TRACKING THE PROGRESS OF THE BIOCOMPATIBLE NANOCOMPOSITES BASED ON POLY(3-HYDROXYBUTYRATE) WITH THE HYPROMELLOSE NANOADDITIVE BY THERMAL ANALYSIS AND BIOLOGICAL STUDIES

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Abstract – The poly (3-hydroxybutyrate) (P3HB) was used to prepare biocompatible composites modified by the hypromellose. The aim of the study was to assess the biological properties and selected thermald parametrs together with the phase contents of the obtained biocomposites. The phase transitions of nanocomposites and polymeric matrix were investigated using differential scanning calorimetry. Moreover, the non-equilibrium and equilibrium thermal parameters of composites and the unfilled nanocomposites were established based on thermal history. Based on equilibrium parameters (i.e. the heat of fusion for the fully crystalline materials and the change of heat capacity at glass transition temperature for the fully amorphous composites), the degree of crystallinity, mobile, and rigid amorphous fractions were estimated. The addition of hypromellose to the P3HB matrix caused a decrease in the degree of crystallinity in reference to the unfilled P3HB. Simultaneously, an increase of amorphous phase contents was noted. It was also noted that the rigid amorphous fraction exists only for nanocomposites containing 2% mass of nanofiller. Thermogravimetric analysis of nanocomposites was also carried out and showed that the thermal stability of all nanocomposites is higher than that of the unfilled P3HB. An additional 1% mass of nanofiller increases the degradation temperature of nanocomposites by about 37°C in reference to the unfilled P3HB. The immunosafety of the tested nanocomposites and the absence of, or very low, endotoxin contamination was confirmed by the the Food and Drug Agency Guidance as well as the European Medicines Agency.

Keywords – Poly (3-Hydroxybutyrate), Hypromellose, Nanocomposite, Biocompatible Nanomaterials

I. INTRODUCTION

Poly-3-hydroxybutyric acid (P3HB) is classified as polyhydroxy alkanoic acid (PHA). The identification of PHB in 1926 initiated a series of reports on the possibility of its use [1]. Its use in many branches of medicine and pharmacy is ensured by such features as: no cytotoxicity, biocompatibility, biodegradability, no mutagenicity or thermoplasticity [2, 3]. The combination of these features with the possibility of synthesis from relatively cheap raw materials make it an extremely attractive material in the context of tissue engineering [4]. P3HB is a semi-crystalline polyester with a high melting point (173-180°C) compared to other biodegradable polyesters [5]. In terms of physical and mechanical properties, P3HB resembles isotactic polypropylene, which made P3HB [6], however, it is not a perfect material due to stiffness, brittleness and low thermal stability, which is close to the melting point [5]. Improving these properties could enable the material to be used on a wider scale.One of the more widely described applications of P3HB is drug delivery systems (DDS). The combination of P3HB with gelatin or various modifications of P3HB is being investigated for possible use as drug carriers, e.g. 5-fluorouracil (FU) and methotrexate (MT), azathioprine (AZA) [7, 8]. Particular attention is drawn to the possibility of using P3HB in tissue engineering. Currently, the gold standard of tissue reconstruction is autologous transplantation due to the lack of stimulation of the recipient's immune response. Autologous grafts are great for the reconstruction of small bone defects; however, in the case of larger defects, it is necessary to use other reconstruction materials. It is crucial that the materials used minimally activate the recipient's immune system, and thus do not cause transplant rejection. P3HB as a biocompatible material is an object of tissue engineering research. Currently, work is underway on P3HB-based materials inoculated with human osteoblasts [9] and mesenchymal stem cells [10], which give promising results. P3HB has also found application in the treatment of bone defects associated with osteomyelitis. Modifications enabling the introduction of antibacterial substances at the site of the bone defect seem to be useful for this purpose [11].One of the compounds that may be an interesting addition to P3HB-based implants is methylcellulose (MC), also known as hypromellose. The substrate for its synthesis is cellulose, commonly

found in plants. MC belongs to the group of ethers obtained by substituting hydrogen atoms from cellulose hydroxyl groups for methyl and hydroxypropyl groups [12]. Solubility in water is a feature that distinguishes MC from cellulose [13]. Cellulose has hydroxyl groups responsible for the formation of hydrogen bonds, enabling the formation of an ordered crystalline structure of this compound. These groups are replaced with methoxy groups, which contributes to changing the properties of MC.

