell line [32].

Thus, in the present study the physico-chemical properties of biocomposites based on PHB and PHBV with 1 % BC and respectively 1% MC were evaluated: reducing substances, acidity, alkalinity, absorbance UV/Vis, residue on evaporation tests were performed on aqueous extracts. The cell viability was evaluated by MTT assay. Cell morphology evaluation of cell culture treated with biocomposites was visualized by light microscopy. Also, thermal properties of biocomposites were investigated by DSC.

Experimental part

Materials and methods

Poly(3-hydroxybutyrate) (PHB) type 319 E, granular form, was used as the polymer matrix. It was kindly supplied by BIOMER, Germany. The material has a density of 1.2197 g/cm^3, MFI 3.63 g/10 min (180^{0}C/2.16 kg). Polyhydroxybutyrate-co-(hydroxyvalerate) (PHBHV) with PHV content 12 mol %, granules form, was purchased from GOOD FELLOW UK. The material has a density of 1.2350 g/cm^3. 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). Microcrystalline cellulose (MC) (dimensions of 10 – 15 mm) was purchased from SIGMA-ALDRICH.

Reagents used were of chemical grade. All the chemicals used for *in vitro* citotoxicity assay are obtained from SIGMA CHEMICALS.

Preparation of PHAs/BC and PHAs/MC biocomposites

PHB with BC 1 % content (wt. %) and respectively MC 1 % in content (wt%) and PHBV with BC 1 % content (wt. %) and respectively MC 1 % content (wt. %) were melted using a BRABENDER Plastograph, under a mixing temperature of 180^{o}C for 10 min and screws rotation rate of 40/70 rpm. Before melting, PHB and PHBV granules were dried in oven at 60^{o}C for 3 h. BC and MC were dried in oven up to 105^{o}C, for 2 h. After blending, the melted samples were pressed into thin films by a laboratory press type POLYSTAT 200 at the following conditions: temperature: 160^{o}C, pressing time 5 minutes and pressure of 200 bars.

Differential Scanning Calorimetry analysis of biocomposites (DSC)

DSC analysis was carried out using a DSC 823^e (Mettler-Toledo). Samples were sealed in pans and heated at 10^{o}C/ min from 30 to 350^{o}C to obtain the melting temperature *(T_m)* and enthalpy of fusion *(ΔH_f)*. The blend *(Blend-X_c)* and PHA *(PHA-X_c)* phase crystallinity were calculated using the following equations [29]:

$$Blend - X_c = \frac{\Delta H_f}{\Delta H_{100\%\ PHA}} x\ 100\% \quad (1)$$

$$PHA - X_c = \frac{Blend - X_c}{(1-w)\Delta H_{100\%\ PHA}} x\ 100\% \quad (2)$$

where: ΔH_f is the melting enthalpy of the specimens (J/g); $\Delta H_{100\% PHA}$ is the enthalpy value for a theoretically 100% crystalline PHA (146 J/g) [7]; *(1-w)* is the weight fraction of polymer in the PHA biocomposites. The $\Delta H_{100\%}$ for PHBV was assumed to be the same as that for PHB [33].

Preparation and characterization of aqueous extract

For the preparation of aqueous extract it was used a method as described in European Farmacopoeia. 25 g of the material to be examined were placed in a borosilicate-glass flask. 500 mL of water were added and heated in an autoclave at 121 ± 2^{o}C for 20 min. In the same conditions, a reference solution was prepared. Within 4 h from preparation of extract aqueous there were carried out: acidity, alkalinity, absorbance, reducing substances and water extractable substances tests.

Acidity or alkalinity test

For acidity test, to 100 mL of solution of aqueous extract add 0.15 mL of BRP indicator solution. It is measured the changing the colour of indicator to blue by adding of 0.01 M sodium hydroxide. Similar, for alkalinity test, to 100 mL of solution aqueous extract add 0.2 mL of methyl orange solution. It is measured the changing of the colour of indicator from yellow to orange.

