

NOVEL BIOCOMPOSITES BASED ON POLYHYDROXYALKANOATES-LAYERED DOUBLE HYDROXIDES FOR TISSUE ENGINEERING APPLICATIONS

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This research study is focused on the synthesis and characterization of novel biocomposites based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and modified layered double hydroxides for tissue engineering applications. The biocomposites were obtained by solvent casting technique from polymer solutions containing dispersed clay particles. After purification and drying, the films were characterized by FTIR spectroscopy and X-ray diffraction to reveal the presence of the clay within the polyester matrix. Thermal characterization involved TGA and DSC measurements. Morphological investigation was employed through SEM analysis on biocomposites. The biocompatibility of these novel biocomposites was studied in relation with human adipose derived stem cells.

Keywords: polyhydroxyalkanoates, layered doubled hydroxides, solvent casting, biocompatibility, tissue engineering

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1. Introduction

In the last few years, a lot of research work has been focused on biodegradable and biosourced polyesters for the purpose of reducing the non-degradable polymer wastes [1-2].

Polyhydroxyalkanoates (PHAs) are a family of natural biodegradable polyesters of 3, 4, 5 & 6-hydroxyacids, obtained by numerous bacteria through the fermentation of sugars, lipids, alkanes, alkenes and alkanoic acid, as a unique intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells [3-5].

PHAs can be degraded in nature by microorganisms using PHA depolymerization, however, the activities of these enzymes vary depending on the composition, crystallinity, additives and the surface area of the polymer [6]. The molecular weight of PHA is dependent on the microorganism in which the polymer is produced and the growth conditions used and this varies in the range 2×10^5 – 3×10^6 g/mole [6]. Poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) are the most studied natural polyesters and they are the first commercial thermoplastics from a bacterial source [7]. Poly(3-hydroxybutyrate) can be biosynthesized by using a variety of microorganisms and this natural polyester has complete biodegradability without any toxic byproducts. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a copolymer of PHB and it found numerous applications in medicine (implants, drug delivery systems, tissue engineering) due to its attractive properties such as good biocompatibility, biodegradability, lack of toxicity, piezoelectricity [9].

Layered double hydroxides (LDHs), also known as anionic clays are a class of two dimensional materials that are well known for their characteristics like high chemical stability, good biocompatibility and anion exchange capacity [10]. The general formula for all LDHs can be written as $[M_{1-x}M'_x(OH)_2](A^{n-})_{x/n} mH_2O$, where M - a divalent metal cation, M'-trivalent metal cation [10-11]. These anionic clays have various applications in fields like biology and medicine: glucose biosensors [12-14], urea biosensors [15], drug and gene delivery [16-17], water treatment- removing fluoride ions from aqueous solutions [18-19].

During the last decades, the development of nanostructured hybrid organic-inorganic materials is of increasing interest. Hybrid systems composed of polymeric matrix and layered materials (MMT in particular and more recently LDH) received more attention because of their unique mechanical, thermal and gas barrier properties compared with those of virgin polymers [2, 20].

This study describes the synthesis and characterization of novel biocomposites based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and modified layered double hydroxides with good biocompatibility for potential in tissue engineering applications. The new biocomposites have been investigated by

FTIR spectroscopy and thermal analyses (TGA and DSC). The structural information can be provided by X-Ray diffraction and the morphological information of the composites samples were obtained by Scanning Electron Microscopy.

2. Experimental

2.1. Materials

Polyhydroxybutyrate/Polyhydroxyvalerate 2% copolymer was purchased from Good Fellow Cambridge Limited, UK. Chloroform, $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and sodium dodecyl sulfate were provided by Sigma Aldrich.

2.2. Methods

2.2.1 Synthesis of modified layered double hydroxide (LDH-SDS)

The modified layered double hydroxide (LDH - SDS) was obtained by coprecipitation method. For this, two solutions were prepared [21]. The first solution was obtained by dissolving 9.6 g $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and 4.7 g $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in 45 ml of H_2O . For the second one, 4 g SDS and 3.4 g NaOH were dissolved in 40 ml H_2O . LDH-SDS was obtained by adding dropwise the two solutions to 50 ml H_2O under stirring. The pH value of the mixture was maintained around 10. The mixture was aged for 22 h at 80 °C. The resulted precipitate was filtered, washed with 2L of hot water (80 °C) and after dried for 48 h at 80 °C in order to remove the excess of sodium dodecyl sulphate.