MC as an easy-to-obtain, biocompatible and biodegradable material has been used in the production of hydrogels promoting the repair of joint cartilage [13] and as a drug carrier in the pharmaceutical industry [14]. Currently, research is underway into the use of MC in the production of bioinks for the bioprinting process [12].The aim of this paper is to examine the effect of hypromellose on the P3HB matrix and to evaluate the properties of the material in terms of applications in regenerative medicine and the biomedical industry. Mechanical tests, thermal properties and biocompatibility with the living organism have been established.

II. METHODOLOGY

3.1. Materials

Poly(3-hydroxybutyrate), P3HB, was supplied by Biomer (Krailling, Germany). Weight average molecular mass of P3HB is M_w = 443,900 g/mol and its molecular mass dispersion M_w / M_n=5.72 measured by the size exclusion chromatography in chloroform. P3HB melt flow index is 0.11 g/10 min (180 °C at 2.16 kg). The molar mass of the repeating unit equals 86.09 g/mol.The used of nanofiller was hypromellose, (methyl cellulose), CAS number: 9004-65-3, was supplied by Sigma Aldrich (Schnelldorf, Germany). The molar mass of hypromellose is 478.49 g/mol.

3.2. Methods and Instrumentation

3.2.1. Nanobiocomposite preparation

The nanocomposites were prepared by melt mixing of P3HB with different amounts of hypromellose i.e. 0.5, 1, 2, and 3 mass % in a co-rotating twin-screw micro-extruder. The screw extruder was equipped with the four temperature controlled zones, i.e.: zone 1, zone 2, zone 3, and feed zone. Before extrusion, the nanofiller was homogenized with the polymeric matrix using an electric grinder.

The conditions of the melt mixing and full characterisation of nanocomposite samples based on P3HB and nanofiller (hypromellose) were given in Table 1. The unfilled P3HB was also meltmixed to prepare a reference material. Sample abbreviations and preparation conditions are given in Table 1.

Sample designation	Content of hypromellose	Temperature of extrusion	Speed of rotation
P3HB	0	zone 1: 125°C zone 2: 135°C zone 3: 150°C zone 4: 160°C head: 166°C	350 rpm
MP3HB-0.5	0.5		
MP3HB-1	1		
MP3HB-2	2		
MP3HB-3	3		

Table 1. Sample abbreviations and preparation conditions of obtained nanocomposites.

3.2.2. Thermogravimetry

Thermogravimetric analysis (TGA) was performed using a Metler Toledo TGA/DSC 3+ thermogravimetric analyzer. The samples were heated at a heating rate of 5°C/min in the temperature range of 25-600°C in an inert gas (nitrogen) atmosphere.

3.2.3. Differential scanning calorimetry

All experiments by calorimetry in the temperature range from -90°C to 195°C were performed using the Differential Scanning Calorimeter (DSC) a Q1000TM from TA Instruments, Inc. (New Castle, DE, USA). This calorimeter is the heat-flux type and is equipped with a mechanical refrigerator to control the heating and cooling of samples. Measurements were carried out in a nitrogen atmosphere with a constant flow rate of around 50 ml/min. The mass of samples used for measurements by DSC was about 10 mg. The experimental heat flow was measured at a heating rate of 10°C/min after previous cooling at a rate of 10°C /min by standard DSC. The temperature and heat flow rate calibration in the DSC apparatus was performed using parameters of melting indium, onset melting temperature, T_m(onset) = 156.6°C, and heat of fusion, ΔH_f= 28.45 J/g (3.281 kJ/mol) [16]. In order to obtain accurate results, the heat capacity was calibrated by sapphire [16]. The heat flow data were collected from the second heating run after the controlled cooling.