Reducing substances test

To 20.0 mL of solution of aqueous extract add 1mL of dilute sulfuric acid and 20.0 mL of 0.002 M potassium permanganate. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of potassium iodide and titrate immediately with 0.01 M sodium thiosulfate, using 0.25 mL of starch solution as indicator. Carry out a blank titration using 20 mL of water for injections.

Water extractable substances test

Evaporate 50 mL of solution of aqueous extract to dryness on a water-bath and dry in an oven at 100-105^{o}C to constant mass. Carry out a blank test with 50.0 mL of water for injections and the difference between those samples is recorded.

Absorbance test

UV/VIS absorption spectra of the samples (10 mL) were recorded using a Spectronic HELIOS ALPHA UV/Vis spectrophotometer in the wavelength range from 250 nm to 310 nm, using the reference solution as compensation liquid.

In vitro biocompatibility test

In vitro cytotoxicity of the new materials was evaluated according to the EN ISO 10993-5:2009 using the direct contact method on a fibroblast cell line (NCTC clone L929). The cell viability was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. This spectrophotometric method is based on the conversion of MTT to insoluble purple formazan crystals under the action of mitochondrial dehydrogenases in living cells. The amount of formazan is proportional to the number of living cells and is spectrophotometrically determined after dissolving the crystals in a suitable solvent. Cell morphology was analyzed on the culture stained with hematoxylin-eosin, after the direct contact of the cells with

the portioned samples (according to the EN ISO 10993-5:2009).

Cell viability assay

Cell culture used in the biocompatibility evaluation of biocomposites was stabilized mouse fibroblast cell line NCTC (clone 929) cultivated in Minimum Essential Medium (MEM) containing 10 % Fetal Bovine Serum (FBS) and 2mM L-glutamine, 100 U/mL penicillin, 100μg/mL streptomycin and 500 μg/mL neomycin. Cell suspension was seeded in 96-well culture plates at a density of 4 x 10^4 NCTC cells/mL and incubated in a humidified 5% CO_2 atmosphere, at 37°C, 5% CO_2 for 24h. The studied composites were added in triplicate manner and plates were incubated in standard conditions for 24h and 48h; the cell viability was quantitatively determined by MTT spectrophotometric assay [34]. Briefly, the culture medium in each well was replaced with MTT solution and the plates were incubated for 3h at 37°C. The MTT solution from the wells was replaced with isopropanol followed by gentle shaking to solubilize the formazan crystals. Absorbance of coloured solution was read at 570 nm using a microplate reader Berthold Mithras LB 940 (Germany). The measured optical density (OD) is directly proportional to the number of viable cells present in the tested cell culture and the results were calculated according to the formula (3):

$$\% \text{ cell viability} = (\text{OD sample} / \text{OD control}) \times 100\ \% \quad (3)$$

Untreated cells served as control group considered as 100% viable cells.

Cell morphology evaluation

For cell morphology examination seeding cells in 12-wells plates with 1mL medium containing 4 x 10^4 cells/mL in suspension were employed. After 24h incubation for the attachment of cells in wells, the medium was replaced with studied biocomposites and incubated for 48h under appropriate conditions. Subsequently the cells were fixed in Bouin and hematoxilin–eosin stained. The cultures were visualized by light microscopy using an inverted microscope Carl Zeiss Axio Observer D1 (×20) and images were taken with the digital camera Axio Cam MRc (Germany).

Results and discussions

Thermal properties

The heat of fusion (ΔH_f), melt temperatures (T_m), and crystallinity of PHA/BC and PHA/MC composites were determined using DSC. The DSC spectra are presented in figures 1 and 2.

As shown in figures 1 and 2, the curves obtained for biocomposites present two endothermic visible peaks, first due to the melting of the polymer and the second related to decomposition associated with rapid loss of weight (about 270°C) overlapping with sample degradation. These effects were similar to those reported in literature [28].

Table 1 gathers the thermal properties of PHAs composites as determined from DSC analysis: melting enthalpies *(ΔH_f)*, maximum of melting *(T_m)*, degradation onset *(T_d)* and crystallinity *(X_c)*; these characteristics were obtained from the first heating run.

