

Improvement of biocompatibility of high molecular weight poly-3-hydroxybutyrate by blending with its functionalized oligomers

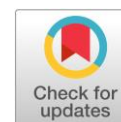
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Abstract

Oligomers of poly-3-hydroxybutyrate (PHB) were prepared by aminolysis of high molecular weight PHB with ethylenediamine and 1,4-diaminobutane. Polymer-oligomer blends (10, 30, and 50% content of the oligomers) were prepared as films by solution casting. As the content of oligomers increased, a gradual increase in the hydrophilicity of the polymer surface was observed, resulting reflected in the water contact angle decrease from 84° to 72–76°. In addition, a moderate decrease in elongation at break, Young's modulus, and tensile strength for the blends were observed as more oligomer was added to the film. Finally, the viability of NIH-3T3 mouse fibroblasts was higher compared to intact PHB when growing in non-prepared polymer/oligomer mixtures. These findings confirm the benefits of the introduction of a hydrophilic functionalized oligomer into the PHB matrix in terms of improving the biocompatibility of the resulting polymer/oligomer blends.

Keywords

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Key findings

- Addition of aminated oligomers of poly-3-hydroxybutyrate (PHB) to the parent PHB improves the hydrophilicity of the obtained blends.
- The biocompatibility of the obtained polymer-oligomer films was demonstrated via an increased adhesion of NIH-3T3 mouse fibroblasts.
- Only moderate influence of the oligomer presence of the mechanical properties of the polymer-oligomer blends was observed.

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1. Introduction

The use of biocompatible polymers is of great importance in many reconstructive fields of modern medicine. Among such biocompatible polymers, polyhydroxyalkanoates (PHAs), polyesters of hydroxy derivatives of fatty acids of microbiological origin, are of special interest [1]. Thus, PHAs are used to develop sutural material, stents, tissue-engineered constructions, and wound dressings. However, the most widely studied representative of PHA, poly-3-hydroxybutyrate (PHB), has such a serious drawback as a relatively high hydrophobicity, which can prevent its effi-

cient interaction with the biological media in the body and upon the cell adhesion [2].

In this study we proposed the approach of the modifying of the polymer structure by obtaining a homopolymer-like blend by introducing its oligomeric functionalized derivatives into the intact polymer. As an example, we used poly-3-hydroxybutyrate (PHB) and its oligomers obtained by aminolysis of PHB with bifunctional amines – ethylenediamine and 1,4-diaminobutane. As a result, we obtained several film samples based on such blends with different ratios of components to evaluate their surface, physical

and mechanical characteristics as well as the ability to maintain cell adhesion and proliferation.

2. Materials and methods

2.1. Materials

Poly-3-hydroxybutyrate (PHB) (weight average molecular weight (M_w) 920 kDa; polydispersity (D) 2.5) was synthesized in Siberian Federal University according to previously described method [3]. N,N-dimethylformamide (DMF) (Roth, Germany), 1,4-dioxane (reagent grade) (ECOS-1, Russia), 1,4-diaminobutane (99 wt.%) (Aldrich, Netherlands), 1,2-ethylenediamine (reagent grade) (Labsintez, Russia), isopropyl alcohol (ACS grade) (ECOS-1, Russia), chloroform (reagent grade) (ECOS-1, Russia) were used for the synthesis and dissolution.

Oligomers of two types were obtained by aminolysis of a high molecular weight PHB with diamines, 1,4-diaminobutane or ethylenediamine, as described below in the Methods section. The oligomer obtained by aminolysis with 1,4-diaminobutane (hereinafter referred as D-PHB) had M_w 4.0 kDa and D 1.5. The oligomer obtained by aminolysis with ethylenediamine (E-PHB) had M_w 5.6 kDa and D 1.7.

2.2. Methods

2.2.1. Preparation of PHB oligomers

To prepare aminated PHB oligomers, 3.44 g of PHB (this amount is equal to 0.04 mol in terms of monomers; the molecular weight of one mole of PHB monomer in the esterified state ($-\text{O}-\text{CH}(\text{CH}_3)-\text{CH}_2-\text{CO}-$) is 86 g/mol) was dissolved in 86 ml of dimethylformamide at a temperature 100 °C to obtain a 4% solution (w/v). After complete dissolution of the polymer, ethylenediamine (molecular weight (M) 60.10 g/mol, density (d) 0.90 g/cm³) or 1,4-diaminobutane (M 88.15 g/mol, d 0.88 g/cm³) were added in the solution in the amount of 0.004 mol (i.e. 268 μl and 402 μl , respectively). The resulting mixtures were kept at 100 °C for 1 h. The separation of oligomers from the mixture was carried out by their precipitation with the addition of a fourfold volume of isopropyl alcohol followed by filtration and triple washing with isopropyl alcohol using a Schott filter. Two types of oligomers (D-PHB and E-PHB) were used for further preparation of polymer films.