3.2.4. X-rays

X-ray diffraction methods (X-ray diffractometer Miniflex II from Rigaku company) were applied to gain information about the structural changes and phases of the obtained composites. A CuKa (λ = 0.1542 nm) ray was used for analysis. The step size was set to 0.02°/3s, the imposed voltage and current were 40 kV and 10 mA, respectively, and the diffraction angle (2q) was varied from 3 to 30°.

3.2.5. Biological studies

3.2.5.1. Cell culture and in vitro biocompatibility assessment

Reference L929 mouse fibroblasts (LGC Standards, Middlesex, UK), human Hs68 skin fibroblasts (CRL-1635™) were used to evaluate biocompatibility tested

materials in vitro.Mouse L929 fibroblasts were cultured at 37°C in a 5% CO_2 in Roswell Park Memorial Institute (RPMI)-1640 medium (Biowest, Nuaillé, France), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biowest, Nuaillé, France), and standard antibiotics: penicillin (100 U/mL) and streptomycin (100 µg/mL) (Biowest, Nuaillé, France), while the human fetal osteoblastic cell line hFOB 1.19 (CRL-11372™) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were cultured in a 1:1 mixture of phenol-free Dulbecco's modified Eagle's medium and Ham's 12-F medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2.5 mL of l-glutamine (Biowest, Nuaillé, France), 10% heat inactivated FBS (Biowest, Nuaillé, France) and 0.3 mg/mL of geneticin (G418) (Sigma-Aldrich, Saint Louis, MO, USA), whereas human bone osteosarcoma Saos-2 (HTB-85™, ATTC) were cultured in McCoy's 5A Medium (Biowest, Nuaillé, France) supplemented with 15% heat-inactivated FBS (Biowest, Nuaillé, France), and standard antibiotics: penicillin (100 U/mL) and streptomycin (100 µg/mL) (Biowest, Nuaillé, France),was used in this study. Cell cultures were supplemented with fresh medium two or three times per week to keep them in log phase.

The confluent monolayer was treated with 0.25% trypsin-EDTA solution (Biowest, Nuaillé, France) to passage.Cytotoxicity of the P3HB, MP3HB-0.5, MP3HB-1 and MP3HB-2, MP3HB-3, was tested using L929, hFOB 1.19 and Saos-2 cell line (density of 2×10^5 cells/mL) according to the ISO norm 10993–5 (International Organization for Standardization, 2009; Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity), based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction test, as previously described [17]. For cytotoxicity testing P3HB, MP3HB-0.5, MP3HB-1 and MP3HB-2, MP3HB-3, corresponding to one-tenth of the well surface area, were prepared.The cell cultures in medium alone, without the P3HB, MP3HB-0.5, MP3HB-1 and MP3HB-2, MP3HB-3, were used as a positive control (PC) for cell viability, whereas cells treated with 0.03% H_2O_2 were used as a negative control (NC), i.e. 100% dead cells due to lysis. The absorbance was measured spectrophotometrically using a Multiskan EX plate reader (Thermo Scientific, Waltham, MA, USA) at 570 nm. MTT reduction relative to untreated cells (%) = (absorbance of treated cells/absorbance of untreated cells × 100%)×100%. The above-mentioned tests were performed in three independent experiments.

3.2.5.2. Monocyte Activation Assay

THP1-Blue™ cells (Invitrogen, San Diego, CA, USA), derived from the human THP-1 monocyte cell line, were used to assess whether the P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2, and MP3HB-3, could stimulate monocytes through induction of nuclear factor kappa B (NF-κB). THP1-Blue™cells were derived from the human THP-1 monocyte cell line by stable integration of anNF-κB-inducible embryonic alkaline phosphatase (SEAP) reporter construct. Activation of NF-κB resulted in the secretion of SEAP, which could be detected spectrophotometrically using Quanti-Blue reagent (Invitrogen, San Diego, CA, USA).THP1-Blue™ cells were cultured at 37°C in a 5% CO_2 in RPMI-1640 medium supplemented with 10% FBS, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), penicillin/streptomycin (100 U/mL), and 2 mM glutamine (all from Biowest,Nuaillé, France), as well as blastidin (10 µg/mL) (Invitrogen, San Diego, CA, USA), as previouslydescribed [18]. For monocyte activation assay THP-1 Blue™ cells (density of 5×10^5 cells/mL) stimulated with P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3, prepared as described above.

