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Effect of Poly(ethylene glycol) on the Ultrastructure and Physicochemical Properties of the Poly(3-hydroxybutyrate)

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Chemical conjugation or blending with poly(ethylene glycols) (PEGs) are established procedures to facilitate solubilisation of hydrophobic compounds. The techniques of bioPEGylation and blending with PEG were applied to poly(3-hydroxybutyrate). In this paper we have examined the properties of copolymer of poly(3-hydroxybutyrate-co-poly(ethylene glycol)) (PHB-PEG) and composite material polyhydroxybutyrate with poly(ethylene glycol) (PHB + PEG) compared to homopolymer of poly(3-hydroxybutyrate) (PHB). It was found that copolymer has significally different mechanical and thermophysical properties with respect to pure PHB: an increased crystallinity but a decreased Young's modulus and elongation at break. Moreover, the creation of the composite, and a copolymer of PHB with PEG results in a change in surface morphology of ultrathin films.

1. Introduction

Intensive development of such biomedical areas as regenerative medicine, bioengineering (including tissue engineering), biopharmaceuticals and nanobiotechnology has increased the demand for new biomaterials, especially biocompatible and biodegradable polymers. A variety of natural and synthetic polymers are used as materials for manufacture of medical devices and formulations, including polyhydroxyalkanoates (PHAs), polyanhydrides, polyalkylcyanoacrylates, polyphosphazenes, polyphosphoesters, polyorthoesters, poly(maleic acid), some polysaccharides (chitosan, hyaluronic acid, agarose, dextran, alginates, chondroitin sulfate), and proteins (collagen, fibrin, silk fibroin, spidroin, gelatin). These polymers are used as medical implants in reconstructive surgery, tissue engineering, for creating new dosage forms in biopharmaceutics, new dental materials, and have other applications.[1]

Despite the fact that a wide range of polymers are used in medicine, the vast majority of them are produced by chemical synthesis or are isolated from natural raw materials (algae, higher plants, mushrooms, crustaceans, tissues of domestic animals). Unfortunately, methods of chemical synthesis and isolation of polymers from natural raw materials cannot provide a full range of properties required for biomedical polymers. The obtained polymers require deep and very expensive purification, and should fulfill very narrow requirements for chemical structure and properties, as well as be biologically safe, etc. Additionally, synthetic polymers and products of their biodegradation may be toxic, while natural polymers may have pronounced immunogenicity or be contaminated with viruses or prion proteins.[2,3]

Biodegradable poly(3-hydroxyalkanoates), poly(3-hydroxybutyrate) (PHB) and its copolymers, attract particular attention among developed and used biomedical polymers. Poly(3-hydroxybutyrate) (PHB) is the most studied member of poly(3-hydroxyalkanoates) family (PHAs) which can be produced microbiologically by different types of microrganisms: Azotobacter chroococcum,[4–8] Azotobacter vinilandii,[9–11] Alkaliphilus oremlandii,[12] Cupriavidus necator,[13–15] Pseudomonas oleovorans,[15] Methylobacterium extorquens,[16] and others.[16–18] In contrast to natural polymers (chitosan, alginate, dextran, collagen, etc.) and chemically synthesized polymers, PHB and its copolymers are produced by biotechnological methods that allow to achieve a high degree of purity, to control and specify physicochemical properties of biopolymers within narrow limits during their biosynthesis.[4–8] PHB has a set of unique properties: high mechanical strength and thermal plasticity that allows to obtain a wide range of products due to easy processing,[11,19–21] ability to form composites with synthetic polymers, inorganic materials and medicinal products,[22–24] complete biodegradability to non-toxic products,[1,25–27] biocompatibility (including hemocompatibility) with human and animal tissues and organs and environmental safety,[1,11,13,19–22,28]

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special diffusion properties that allows to produce devices and formulations for drugs sustained release.[29] Therefore, PHB is widely used for biomedical applications in regenerative medicine and tissue engineering.[19–24,26–30] However, PHB homopolymer has certain disadvantages as well: high hydrophobicity and crystallinity, long-term biodegradation and low plasticity, which in some cases severely limits their use as bioengineered materials in medicine, for example for the manufacture of vessel grafts.[11,13,31–33]