2.2.2. Preparation of polymer blend films

For preparation of the blends, PHB, D-PHB and E-PHB were separately dissolved in chloroform in a 2% amount (w/v). Then the resulting solutions were mixed in 9:1 (10%), 7:3 (30%) and 1:1 (50%) ratios of PHB to its aminated oligomers, respectively, and incubated for three hours with occasional agitation. The blends as well as a PHB solution were further used to prepare films by solution casting. Specifically, 20 ml of the respective solution was preheated to 35 °C and poured on a degreased Petri

dish and dried at room temperature. The following studies were carried out no earlier than ten days after the films were prepared. The samples prepared from the PHB solution were used as a control.

2.2.3. Molecular weight analysis

Molecular weight and molecular weight distribution of the polymers were analyzed using gel permeation chromatography using Agilent 1260 Infinity chromatograph (Agilent Technologies, U.S.) with Agilent PLgel Mixed-C column equipped with an isocratic pump, an autosampler and a differential refractometer. 50 μl of polymer solution in chloroform (5 mg/ml) was injected at a 1.0 ml/min flow rate and at 40 °C using chloroform as an eluent. The calibration curve was plotted based on the measurement of Agilent EasiVial PS-H polystyrene standards.

2.2.4. Surface analysis

Surface properties of the films were tested with DSA-25E drop shape analyzer (Krüss, Hamburg, Germany) using DSA-4 software for Windows. Drops of water and diiodomethane, 1.5 μL each, were alternately placed on the film surface, and contact angles (CA) of the liquids were measured in a semiautomatic mode by the sessile drop technique. The results of the measurements were used to calculate surface free energy (SFE) and its dispersive (DSFE) and polar (PSFE) fractions by the Owens, Wendt, Rabel and Kaelble method [4, 5].

2.2.5. Mechanical testing

Mechanical properties of the prepared films were studied using Instron 5565 electromechanical tensile testing machine (UK). Dumbbell-shaped samples 50 mm long, 6.1 mm wide, and 25–30 mm thick were prepared for studying mechanical properties of the films. The thickness of the films was measured prior to testing, using LEGIONER EDM-25-0.001 electronic digital micrometer. The samples were stored in standard conditions for at least two weeks to reach equilibrium crystallization. At least five samples were tested for each type of films. Measurements were conducted at room temperature, and the clamp-length of the samples was 30 mm. The crosshead speed was 3 mm/min at room temperature. Young's modulus (E , MPa), tensile strength (s , MPa) and elongation at break (e , %) were automatically calculated by Instron software Bluehill 2 (Elancourt, France). To obtain Young's modulus, the software calculated the slope of each stress-strain curve in its elastic deformation region. Measurement errors did not exceed 10%.

2.2.6. Analysis of biological properties

For the biotests, the studied films in the form of discs 10 mm in diameter were placed in 24-well plates (TPP, Switzerland) and sterilized in Sterrad NX plasma sterilizer (Johnson&Johnson, USA). NIH-3T3 mouse fibroblast culture cells were then seeded onto the polymer discs with $1.0 \cdot 10^4$ cells/well/ml.

Table 1 Surface properties of polymer films.

Oligomer	Content, %	CA _w , ° ^a	SFE, mN/m ^a	PSFE, mN/m ^a	DSFE, mN/m ^a
Control	0	84.7±4.5	40.9±0.8	2.7±0.2	38.2±0.6
D-PHB	10	84.8±1.6	40.5±0.3	2.7±0.1	37.8±0.2
D-PHB	30	82.6±1.6	41.9±1.0	3.2±0.2	38.7±0.8
D-PHB	50	76.0±0.6	45.2±0.3	5.3±0.1	40.0±0.2
E-PHB	10	89.1±4.7	38.5±1.0	1.8±0.8	36.7±0.2
E-PHB	30	81.0±0.4	42.5±0.4	3.7±0.1	38.7±0.4
E-PHB	50	71.9±2.6	47.1±0.6	6.9±0.2	40.3±0.4

^a CA_w contact angle of water; SFE – surface free energy; PSFE and DSFE – polar and dispersive fractions of SFE, respectively.

Table 2 Mechanical properties of polymer films.