Untreated cells in culture medium alone served as a negative control, whereas cells treated with Escherichia coli lipopolysaccharide (LPS) (1 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) were taken as a positive control. The SEAP activity was measured spectrophotometrically using a Multiskan EX plate reader (Thermo Scientific, Waltham, MA, USA) at 650 nm. The above-mentioned tests were performed in three independent experiments.

3.2.5.3. Statistical analysis

Statistical significance was accepted at a p-value < 0.05 nonparametric U Mann-Whitney. Data are presented as mean values ± SD. For statistical analysis the STATISTICA 123PL software was used (Stat Soft, Poland).

IV. RESULTS AND DISCUSSION

Figure 1 shows diffractograms of unfilled P3HB, and its nanocomposites with 0.5, 1, 2 and 3 wt.-% of hypromellose (designated P3HB-0.5, P3HB-1, P3HB-2, and P3HB-3, respectively). The XRD analysis of P3HB and its composites obtained with the use of hypromellose allowed us to observe the effect of introducing the nanoadditive on changing the phase composition. A change in shape of the peaks can be observed in the range of angle 2 theta form 18 to 24. The wide indistinct peak on the P3HB diffractogram transformed into 3 sharp peaks on the composite diffractograms.

The effect of raising background was visible in the increase of the the amount of Hypromellose used. Therefore, it can be clearly stated that the introduction of hypromellose increased the amorphous phase content and P3HB inreases its degree of crystallinity in nanomaterials. It can be seen by the increase in the intensity of the peaks.In order to examine the durability, processing and functional

properties of the obtained nanomaterials, a thermogravimetric analysis, TGA, (Fig. 2) was carried out. TG curves of nanocomposites showed that thermal stability of all nanocomposites is higher than that of the unfilled P3HB.

Introduction of hypromellose into P3HB matrix causes the increase of the degradation temperature of nanocomposites in reference to the unfilled P3HB e.g. 2% mass loss takes place at temperature 222.8°C. For the obtained nanocomposites were reported in the same conditions 245.6, 260.1, 250.2, and 249.6°C in the case of MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3, respectively. It can be seen that the weight loss temperature of all nanocomposites is higher than unfilled P3HB. The most desirable is the MP3HB-1 sample with the highest degradation temperature. The addition of 1% mass of nanofiller increases the degradation temperature of nanocomposites by about 37°C in reference to the unfilled P3HB.

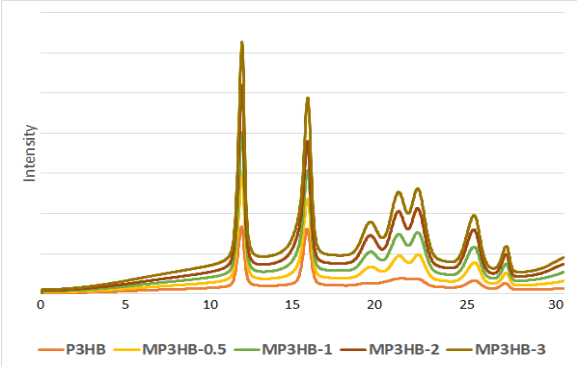


Figure. 1. Diffractograms of the unfilled P3HB, and its nanocomposites with 0.5, 1, 2 and 3 wt.-% of hypromellose (designated MP3HB-0.5, MP3HB-1, MP3HB-2, and MP3HB-3, respectively).

Figure 3 shows the heat flow rates of obtained nanocomposites (0.5, 1, 2, 3 wt %) versus temperature which was measured using the standard differential scanning calorimetry (DSC). The measurements were carried out with the heating rate of 10°C/min in the temperature range of -90°C to 195°C after prior cooling in the same rate from the temperature range of 195°C to -90°C.