It is generally accepted that biodegradation of PHB both in living systems and in environment occurs via enzymatic and non-enzymatic processes that take place simultaneously under natural conditions. Opposite to other biodegradable polymers (e.g. PGA and PLGA), PHB is considered to be moderately resistant to degradation in vitro as well as to biodegradation in animal tissues. The rates of degradation are influenced by the characteristics of the polymer, such as chemical composition, crystallinity, morphology and molecular weight.[1,13,26,27,34,35]

Polymer chemistry is one of the rapidly developing field of natural sciences, because at the moment it can be argued that the polymeric materials are used in all spheres of human activity. The polymeric materials are of great importance in medicine including transplantation and tissue engineering. Nowadays, these fields represent a major medical specialties and selection of materials for the solution of various biological problems is urgent.

The most widely used materials in clinical medical practice are synthetic polymeric materials such as PLA, PGA, PCL, and others. Sufficiently available raw, the possibility of production in industrial scale and low toxicity are the most significant advantages of these materials.

Also, they have an advantage of possessing initially enhanced mechanical properties, but their relatively quick degradation profile diminishes these properties. The disadvantages of such materials are the chemical method of production, which leads to a residual content of inorganic impurities in the production process and sufficiently rapids biodegradation associated with an acidification.

Thereby the polymer materials of microbial origin are gradually coming to the fore, and their study is becoming more in demand. The family of polyhydroxyalkanoates, which producers are more than 100 strains, include polymers such as PHB, PHBV, PHBHx, etc. The most studied member of the PHA family is poly(3-hydroxybutyrate) (PHB), a homopolymer comprised of the monomer 3-hydroxybutyric acid. It is well known for its good biological properties, such as high compatibility with biological tissues and biodegradability without toxic products.

PHB remains the only PHA approved by the Food and Drug Administration (FDA) in the USA and has been used in a variety of biomedical applications including sutures, bone plates and tissue scaffolds.[36]

However, the PHB homopolymer has some physicochemical properties that limit its biomedical usefulness. Namely, the solution cast films of PHB have brittle properties, a high crystallinity degree, high hydrophobicity and low rate of biodegradation. One way to improve certain material parameters is their combination with other materials possessing the missing properties, or in other words, a composite materials production. To improve indicators such as hydrophilic properties and plasticity, PEG seems to be the most suitable composition additive.

PEG is a nontoxic polymer approved by the U.S. Food and Drug Administration (FDA) for internal consumption. Several techniques have been used to immobilize PEG onto a variety of polymer surfaces, including physical adsorption, graft copolymerization, covalent grafting, etc.[37]

Chemical grafting of poly(ethylene glycol), called 'PEGylation', is a standard process to correct some properties of different biological agents. PEGylation may be an effective method of delivering therapeutic proteins and modifying their pharmacokinetic properties, in turn modifying pharmacodynamics, via a mechanism dependent on altered binding properties of the native protein.[38] Recent studies where similar copolymers were derived from other strains, mentioned significantly different physiochemical and material properties. In particular, the elongation at break increased from 8.4 to 20.6%. V contact angles decreased from 89° to 75° depending on the molecular weight of the PEG end group and others. The mini-review on the subject presented in the work.[39]

Earlier in our laboratory a copolymer of PHB-PEG was obtained by adding PEG to the growth medium of producing strain. It has been shown that the EG components occurrence is 0.33% by weight of the polymer. The preliminary experiments of this copolymer in comparison to the original homopolymer showed some variation of the copolymer properties, for example, a decrease in crystallinity and the contact angle, which indicates a lowering of the material hydrophobicity.

However, blending is a comparatively more convenient and cost-effective method to manipulate physiochemical and material properties of biomaterials, which has been used in a range of PHB-based devices.[9]

In this paper, we aimed to examine homopolymer PHB from strain Azotobacter croococcum 7B, its copolymer with PEG, PHB-PEG, produced biotechnologically and its composite material obtained by blending with PEG. We studied and compared their mechanical properties and biological potential as a base for matrices intended for cell growth.