2.2.2 Obtaining of PHBHV/LDH-SDS biocomposites

LDH-SDS powder was dispersed in PHBHV chloroform solutions by sonication for 45 minutes and the composite films were obtained by solvent evaporation. Films were left to dry at room temperature for 24 h. For comparison PHBHV films were prepared by casting from chloroform solutions on Petri glass plates.

2.3. Characterization

Fourier Transform Infrared (FT-IR) spectra were taken on a Bruker Vertex 70 spectrometer in 4000-600 cm^{-1} wavenumber region equipped with a attenuated total reflectance (ATR) accessory, using a resolution of 4 cm^{-1} and an accumulation of 32 spectra. X-Ray Diffraction (XRD) patterns were obtained using a RIGAKU miniflex II diffractometer with $\text{CuK}\alpha$ radiation. Morphological information of the composites samples was obtained through the scanning electron microscopy (SEM) 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 800 °C, using TGA Q500 equipment (TA Instruments). The samples weight was 2.4 ± 0.1 mg.

A NETZSCH Differential Scanning Calorimetry (DSC) 204 was used for analyzing the thermal characteristics of the samples. Nitrogen (99.99% purity) was the purge gas used and flowed at 10 mL/min. Samples of 4 mg was weighed into aluminum pans covered and fixed on the sample platform.

2.3.1 Biological tests

Cell culture model

hASCs were previously isolated from human subcutaneous adipose tissue as described by Galateanu *et al.*[22]. Briefly, the lipoaspirates (LAs) were processed by collagenase digestion and the cells obtained were resuspended in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Co), supplemented with fetal bovine serum.

The human adipose tissue was obtained from female patients undergoing elective liposuction and all the medical procedures were performed in compliance with the Helsinki Declaration, with the approval of the Emergency Hospital for Plastic Surgery and Burns Ethical Committee (reference no. 3076/10.06.2010).

LIVE/DEAD assay

Live/Dead fluorescence microscopy assay was performed to evaluate hASCs viability in direct contact with PHBHV film (B) and PHBHV_LDH-SDS composites with 1% (B1), 2% (B2) and 3% (B3) LDH-SDS biomaterials, using the Live/Dead Kit (Invitrogen, Life Technologies, Foster City, CA). This fluorescence-based kit combines calcein AM and ethidium bromide to yield two-color discrimination of the population of live cells from the dead-cell population. Briefly, at 24 h post seeding, hASCs/B0, hASCs/B1, hASCs/B2 and hASCs/B3, bioconstructs were incubated with the staining solution prepared according to manufacturer's instructions, for 15 minutes in the dark. Next, the stained biohybrids were analyzed by confocal fluorescence microscopy using a Carl Zeiss LSM710 laser-scanning microscope and images were captured with Carl Zeiss Zen 2010 software version 6.0.

3. Results & Discussion

The FTIR-ATR characterization was performed to confirm the structure of the new synthesized composite films. FTIR-ATR measurements of all specimens are shown in Fig. 1.