As it is shown in table 1, the neat PHB sample has an endothermic melting peak at 174.02°C since the neat PHBV sample has a melting temperature of 163.67°C. 12% valerate content from PHBV grade displays lower melting enthalpy and lower melting point than of PHB. The melting temperature *(T_m)* decreased with adding of BC or MC for both PHA composites and the decrease was more noticeable for PHBV/MC. The lower T_m may have been caused by MC lowering the melting viscosity of PHA [35]. Addition of BC or MC into PHA matrix did not lead to a

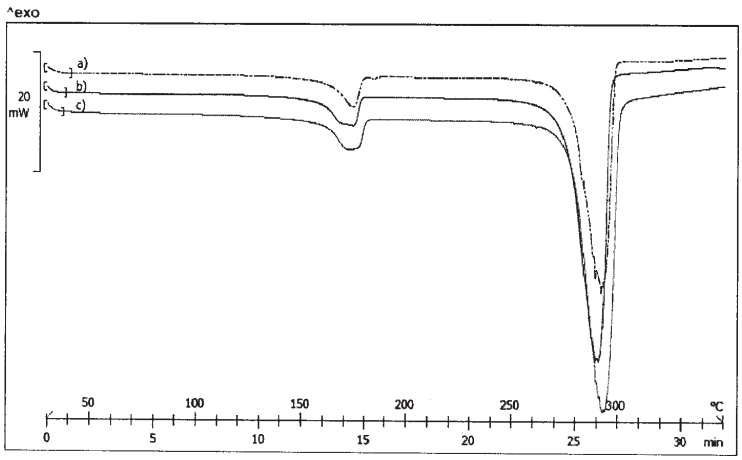


Fig.1. DSC curves for PHB composites *a)PHB 319E; b) PHB 319E/1% BC; c) PHB 319E/1% MC*

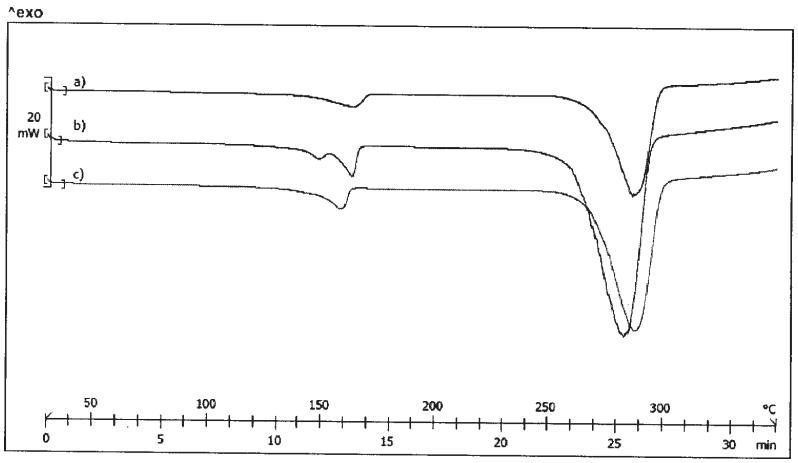


Fig.2. DSC curves for PHBH composites *a)PHBV (12%HV); b) PHBV/1% BC; c) PHBV/1% MC*

Sample	ΔH_f, J/g	T_m, ^{0}C	T_d, ^{0}C	X_c, %
PHB	77.67	174.02	276.34	53.19
PHB/BC	74.10	174.01	272.21	50.75
PHB/MC	78.06	173.46	272,50	53.46
PHBV	59.49	163.67	268.54	53.46
PHBV/BC	49.00	162.80	258.69	33.56
PHBV/MC	48.91	158.08	268.85	33.50