2. Experimental Section

2.1. Materials

The following materials were used: native poly(3-hydroxybutyrate) (PHB) Mw = 4.85 × 105, its copolymer poly(3-hydroxybutyrate)-poly(ethylene glycol) (PHB-PEG) Mw = 2.17 × 105, PEG Mw = 1500.[8]

2.2. Films and Ultrathin Films Preparation

Samples of polymers were dissolved in chloroform (3% w/v) and fabricated into thin films by casting onto clean, dry, sterile glass Petri dishes. Film thickness was measured using a digital micrometer (Starrett, 796XFL-1, USA). Ten locations from the edges and centers of each film were randomly selected and their means determined (n = 10). The thickness of polymer films was 50 ± 4.5 μm.

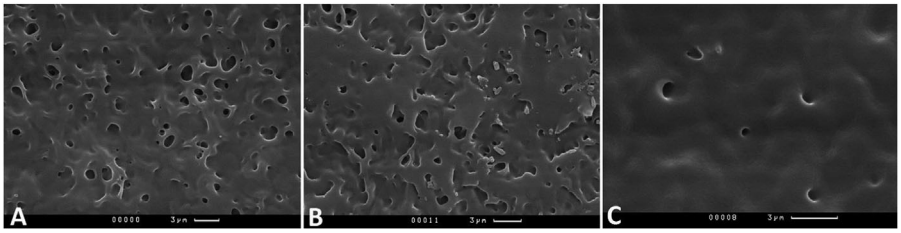

Figure 1. Microstructure of polymer films (SEM): (A) PHB, (B) PHB + PEG, (C) PHB-PEG.

Ultrathin films were prepared by spin-coating at a rotation speed of 3000 rpm for 30 sec, using a Eppendorf microspin centrifuge.[40] Dichloromethane was used as solvent. The concentration of solution was 0.5 mg/ml.

2.3. Microscopy

Microstructure of polymer films surface was studied by scanning electron microscopy (SEM) and atomic force microscopy (AFM).

The samples were mounted on aluminium stumps, coated with gold in a sputtering device for 15 min at 15 mA (IB-3,Giko, Japan) and examined under a scanning electron microscope (JSM-6380LA, JEOL, Japan).

Diameter of micropores was calculated by Image J software. The results are presented as mean (n = 15). Microphotographs of the surface of PHA films were obtained by AFM. The AFM imaging was performed with Solver PRO-M (Zelenograd, Russia). For AFM imaging a piece of the PHB film ($\sim 2 \times 2\,mm^2$) was fixed onto a sample holder by double-sided adhesive tape. Silicon cantilevers NSG11 (NT-MDT, Russia) with a typical spring constant of 5.1 N/m and ETALON (spring constant 3.5 N/m for 80 μm cantilevers and 12 N/m for 110 μm cantilevers) were used. The images were recorded in semi-contact mode, a scanning frequency of 1–3 Hz, scanning areas from $500 \times 500\,nm^2$ to $20 \times 20\,\mu m^2$, and topography and phase signals were captured during each scan. To select the scan area an optical system combined with the AFM was used. The images were captured with 512×512 pixels. Image processing was carried out using Image Analysis (NT-MDT, Russia) and FemtoScan Online (Advanced technologies center) software.

The average roughness, R_a, was calculated to describe film surfaces

$$R_a = \frac{1}{N}\sum_{n=1}^{N} |r_n| \quad (1)$$

This parameter was calculated in three scan areas of $20 \times 20\,\mu m^2$ (512×512 points). Additionally, several scans at higher resolution were obtained for each sample for more detailed description of the polymer surface.