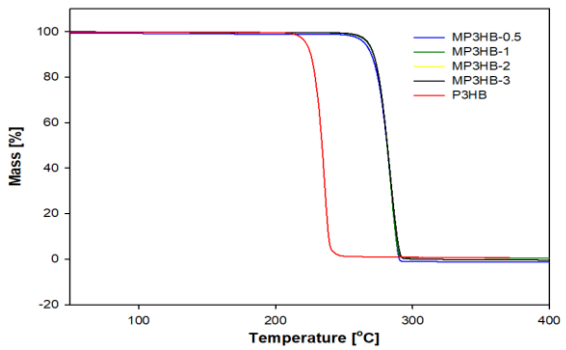


Figure 2. Thermogravimetric analysis of the unfilled P3HB and their nanocomposites containing 0.5, 1, 2 and 3 mass % of hypromellose (nanocomposites were marked as MP3HB-0.5, MP3HB-1, MP3HB-2, MP3HB-3, respectively).

Sample designation	T_g [°C]	ΔC_p [$J \cdot g^{-1} \cdot °C^{-1}$]	T_{m1} [°C]	T_{m2} [°C]	ΔH_f [$J \cdot g^{-1}$]
P3HB	7.7	0.1768	-	165.80	100.36
MP3HB-0.5	0.8	0.4547	155.45	166.80	21.95
MP3HB-1	0.6	0.5150	155.20	166.50	0.40
MP3HB-2	0.4	0.6244	155.20	166.90	4.43
MP3HB-3	0.10	0.6120	153.30	165.30	11.40

Table 2. Comparison of the thermal parameters of the unfilled P3HB nanocomposites with hypromellose of representative samples obtained while heating at a rate of 10°C/min after cooling at the same rate.

Qualitative thermal analysis was carried out based on the heat flow rate of the semicrystalline P3HB and its nanocomposites. The glass transition and the melting were observed during the heating scan for all materials. Cold crystallization in the form of an exothermic peak was recorded between the glass transition and melting region for all nanocomposites (see Figure 3). Thermal parameters of phase transitions were estimated and listed in Table 2. The temperature glass transition, T_g, and the change of heat capacity at T_g, ΔC_p, were established based on the study of the glass transition region under heating and the heat of fusion, ΔH_f, and the melting temperature, T_{m1} and T_{m2}, were also estimated based on the analysis of melting region. Additionally, it can be noted that the double melting peak was maintained as with the P3HB analysis. It is probably related to the presence of crystals of different lamella thickness [19].

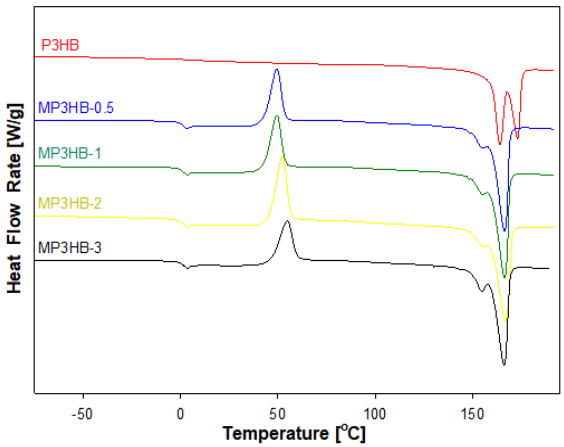


Figure 3. Heat flow rate vs temperature obtained based on the DSC analysis of the unfilled P3HB and its nanocomposites containing 0.5, 1, 2 and 3 mass % of hypromellose (they were marked as MP3HB-0.5, MP3HB-1, MP3HB-2, MP3HB-3, respectively). The results were obtained during heating at 10°C/min after cooling the sample at a constant rate of 10°C/min from melt.

The influence of the hypromellose additive on the glass transition temperature (T_g), and melting temperature(T_m), and the variations of heat of fusion and the heat capacity at T_g were investigated. Figure 3 shows the curves of the unfilled P3HB and its nanocomposites with a different content of hypromellose. Based on a qualitative analysis (analysis of heat flow rate) of the unfilled semi-crystalline P3HB and its nanocomposites, the changes of heat capacity at T_g and heat of fusion at T_m were estimated.