Oligomer	Oligomer content, %	Young's modulus, MPa	Tensile strength, MPa	Elongation at break, %
Control	0	3262.3±139.0	33.5±0.2	1.23±0.09
D-PHB	10	3692.8±203.4	26.7±3.5	0.83±0.07
D-PHB	30	3308.4±108.2	16.8±0.7	0.59±0.04
D-PHB	50	3359.8±208.5	7.4±2.7	0.32±0.08
E-PHB	10	3269.1±244.5	25.4±3.4	0.89±0.03
E-PHB	30	3370.0±302.0	21.9±2.0	0.74±0.06
E-PHB	50	2938.6±220.7	7.8±2.9	0.36±0.11

The cells were cultivated in a DMEM medium with the addition of 10% fetal bovine serum, antibiotic solution (streptomycin 100 µg/ml, and penicillin 100 U/ml (Gibco, Invitrogen)), and incubated in a CO₂ incubator at atmospheric CO₂ concentration of 5% at 37 °C. The medium was renewed every three days. The viability of cells was assessed by the MTT assay based on the ability of mitochondrial dehydrogenases of living cells to reduce soluble (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) into crystals of 3-(4,5 -dimethylthiazol-2-yl)-2,5-tetrazolium-formazan. The optical density was determined after dissolving MTT formazan in DMSO using iMark microplate photometer (BioRad, USA). The number of cells was determined by the calibration curve after fixing with a 4% formaldehyde solution.

2.2.7. Statistical processing

Statistical processing of the results was performed using Microsoft Excel 2003. Arithmetic means and standard deviations were found as a result. Significant differences between the mean values were tested using Student's *t* test (significance level: *p* = 0.05) by the standard methods.

3. Results and Discussion

Low molecular weight PHB was unsuitable for preparation of polymer films due to its fragmentation in the small lamelli as the solvent evaporated. In general, a decrease in the molecular weight of polymers leads to a decrease in their mechanical characteristics. For PHB, a significant decrease in the mechanical characteristics of the polymer occurs when the molecular weight decreases below 160 kDa [6], and primarily occurs simultaneously with the a significant reduction on the tensile strength and elongation at break without significant changes of the elastic modulus [7]. Thus, due to poor mechanical properties of low molecu-

lar weight PHB, it was infeasible to obtain the films of any sustainable strength. However, its blending with high molecular PHB made the production of such films feasible.

At a low content of D-PHB or E-PHB in the obtained blends (10%), the values of the water contact angle (CA_w), PSFE and DSFE were at the levels comparable to those of the pure PHB solution (Table 1). As the content of D-PHB increased up to 30–50%, CA_w value reduced from 82.6(±1.6)° to 76.0(±0.6)°, and PSFE and DSFE values increased from 2.7(±0.2) mN/m and 38.2(±0.6) mN/m to 3.2(±0.2)–5.3(±0.1) mN/m and 38.7(±0.8)–40(±0.2) mN/m, respectively. In the case of films with E-PHB, an increase in the E-PHB content to 30–50% was also accompanied by an increase in PSFE (from 3.7(±0.1) to 6.9(±0.2) mN/m) and DSFE (from 38.7(±0.4) to 40.3(±0.4) mN/m) values as well as by an increase in hydrophilicity of the film surface reflected in CA_w changing from 81.0(±0.4)° to 71.9(±2.6)°.

According to the mechanical testing, mechanical properties of the obtained polymer films vary significantly depending on the ratio of oligomer components (Table 2). The films based on the pure PHB had the highest tensile strength (33.5±0.2 MPa). As the content of oligomers increased, the tensile strength decreased down to 7.4±2.7 MPa and 7.8±2.9 MPa for D-PHB and E-PHB, respectively. In the study where the oligomers obtained by thermal degradation of PHB in the presence of ethylene glycol were used [8], a similar dependence was observed: with 25 wt.% of oligomer there was a decrease in tensile strength by 52%, whereas in the present study, a 30 wt.% content of aminated oligomers led to a drop in the respective value by 35–50%. Polymer films with 10% D-PHB had the highest value of Young's modulus (3.7±0.2 GPa), which can be a result of plasticization by addition of oligomer fraction. For the majority of prepared polymer blend films, this value did not differ significantly from that of the sole

PHB film (it varied around 3.3 GPa). The lowest value (around 2.9 GPa) was for E-PHB 50%.

The elongation at break was $1.23(\pm 0.09)\%$ for the sole PHB films and less than 1% for the rest of prepared films, which is a distinctive feature of PHB films compared to that from other PHAs, which are usually more elastic. For all blended films, with an increase in the content of amino oligomers, a decrease in elongation at break was observed.