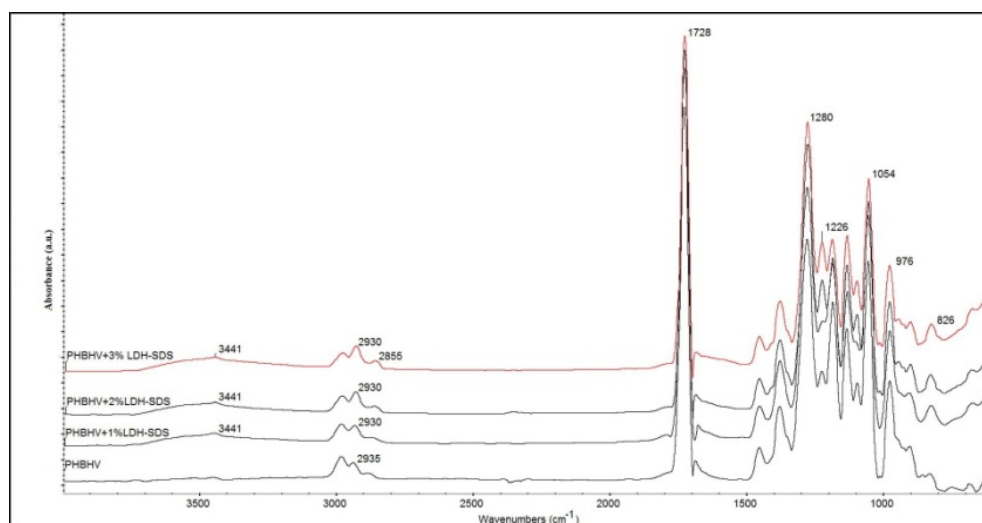


Fig.1. FTIR-ATR Spectra of neat PHBHV and PHBHV-LDH-SDS composite films

All spectra present an intense peak at 1728 cm^{-1} which is attributed to the specific vibration of $\text{C}=\text{O}$ from copolyester. Certain changes in the intensities of some characteristic band could be observed. That is the intensity changes in the crystalline $\text{C}-\text{O}-\text{C}$ stretching band at 1226 cm^{-1} , as well as the amorphous $\text{C}-\text{O}-\text{C}$ band at 1187 cm^{-1} could be observed.

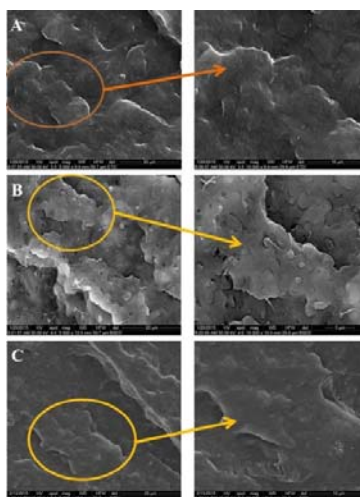


Fig.2. SEM microphotographs reveal the morphology of: PHBHV-film (A), PHBHV/LDH-SDS 1% (B) and PHBHV/LDH-SDS 3% (C)

SEM analysis is performed on PHBHV film, LDH-SDS particles and PHBHV/LDH-SDS composites. Fig. 2 (A) shows the surface of the neat polyester

film obtained by casting from the chloroform solution. PHBHV microparticles are dispersed in a compact structure. LDH-SDS particles are presented in Fig. 2 (B). SEM image of PHBHV/LDH-SDS composite shows a compact surface where the LDH-SDS particles are embedded into the polyester film.

Fig. 3 shows TGA (3.A) results with DTG (3.B) curves also. TGA curve of PHBHV film in CHCl_3 shows two weight loss events. The initial weight loss is up to 200 °C due to residual solvent from the casting technique used for obtaining of PHBHV films. The last mass loss starting at around 270 °C up to 300 °C corresponding to the degradation of the natural polyester. Almost no residue is observed.