Table 1
THERMAL CHARACTERISTICS OF PHA COMPOSITES

substantial decreasing of degradation temperature, so did not influence the melting processing temperature. It was interesting to emphasize that the introduction of BC and respectively MC strongly influenced the heat of fusion and cristallinity of composites. The enthalpy values were calculated taking into account the actual weight fraction of PHB and PHBV used in the formulations. According to formulas (1) and (2), the ΔH_f of PHB was 77.67 J g^{-1}, whereas that of PHB/BC was 74.10 J g^{-1}. Also a lower ΔH_f for PHBV/BC and PHBV/MC composites (49.00 J/g and respectively 48.91 J/g) is observed in comparison with pure PHBV (ΔH_f of 59.49 J/g). Decreasing of ΔH_f of PHA composites indicates a decrease in crystallinity of composites which can be explained by the enhanced chain-end mobility. A reduction in crystallinity of PHBV in comparison with PHB is attributed to the hydroxyvalerate units inhibiting the crystallization through hydrogen bonding [8]. These results are similar to those obtained elsewhere with composites of polymers and natural fiber [35]. An unexpected behaviour is recorded by formulation that contains 1 % microcrystalline cellulose into the PHB matrix. PHB/MC composite records an increase both in the melting enthalpy (ΔH_f of 78.06 J/g) and crystallinity (53.46%). This result suggests that MC acts as nucleating sites promoting the development of smaller crystals for PHB/MC composite.

Characterization of aqueous extract

Figures 3-7 show the physico-chemical testing performed on aqueous extracts from PHAs composites.

Compared with PVC, the most useful polymer for biomedical purpose, PHA composites show values maintaining in acceptable range of European Pharmacopoeia. Therefore, for acidity, not more than 1.5 mL of 0.01 M NaOH is required to initiate the color change of the BRP indicator solution to blue, since for alkalinity, maxim 1.0 mL of 0.01 M HCl is required to initiate the colour change of the methyl orange solution from yellow to orange. According to European Pharmacopoeia, for reducing substances the value must not exceed 2 mL 0.01 M $Na_2S_2O_7$, since for water extractable substances the maxim value is 7.5 mg.

Absorbance by UV/Vis of biocomposites is presented in figure 7.