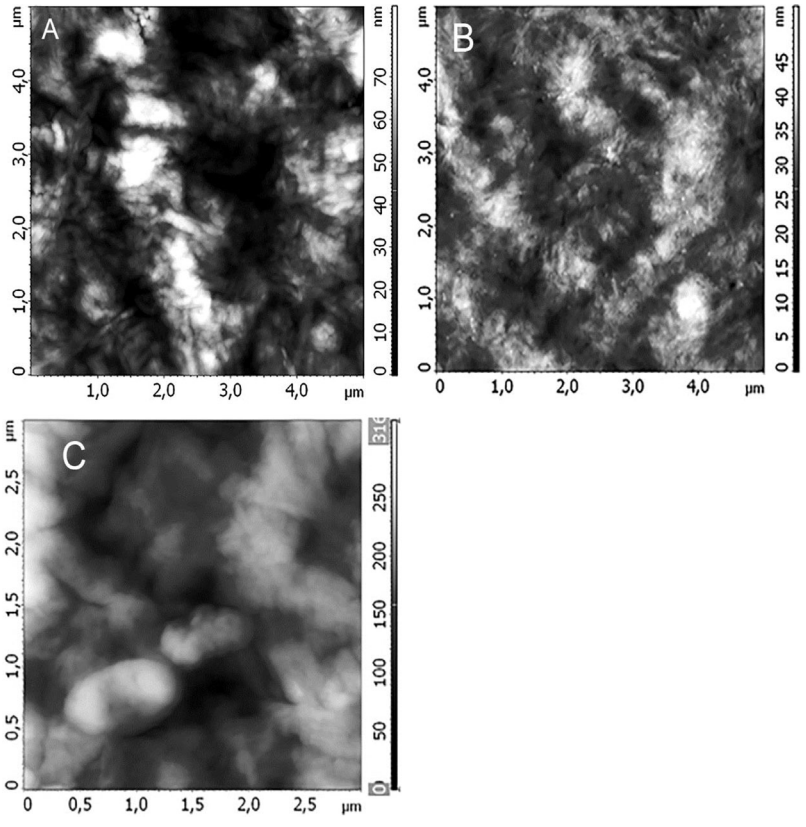

Figure 2. AFM microphotography of film surface of produced PHAs. AFM microphotography of film surface of produced PHAs: (A) PHB; (B) PHB + PEG; (C) PHB-PEG.

2.4. Differential Scanning Calorimetry (DSC)

The PHB, PHB + PEG and PHB-PEG thermal properties were measured by means of differential scanning calorimetry using a DSC 204 F1 Phoenix (Netzsch, Germany) equipment. About 1–4 mg of polymer film was sealed in a 25 μl aluminium crucible. The samples were heated from 25 to 200 °C at a heating rate of 10 °C/min in nitrogen atmosphere. The onset and peak temperature of the change in heat capacity was designated as the Tmonset and Tpeak m melting points. The accuracy of determination did not exceed 1 °C for the temperature measurement and 2 J/g for the melting enthalpy. The crystallinity of PHB component (Xc)

Table 1. Average roughness and pores size of polymer film surface.

Polymer	Average roughness (R_a, nm ± SD)	Average size of pores (μm ± SD)
PHB	15.0 ± 2.0	0.93 ± 0.33
PHB + PEG	53.5 ± 6.3	0.74 ± 0.28
PHB-PEG	6.6 ± 1.4	0.65 ± 0.19

Table 3. The mechanical properties and its derivatives.

	Tensile strength, MPa	Elongation at break, %	Young's modulus, E, MPa
PHB	36.53 ± 6.44	4.52 ± 1.71	1458.33 ± 154.46
PHB + PEG	13.48 ± 1.21	7.91 ± 1.99	424.36 ± 152.31
PHB-PEG	35.77 ± 0.26	1.61 ± 0.05	758.37 ± 204.52

can be calculated by the following equation[41]:

$Xc = \Delta Hm/\Delta H_0 m(PHB)$ (2) for homopolymer and copolymer,

and $Xc = \Delta Hm/\Delta H_0 m(PHB)*\omega(PHB)$ (3) for composite,

where H_0m(PHB) is the theoretical value for the thermodynamic melting enthalpy, which would be obtained for a 100%-crystalline PHB sample (146.6 J/g), Hm(PHB) is the apparent melting enthalpy corresponding to PHB component and ω (PHB) is the weight fraction of PHB in the blend. All calculations were performed for the second heating cycle. Data are presented as the average of three measurements, P values <0.05 were considered statistically significant.