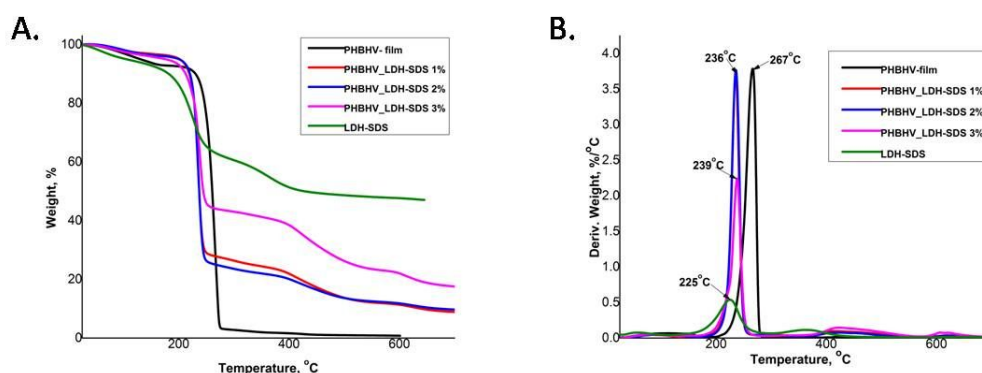


Fig.3. TGA (A) and DTG (B) curves for neat PHBHV film, LDH-SDS and PHBHV_LDH-SDS composites with 1%, 2%, 3% LDH-SDS

TGA curves of PHBHV-LDH-SDS (1% and 2% LDH-SDS) express that the presence of the clay does not influence the thermal properties of the composite films. In the range of temperature (20 °C ÷ 600 °C), PHBHV-LDH-SDS composites exhibit three endothermic effects. The first step of decomposition is in the temperature range of 20 °C ÷ 200 °C and represents the loss of residual solvent from the casting technique. The weight loss on both second step of decomposition (in the range of temperature 200 °C ÷ 400 °C) and the third step of decomposition (in the range of temperature 400 °C ÷ 600 °C) are characteristic for LDH-SDS decomposition. The composites start to decompose at lower temperatures than PHBHV, probably due to the presence of dodecyl sulphate from LDH-SDS. We can observe that the residue of these samples increases as compared to the neat polymer.

The influence of the inorganic filler on the crystallization behavior of PHBHV is studied by DSC analysis. DSC curves for PHBHV and composite materials with 1%, 2% and 3% LDH-SDS are shown in Fig. 4.

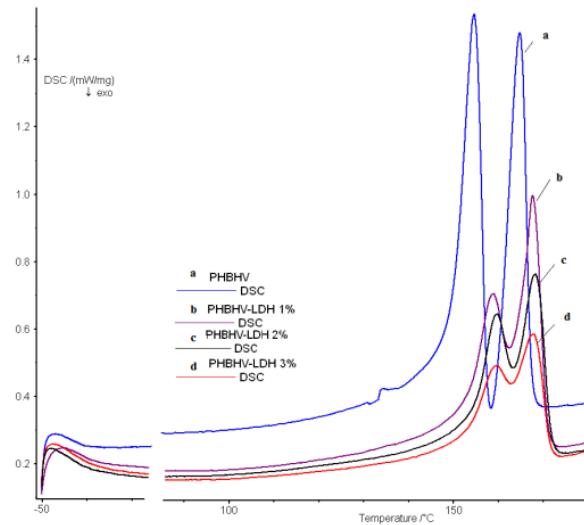


Fig.4. DSC results for neat PHBHV film and PHBHV_LDH-SDS composites with 1%, 2% and 3% LDH-SDS

DSC analysis of PHBHV and nanocomposites shows double melting curves and the melting temperature increases with the introduction of the LDH-SDS within the polymer matrix. Nevertheless, the melting temperature of the clay biocomposites is not influenced by the increase of the LDH-SDS concentration.

The X-Ray diffractograms of LDH-SDS, PHBHV-film and PHBHV-LDH-SDS composites are presented in Fig.5.

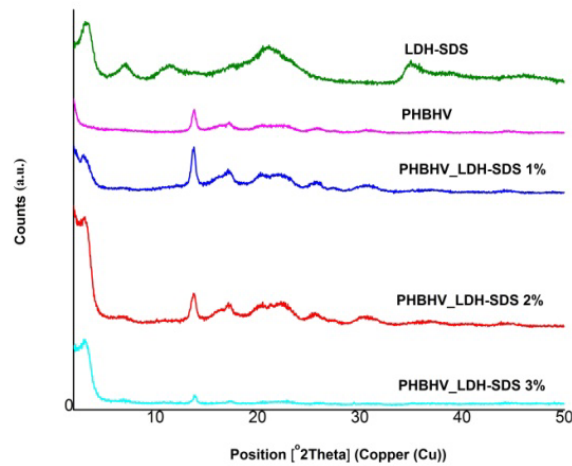


Fig.5. XRD diffractograms for LDH-SDS, neat PHBHV and PHBHV_LDH-SDS composites with 1%, 2% and 3% LDH-SDS

LDH-SDS shows an important peak at $2\theta = 3.47^\circ$ corresponding to a basal space of 2.54 nm. The next peaks of LDH-SDS correspond to the higher harmonics of the interlayer distance. We can observe that peaks are sharp, which can indicate a lamellar structure of the anions intercalated in the nanofiller interlayer galleries.