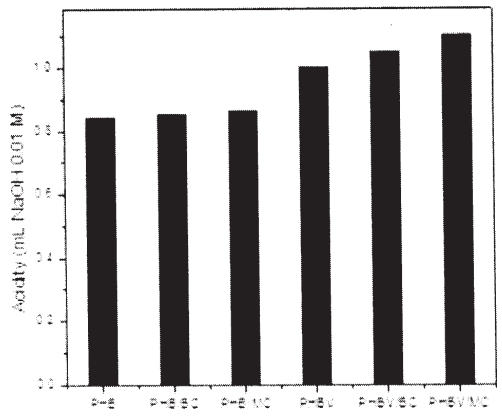


Fig.3. Acidity of PHAs composites

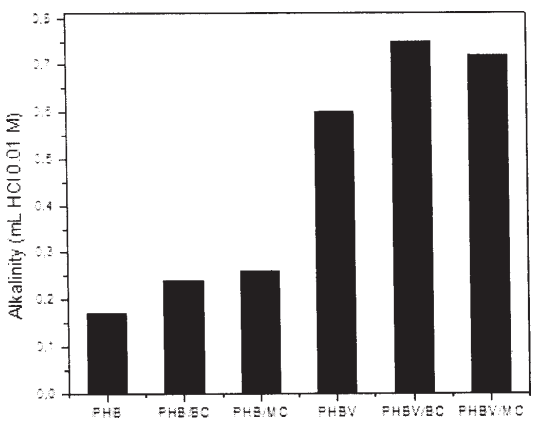


Fig.4. Alkalinity of PHAs composites

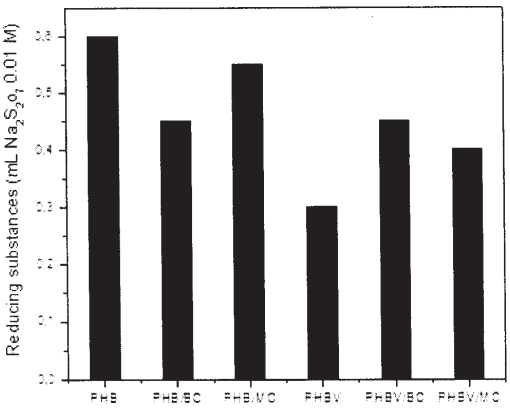


Fig.5. Reducing substances of PHAs composites

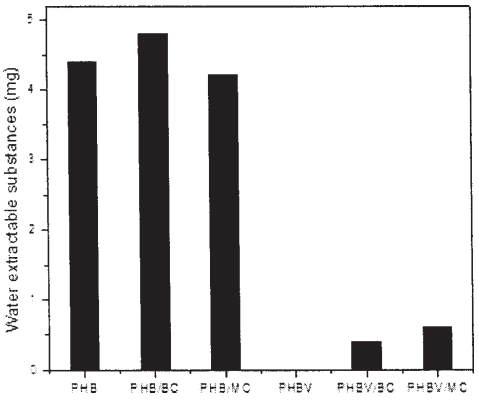
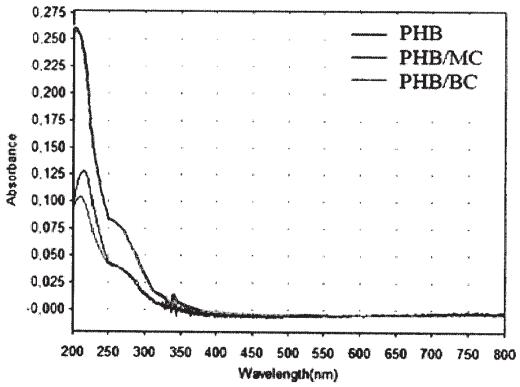


Fig.6. Water extractable substances of PHA's composites



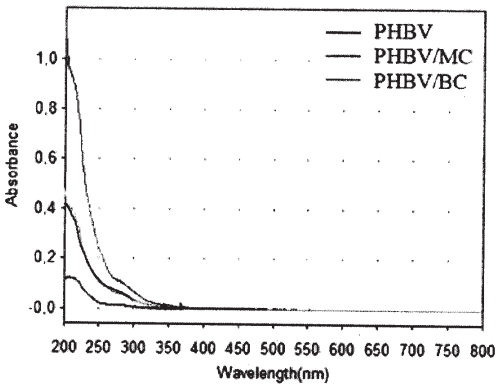


Fig.7. UV/VIS absorbance of PHA biocomposites
a)PHB composites; b) PHBV composites

As shown in figure 7, examined from 250 nm to 310 nm, using the reference solution as compensation liquid, solutions of aqueous extract obtained by PHA/BC or MC composites show no absorbance greater than 0.25, as is specified in European Pharmacopoeia.

From data indicated in figures 3-7, all studied biocomposites are in the range of acceptable values regarding physico-chemical testing.

In vitro biocompatibility assay

Cell viability assay

MTT viability assay was done in order to find out if the composites have cytotoxic effects on NCTC cells. Quantitative determination by MTT method led to the following values of cell viability at 24 and respectively 48 h, according to figure 8.

The tested biocomposites were not found cytotoxic compared with viability of cell cultures exposed to the

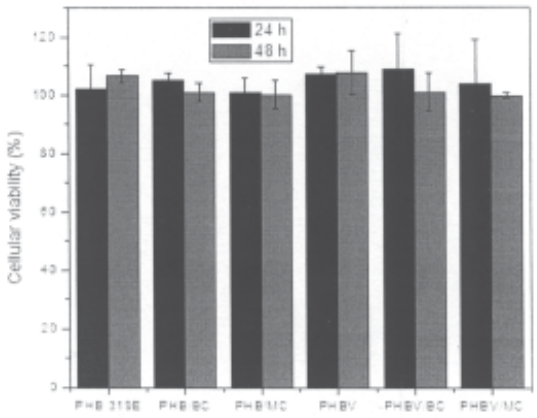


Fig.8. Cell viability of L929 cell after direct contact with composites for 24 and respectively 48 hours evaluated by MTT assay. Measurements represent mean of three determinations ± S.D

control group. All composites variants led to a cell viability percentage over 100%. According to EN ISO 10993-5:2009, the cytotoxicity scale signifies a high degree of biocompatibility.

There is know, that the family of PHA has exhibited good *in vitro biocompatibility. However,* it has been reported that PHBV had better biocompatibility than PHB did [36].

Morphology evaluation

The morphology of the samples at 48h was examined by optical microscopy and is presented in figure 9.