According to the MTT test (Figure 1), the inclusion of oligomers led to a significant increase in the fibroblast growth compared to the pure PHB films. In case of the D-PHB blends, the highest number of viable cells was observed for 50% oligomer concentration. In polymer blend films with E-PHB, the highest number of viable cells was observed for 10 and 30% oligomer concentrations. At the same time, at 50% oligomer concentration, practically no change in the cell growth was observed as compared to the control sample. It is considered that extreme values of both hydrophobicity and hydrophilicity are not favorable for cell adhesion, whereas their moderate values contribute to more effective protein adhesion, which ensures successful cell adhesion [9]. The decrease in cell growth on PHB films may be due to an excessive level of hydrophilicity (PSFE values) beyond the optimal range.

Various approaches can be applied to increase PHB biocompatibility. The most common methods include modification of the surface of final products to increase their hydrophilicity and, subsequently, to increase the efficiency of their interaction with surrounding cells and tissues. A number of methods, such as plasma treatment [10, 11], laser radiation [12, 13], reactive gases fumigation (e.g., ozone [14]), ultraviolet radiation [15], and chemical reagents treatment [16] may be used for this purpose. These treatments mainly affect the surface without changing the structure of the polymers, which limits their use to final products only. Modification of polyester chains is limited by the tendency of ester bonds to degrade as a result of being exposed to extreme physical and chemical conditions. Another approach is to introduce the modifying additives directly in the structure of the polymer matrix. Such additives include, e.g., plasticizers [17] and oligomers of various origin. As the examples of the latter, there are known copolymers of lactic and adipic acid with 1,2-propanediol [18, 19] or copolymers of adipic acid with ethylene glycol, 1,4-butanediol, and 2-ethyl-1-hexanol [19], which are used for the improvement of mechanical properties of the resulting products.

At the same time, there are many studies on the preparation of polyhydroxyalkanoate-derived oligomers, including simple thermal degradation of these polymers at a relatively low temperature, resulting in the formation of both residues of unsaturated acid (in the case of PHB, *trans*-2-butenic (crotonic)) at the O-terminus and carboxyl groups at the C-terminus of the oligomers [20]. Methods for the preparation of oligomers bearing two terminal hydroxyl groups by means of alcoholysis of high molecular weight

PHAs mediated by diols and triols [21] or by reduction with sodium borohydride [22] were also developed. The main drawback of the abovementioned methods is the possibility to generate, on the oligomer backbone, only a relatively small range of functional groups, such as carboxyl, hydroxyl, or double bonds. This restricts the spectrum of the reactions or necessitates the introduction of additional stages that adversely affect the yield, the purity of the products, or both.

Aminolysis of esters with amines is one of the known methods for the preparation of amides. In the present conditions, when using diamines in DMF, the aminolysis of PHB leads to a scission of the main polymer chain resulting in the formation of terminal amide moieties on the head end of polymer chains and different (3-hydroxybutyric, crotonic and isocrotonic) acids residues – on their tail end (Figure 2) [23]. Although in standard conditions the reaction proceeds quite slowly, even breaking few ester bonds is sufficient to obtain oligomers of 4–6 kDa. The significant increase in hydrophilicity (expressed in a decrease in contact angles of water and in an increase in PSFE value) of polymer matrices containing aminated oligomers in relation to sole high-molecular-weight PHB can be explained by the presence of hydrophilic amino groups.

It is known that treatment of polyesters with diamines, resulting in the aminolysis of ester bonds with the formation of amino groups on polyester surface improves the cell adhesion of the obtained polymers, as well as proliferation and cellular functions, prepotency of stem cell differentiation, and even isolation of certain subgroup of cells. Another method of polymer surface amination is its treatment with radio-frequency ammonium plasma.