All the composites show a decrease of the 2θ angle but the best results were obtained for composite with 1% LDH-SDS. The presence of sharp Bragg peaks after solution casting indicates that the inorganic component retains an ordered structure. Composites with intercalated, partially exfoliated lamellar structure were obtained. By this type of test we can examine the influence of the LDH-SDS on the biopolymer matrix. We can say that the polyester is a semi-crystalline material and its characteristic peak are at $2\theta = 13.79^\circ$, 17.26° , 20.22° , 22.25° and 25.95° with peaks corresponding to a rhombic unit cell.

The biocomposite films show diffraction maxima at the same values as for the neat biopolymer, indicating that, in the composites, PHBHV crystallizes in its typical crystalline form. Its unit cell is not influenced by the adding of LDH-SDS.

LIVE/DEAD fluorescent microscopy assay

In order to examine cell survival on the tested biomaterials, the viability of hASCs was evaluated at 24 h post-seeding by confocal fluorescence (Fig. 6) microscopy, based on the simultaneous staining of live (green labeled) and dead (red labeled) cells.

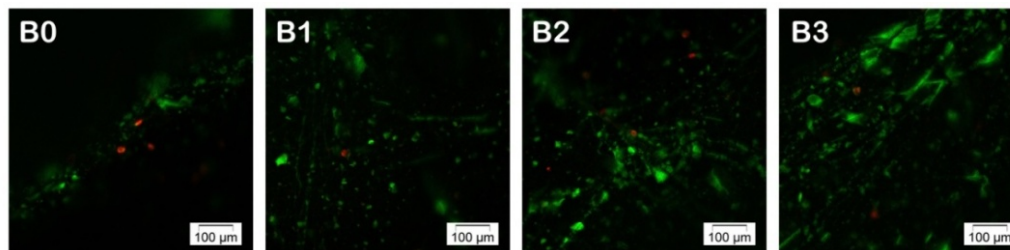


Fig.6. Confocal fluorescence microscopy micrographs revealing live and dead cells on PHBHV film (B) and PHBHV_LDH-SDS composites with 1% (B1), 2% (B2) and 3% (B3) LDH-SDS, after 24 h of culture

After 24 h post-seeding bright green-labeled cells were observed on the surface of all tested biomaterials, but in a greater amount on PHBHV_LDH-SDS composites with 1% (B1), 2% (B2) and 3% (B3) LDH-SDS as compared to PHBHV film (B0) biomaterial. Additionally, the ratio between the green (living) and red (dead) cells was constant in all the tested biocomposites.

Fluorescent labeling of both living and dead cells showed that hASCs survived after 24 h of culture in contact with all tested biomaterials. Additionally, no significant differences were detected in the ratio between live and dead cell on

PHBV film (B) and PHBV/LDH-SDS composites with 1% (B1), 2% (B2) and 3% (B3) LDH-SDS biomaterials.

4. Conclusions

Novel biocomposites based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and organophyllized layered double hydroxides (LDH-SDS) with various compositions were synthesized. SEM images show the morphology of the PHBV/LDH-SDS composites, where LDH-SDS particles are dispersed. Biological tests revealed a good biocompatibility of the tested composites expressed by LIVE/DEAD analysis. Furthermore, PHBV/LDH-SDS composites morphology seem to better mimic the physiological microenvironment of the cells, as hASCs viability was increased after 24 h of culture as compared to the reference biomaterial. Thanks to the good biocompatibility, these materials could be promising candidates for *in vivo* testing for wound dressing applications.

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