Qualitative data (cell morphology) for *in vitro* cytotoxicity evaluation presented in figure 9 reveals cell proliferation of the cells grown in direct contact with composites compared with the control culture. At 48h the NCTC control culture has the splitting phase near confluence, the culture consists by round and spindly fibroblast cells, with circular nucleus and fine cytoplasmic granulation. From comparison of morphology images of the treated cells and the control it stands out the aspect of culture treated with PHB and PHBV composites, which show a great cellular proliferation in the presence of biocomposites, indicating a high degree of biocompatibility. With the same variations of cellular density, all the images representing the cells after 48h of direct contact with composites have the specific appearance of the control culture without any degradation aspects of the cell morphology; in conclusion all the samples are biocompatible. The morphology aspects of treated cells are in good agreement with results obtained in the MTT viability assay.

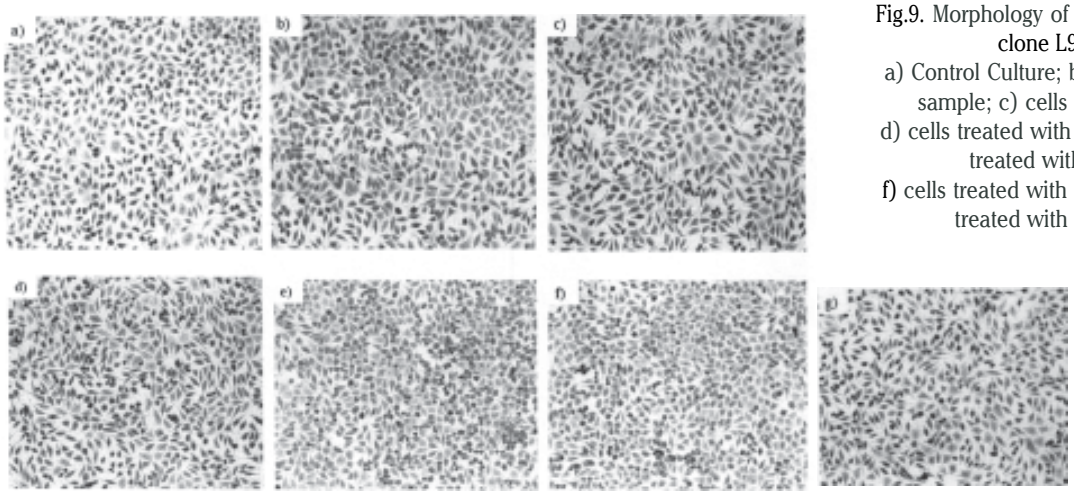


Fig.9. Morphology of fibroblast cell line (NCTC clone L929) after 48h
a) Control Culture; b) cells treated with PHB sample; c) cells treated with PHB/BC; d) cells treated with PHB/MC sample; e) cells treated with PHBV sample; f) cells treated with PHBV/BC sample; g) cells treated with PHBV/MC sample

Conclusions

In this work, biocompatible composites based on poly (3-hydroxybuthyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) loaded with bacterial cellulose (BC) and microcrystalline cellulose (MC) were obtained via melt processing.

As evidenced by DSC analysis, addition of BC or MC into PHA matrix led to decrease of melting temperature (T_m) and strongly influenced the heat of fusion and crystallinity of composites

Biocompatibility was tested by physico-chemical and *in vitro* methods. Tested biocomposites met the requirements of European Pharmacopoeia regarding acidity, alkalinity, absorbance, reducing substances and water extractable substances tests.

The testes biocomposites were not found cytotoxic compared with viability of cell cultures exposed to the control group. All composites variants led to a cell viability percentage over 100%. Qualitative data (cell morphology) for *in vitro* cytotoxicity evaluation revealed cell proliferation of the cells grown in direct contact with composites compared with the control culture. The morphology aspects of treated cells are in good agreement with results obtained in the MTT viability assay and, as conclusion, composites based on poly (3-hydroxybuthyrate) (PHB) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) loaded with bacterial cellulose (BC) and microcrystalline cellulose (MC) are biocompatible.

These materials could have potential applications in tissue engineering where they envisage biocompatibility property.

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