2.5. Thermogravimetric Analysis (TGA)

Thermogravimetric analyses were performed on a TG 209 F1 Libra (Netzsch, Germany). Samples were initially heated by using a heating rate of 10° C/min from 30 to 300 °C in a dynamic argon atmosphere. Decomposition temperatures were taken at the peak maximum of the first derivative of weight remaining (%) against the temperature (°C) curve.

2.6. Mechanical Testing

Mechanical testing of polymeric materials was performed on an Instron Zwick/Roell BZ 25/TN1S (Zwick Roell, Germany) with the following parameters: pre-load – 0.05 N, pre-load speed of 1 mm/min, the beginning and the end of the determination of the Young's modulus – 0.1 and 0.15N respectively, the speed determining Young's modulus – 2 mm/min.

2.7. Statistical Analysis

Statistical evaluation of data was performed using the software package SPSS/PC + Statistics™ 12.1 (SPSS). Non-parametric Kruskal–Wallis test was used for all statistical analyses. Data were averaged with the standard deviation (±SD) and considered significant for $P < 0.05$.

3. Results and Discussion

3.1. Surface Morphology of Copolymers Films

Polymer films were examined with SEM and AFM. We studied morphology of the smooth side of polymer films, because it was shown previously that a smooth film surface reflects the properties of the polymeric material much better than the rough side, the structure of which is highly dependent on the film preparation technique.[7] The surface microstructures of PHB, PHB + PEG, and PHB-PEG are presented at **Figure 1**. The micropore size of PHB homopolymer film was 0.93 ± 0.33 μm, PHB + PEG composite film – 0.74 ± 0.28 μm, and PHB-PEG copolymer film – 0.65 ± 0.19 μm.

PHB and PHB + PEG films, presented at the Figure 1, were the similar microstructure. It may indicate that PEG completely embeds into PHB fibers and follows their layering. The PHB-PEG copolymer films have a minimal number of pores, and size of these pores is less than the other two samples. This may indicate a quite different submolecular structure of PHB-PEG copolymer, which defines polymer material microstructure.

The surface morphology of various biopolymer films did not differ significantly. As seen in Table 3, the average roughness of the smooth surface of PHB copolymers films has slightly decreased as compared to the PHB homopolymer; the rough surface of polymer films did not differ significantly (**Figure 2**).

Table 2. Physico-thermal properties of polymers.

Polymers	ΔHm, J/g	T_m^{onset}/T_m^{peak}, °C	Crystallinity (X_c, %)	Degradation temperature, °C
PHB	99.6	167.4/175.0	67.94	218
PHB + PEG	76.12	150.5/173.0	74.18[a)]	240
PHB-PEG	102.6	164.4/172.0	69.99	226

[a)] X_c(PHB), crystallinity of PHB component (calculated by Eq. (3)).

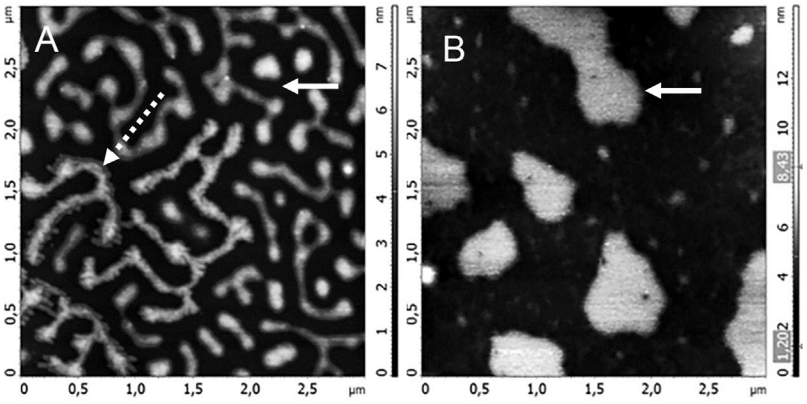

Figure 3. Ultrathin films of PHB (A) and PEG (B). Dotted arrow indicates the crystalline PHB, solid arrows indicate the amorphous regions.

If the film morphology looks almost identical, their roughness differs (**Table 1**). The table shows that the smoothest film is a copolymer of PHB-PEG, and most rough is a composite.