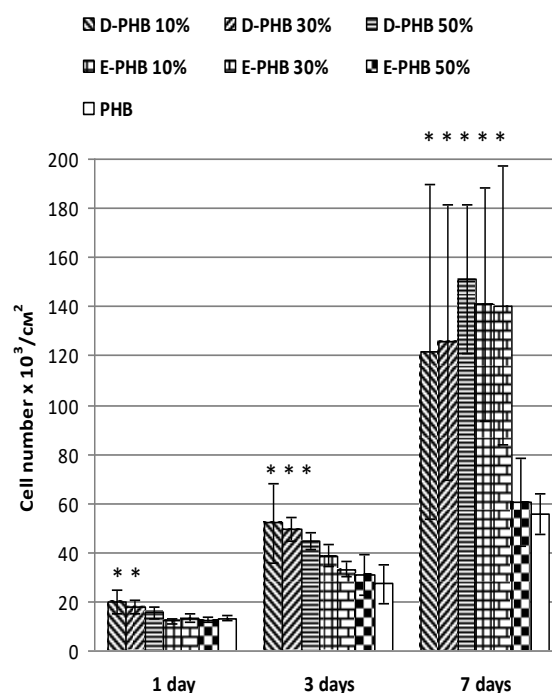


Figure 1 NIH-3T3 fibroblast growth test on PHB-based matrices (E-PHB, D-PHB). The data are shown as the mean \pm confidence interval ($p \leq 0.05$); the asterisks denote the significance in relation to control (pure PHB); $n = 3$.

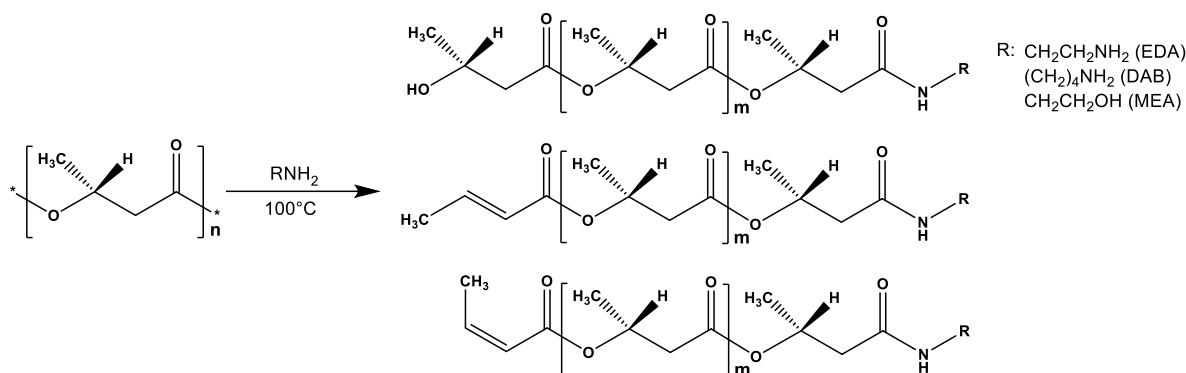


Figure 2 Scheme of PHB aminolysis [23].

Thus, it was shown that the treatment of the surface of PHB with radio-frequency plasma of pure ammonia [24] or its mixtures with argon [7] increases the hydrophilicity of the surface and significantly improves cell adhesion. This demonstrates the prospects of such method of surface modification for biomedical applications [25].

According to the results obtained in this study, similar effects can be obtained by introducing amino-functionalized oligomers of the respective polymers to the parent polymers. It should be noted that the observed increase in the viability of fibroblasts (which is one of the biocompatibility criteria) is much stronger, which could be expected after a relatively small increase in surface hydrophilicity. This is probably the result of a change in the chemical characteristics of the polymer-oligomer blends due to the introduction of PHB oligomers bearing amino groups. Meanwhile, the mechanical properties of functionalized polymer samples, although inferior to those of intact PHB, remain acceptable for the practical application of the resulting polymer-oligomer blends.

4. Limitations

In the framework of this research only the samples of polymer-oligomer blends prepared by solution casting were studied. For the preparation of polymer blends by extrusion, which in fact is more preferable for many applications, further studies on the effect of oligomeric additives on mechanical properties are required. Regarding the assessment of biocompatibility, we did not consider cell morphology on the studied substrates. The additional research may be also required on the interaction of the herein reported materials with other living tissues, such as muscle, nervous or epithelial ones.

5. Conclusions

PHB oligomers were prepared *via* aminolysis of high molecular weight PHB with ethylenediamine and 1,4-diaminobutane, and then subsequently blended with the parent high molecular weight PHB. From the resulting polymer-oligomer blends, film samples were prepared and studied. It was found that the introduction of oligomers in high concentrations into the polymer led to an increase in

the hydrophilicity of the surface of the obtained materials. This also resulted in an increase in the biocompatibility of the samples, which was confirmed by the tests using NIH-3T3 mouse fibroblasts, and only moderate influence of the oligomer presence of the mechanical properties was observed. Thus, the inclusion of a functionalized oligomer of the same nature as a respective high-molecular-weight polymer has been shown to be promising for improving the biocompatibility of biopolymer products.

• Supplementary materials

No supplementary materials are available.

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• Conflict of interest

The authors declare no conflict of interest.

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