It can be observed that the values of glass transition are lower than unfilled P3HB. This is proof of the plasticizing effect of hypromellose on the P3HB matrix.The change of glass transition temperature for nanocomposite based on the highest amount of nanofiller in comparison with the unfilled P3HB equals 7°C and the melting temperature is established on this same level like T_m of P3HB.Particularly interesting and useful is the substantial difference between values of the degradation and melting temperature that facilitates processing and prevents the degradation of the material.

It can be noted that the best thermal properties and the widest processing window expressed as the difference between the degradation temperature and the melting temperature were obtained for P3HB-1 and equals 93.6°C. This movement apart from melting and decomposition temperatures of nanocomposites based on P3HB facilitates processing and prevents the degradation of the material. Moreover, the changes of heat of fusion, and changes of heat capacity were analyzed in relation to the values expressed as percentages of the crystalline phase (W_c) and the mobile (W_a) and rigid amorphous (W_{RAF}) phases (please see table 3).Figure 4 shows the analysis example of the changes of heat capacity at the glass transition temperature and the measured heat of fusion for semi-crystalline MP3HB-2 samples with different thermal histories, after different cooling rates.

Depending on different cooling rates, semi-crystalline MP3HB-2's glass transition can be situated between -0.2°C and 15.6°C with a ΔC_p (changes of heat capacity between liquid and solid states at T_g) in the range 0.2–0.6 $J \cdot °C^{-1} \cdot g^{-1}$. The melting endotherm of semi-crystalline MP3HB-2 occurs between 151.2 and 170.4°C with a heat of fusion in the range 0.9 – 97.1 $J \cdot g^{-1}$. It should be noted that the changes of heat capacity values were estimated from qualitative thermal analysis (analysis of heat flow rate).

The red points are characteristic of a two-phase model. In turn, the black squares confirm the existence of the rigid amorphous fraction and characterized the three-phase model. The solid straight line is designated between full amorphous and full crystalline MP3HB-2 nanocomposite represented by yellow star points. The change of heat capacity for fully amorphous MP3HB-2 equals 0.6393 $J \cdot °C^{-1} \cdot g^{-1}$, and the heat of fusion for fully crystalline samples equals 142 $J \cdot g^{-1}$.

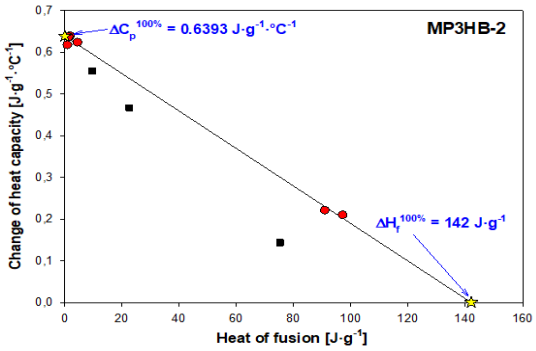


Figure 4. Dependence of the heat capacity change at the glass transition temperature versus the heat of fusion of the semi-crystalline nanocomposites P3HB containing 2 mass % of hypromellose (denoted as MP3HB-1). The red points are characteristic of the two-phase model of nanocomposite, and the black squares show the three-phase model of MP3HB-2. The solid straight line is designated between full amorphous and full crystalline MP3HB-2 samples represented by yellow star points.

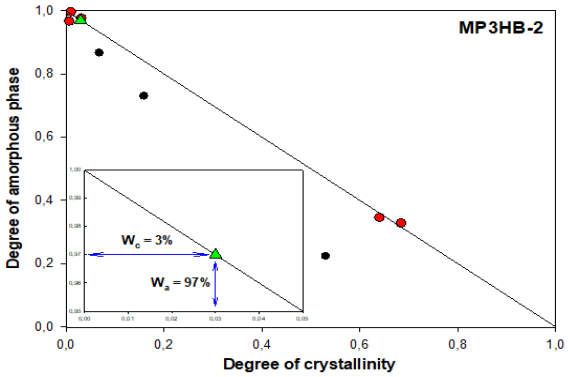


Figure 5. Dependence of the amorphous fraction as a function of the degree of crystallinity of the semi-crystalline P3HB nanocomposite containing 2 mass % of hypromellose (denoted as MP3HB-2).