This is because the copolymer forms supramolecular structures, that are similar to the homopolymer. At the same time, a composite of PHB and PEG tends to form a structure favorable to them energetically, while sterically hindering each other's position. This is partly confirmed by images of ultra-thin films, where the composite structure differs from the structures of the copolymer and homopolymer.

Thermal analysis showed that the melting temperature is reduced by 3 °C in the copolymer and 16.9 °C at composite, the crystallinity is increased by 2.9% in copolymer and 9.2% in composite with respect to pure PHB. Thermogravimetric analysis revealed an increase in the degradation temperature at 8 °C in copolymer and 22 °C in composite (**Table 2**).

Mechanical properties are a part of the main characteristics of the materials. A 25–60 micron-thick film was used in this study. The data presented in **Table 3**. Average Young's modulus of the material for the homopolymer PHB was about 1.46 GPa, which corresponds to the literature data. Modulus of the copolymer PHB-PEG was about 0.76 GPa and the composite material for this value was 0.42 GPa.

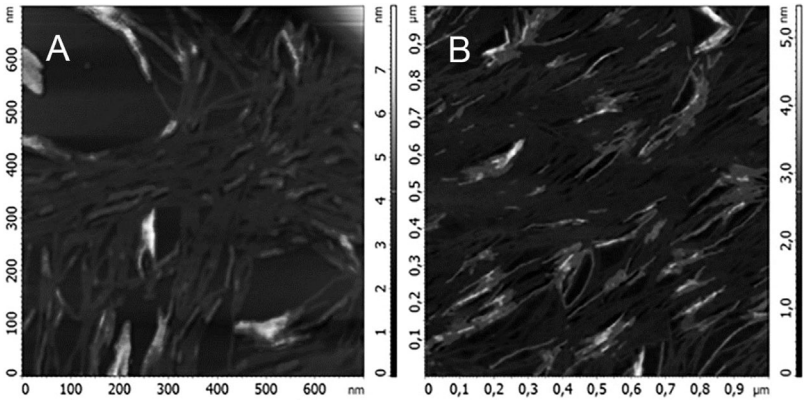

Figure 4. Ultrathin films of copolymer (PHB-PEG) (A) and composite (PHB + PEG) (B).

Elongation was for the PHB, the PHB + PEG and PHB-PEG of 4.52, 7.91 and 1.61%, respectively.

The data obtained indicate the change of mechanical properties of the copolymer PHB-PEG compared to the homopolymer, which is irrefutable proof of the difference of the two materials produced by similar techniques from the same bacterial strain. The reason for the differences in mechanical properties may vary depending on the structure of polymer strands resulting in the loss of elasticity. We can see there is no direct correlation with crystallinity parameter.

The composite material shows a significant increase in elongation and a significant reduction in Young's modulus, which indicates an increase in elasticity and is quite logical in the presence of plasticizer PEG.

3.2. Ultrathin Films of PHB, PHB-PEG, PHB + PEG

Ultra-thin films are a model object, which allows to see how the presence of another polymer affects the morphology of the surface of the samples.

Figure 3 shows ultrathin films of PHB and PEG.

On these images PHB and PEG represent a shapeless, amorphous formation (solid arrows) with an average height of 4.1 ± 0.7 nm for PHB and 4.4 ± 0.5 nm for PEG. Also, crystalline regions that were found on the film of PHB, are marked by dotted arrow.

Films of copolymer (PHB-PEG) and composite (PEG + PHB) have amorphous droplets and crystalline regions too, but also another polymer morphology was observed. Such structures interpreted as monolayer lamellae, can be seen in **Figure 4**, because they have common features with the structures described in the literature (**Figure 5**).[25]

Part of the polymer lamella lies on each other, creating a second layer. The heights of the lamella in copolymer and composite was the same. The height of the monolayer lamella is 0.7 ± 0.1 nm, and the thickness of the double-layer 1.5 ± 0.3 nm.

In the study of ultra-thin film composite PHB + PEG, the processes of diffusion and self-assembly of lamella were observed, like analogous processes observed in peptides.[42]

Figure 6 shows that during the obtaining of the frame (4 min), polymer molecules are moving and creating a new lamella. By analogy with the peptides the movement of polymer molecules and their self-assembly is possible in water nanofilms. This indicates high mobility of composite molecules. In addition, the emerge of the next layer of lamella was shown (dotted arrow). The height of this layer is equal to 1.6 ± 0.1 nm, which means that the height of the subsequent layer is 1 ± 0.1 nm.

The average height of the polymer chains is 0.5 ± 0.1 nm, which corresponds to the diameter

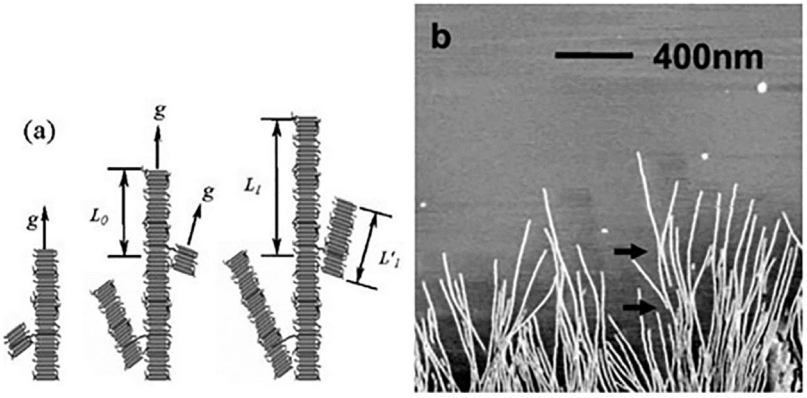

Figure 5. The image of poly(bisphenol A-co-decane).[25]

of the polymer chain. They are not an artifact of the scanning, as described in the literature for polymers where the polymer chain has the same diameter.[43,44]

4. Conclusion

Thus, despite the low percentage of EG monomers the changes in physicochemical properties were noticed. As compared to homopolymer, copolymer PHB-PEG has higher hydrophility parameters, as demonstrated early,[7] it demonstrates a significant decrease in Young's modulus and elastic properties. The composite PHB + PEG obviously has better hydrophility and mechanical properties.

It has properties of hydrophilic and amorphous polymers, despite the fact that the calculation showed an increase in crystallinity of polyhydroxybutyrate component.

Moreover, it was found that the creation of the composite and a copolymer of PHB with PEG results in a change in surface morphology: lamellas appear as a monolayer with an average height of 0.7 ± 0.1 nm and the appearance of subsequent secondary layers have been also identified. In the composite films the process of self-assembly of lamella was shown as well as the emerging of the second layer of lamella.

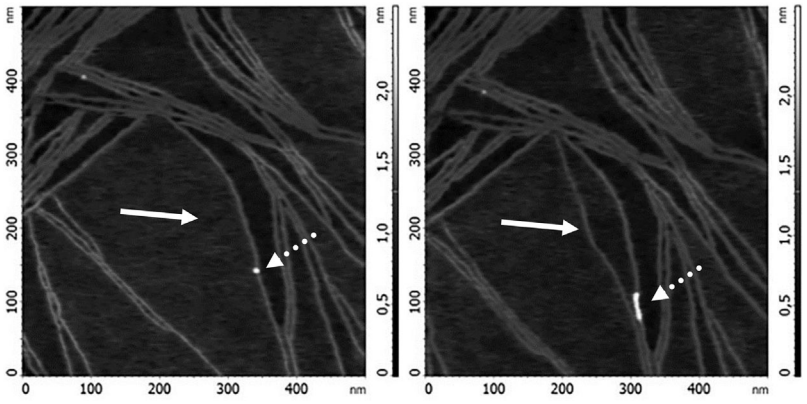

Figure 6. The process of diffusion and self-assembling composite lamellae on mica. Pictures were taken with an interval of 4 min. The solid arrow indicates the movement and assembly of lamellae. The dotted arrow – an appearance of the second layer of lamella.

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Keywords

biopolymers, mechanical properties, PHA, poly(3-hydroxybutyrate) (PHB), poly(ethylene glycol) (PEG)

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