The dependence of the mobile amorphous fraction on the degree of crystallinity of semi-crystalline MP3HB-2 is shown in Fig. 5. The data obtained from Fig. 4 were converted to corresponding degree of amorphous phase using $W_a = \Delta C_p/\Delta C_p^{100\%}$ and degree of crystallinity using $W_c = \Delta H_f/\Delta H_f^{100\%}$.

The degree of crystallinity (W_c) was defined as the ratio between experimental heat of fusion (ΔH_f) of the semicrystalline sample and heat of fusion of the fully crystalline MP3HB-2 sample ($\Delta H_f^{100\%}$). ΔC_p and $\Delta C_p^{100\%}$ are variations of heat capacity at T_g (mobile) of semi-crystalline and full amorphous MP3HB-2, respectively.

For computation, the value of $\Delta C_p^{100\%}$ equals 0.6393 $J \cdot °C^{-1} \cdot g^{-1}$ was used. The green triangle point derived from qualitative analysis of the MP3HB-2 is also plotted in Fig. 5.

The data of this green triangle point was obtained during heating at 10°C/min after cooling the sample at constant rate 10 °C/min from the melt.

It has been calculated that the selected MP3HB-2 sample (green triangle) remains in a two-phase model which is confirmed in Fig. 5 (the sample didn't show deviation from a straight line). As illustrated in Fig. 5, MP3HB-2 contains 97% amorphous, 3% crystalline phase and the rigid amorphous fraction is not observed.Similar analyses for other nanocomposites were carried out and listed in Table 3.

The change of heat capacity for fully amorphous material and the heat of fusion for fully crystalline material were also obtained using the same method as for MP3HB-2. In Table 3, the phase contents are calculated for samples which were cooled at a controlled rate of 10°C/min and after that heated at a rate of 10°C/min. Based on those results, it can be concluded that the addition of hypromellose leads to the decrease of the degree of crystallinity of nanocomposites.

Sample designation	$C_p^{100\%}$ [$J \cdot g^{-1} \cdot °C^{-1}$]	$H_f^{100\%}$ [$J \cdot g^{-1}$]	W_a [%]	W_c [%]	W_{RAF} [%]
P3HB	0.4900	145.0	33	63	4
MP3HB-0.5	0.6398	145.4	71.1	15.1	13.8
MP3HB-1	0.5770	147.9	89.3	0.3	10.4
MP3HB-2	0.6393	142.0	97	3	0
MP3HB-3	0.7345	120.4	83.3	9.5	7.2

Table 3. Characterization of phase contents for the unfilled P3HB and its nanocomposites with hypromellose

In order to determine the application possibilities of the newly obtained nanocomposites, the cell viability and immunocompatibility in the milieu of tested materials were also carried out. The influence of P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3 the viability of the reference L929 mouse fibroblasts and human hFBO 1.19 and Saos-2 was assessed by MTT reduction assay, based on the measurement of mitochondrial dehydrogenase activity in the presence or absence of the tested substances. In this assay, the biochemical activity of mitochondrial enzymes was positively correlated with cell viability.

The viability of L929 hFOB 1.19, Saos-2 cells incubated in the presence of P3HB, MP3HB-1, MP3HB-2 and MP3HB-3.0 are higher than 70%, meeting the biological safety standard (Fig. 6), thus these composites can be used in contact with human tissue. The L929 and Saos-2 cell lines also showed viability above 70% after contact with MP3HB-0.5. Only the hFOB 1.19 cell line did not meet the safety criterion in vitro (MP3HB-0.5 composite) (Fig. 6).

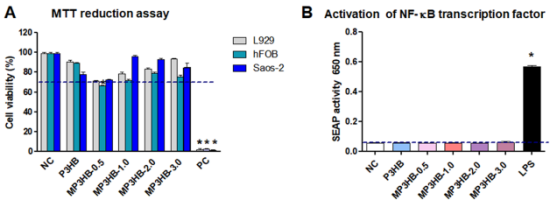


Figure 6. Biocompatibility and immunocompatibility of P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3.

A - Cytotoxicity the P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3 towards mouse L929 and human hFob 1.19 and Saos-2 was estimated as percent of cells, which were able to reduce tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT). NC – negative control (cells treated with H_2O_2), PC-positive control (cells in medium alone). Results are shown as mean ± standard deviation (SD).

Three experiments were performed in triplicates for each experimental variant. The red line indicates the minimal percentage of viable cells (70%) required to confirm the extracts as non-cytotoxic at the in vitro level. Statistical analysis was performed using the nonparametric U Mann-Whitney test with significance, $p < 0.05$ (*unstimulated cells vs. stimulated cells).B- Activation of embryonic alkaline phosphatase (SEAP) in THP-1 x Blue monocytes. THP-1 x Blue monocytes were stimulated were stimulated with lipopolysaccharide (LPS) E. coli alone, or P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3. Control cells were subcultured in the medium alone. The supernatants were collected for assessment SEAP calorimetrically.
The experiments were performed three times in triplicate for each experimental variant. The results are shown as mean ± standard deviation (SD). Statistical analysis was performed using the nonparametric U Mann-Whitney test with significance, $p < 0.05$ (*unstimulated cells vs. stimulated cells)THP1-Blue™ cells, like other monocytes, are very sensitive to increased inflammatory response in reaction to contact with a foreign antigen.

Activation of the inflammatory response is aimed at recognizing and destroying the threat and restoring balance in the body. Excessive inflammatory response as a result of excessive stimulation of monocytes (e.g. by the components of the biomaterial or contaminants such as endotoxins) may provide to tissue degradation, and disintegration of cell barriers [20].

THP1-Blue cells, have receptors on their surface respond to ligands, such as peptides, endotoxins, and glycoconjugates, through toll-like receptors (TLR): TLR-2, TLR-4, TLR-5, TLR-6, and TLR-

8. Stimulation TLR provided to NF-κB is activated, and the SEAP enzymatic marker is subsequently secreted into the cell culture milieu. In this study we show that In the milieu of LPS THP-1xBlue monocytes responded by a high level of SEAP indicating the activation of nuclear transcription factor NF-kappa B.

The P3HB, MP3HB-0.5, MP3HB-1, MP3HB-2 and MP3HB-3 did not induce SEAP activity in any of the test concentrations beyond the negative control value of cells grown in medium alone, which confirmed the immunosafety of the tested extracts and the absence of, or very low, endotoxin contamination.

According to the Food and Drug Agency Guidance as well as the European Medicines Agency, concentration endotoxin cannot be higher than 0.25 EU in biomaterials in contact with human blood/tissue.

In our study we showed that the activation of THP1-Blue™ monocytes with 0.25 EU of endotoxin (positive control of monocyte activation) resulted in a significant increase in SEAP production, as shown by an absorbance of 1.5 ± 0.02 at 620 nm (Fig. 6B).

V. CONCLUSION AND FUTURE SCOPE

The nanocomposites of P3HB and hypromellose were obtained during direct mixing in a co-rotating twin-screw micro-extruder. The conducted studies revealed clearly the influence of nanofiller in the P3HB matrix on the selected thermal and biological properties of the prepared nanocomposites. It has been observed that the introduction of hypromellose into a polymeric matrix influences the improved thermal stability of the nanocomposites, measured by TG, under non-oxidative conditions.

The best thermal stability was observed for the MP3HB-1 and was comparable to the degradation temperature value of 2% weight loss. The presented thermal analysis showed a decrease in the glass transition with an increasing amount of nanofiller in the obtained P3HB composites and it confirmed plasticized effect of hypromellose. The increase in the degradation temperature of the nanocomposites is preferred due to a widened processing window, which gives better processing possibilities of the obtained nanocomposites in relation to unfilled P3HB.Additionally, introducing nanocrystalline cellulose into a P3HB matrix leads to a decreased degree of crystallinity of the nanocomposites and increased of amorphous fraction. The analisis was confirmed using the X-ray spectra and differential scanning calorimetry. Biological studies in terms of implant applications have shown that theimmunosafety of the tested extracts and the absence of, or very low, endotoxin contamination.

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