Properties of degradable polyhydroxyalkanoates with different monomer compositions

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ARTICLE INFO

ABSTRACT

Purpose: To synthesize and investigate polyhydroxyalkanoates (PHAs) with different monomer composition and percentages and polymer films prepared from them.

Results: Various PHAs: homopolymer poly-3-hydroxybutyrate P(3HB) and 2-, 3-, and 4-component copolymers comprising various combinations of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), 4-hydroxybutyrate (4HB), and 3-hydroxyhexanoate (3HHx) monomers were synthesized under specialized conditions. Relationships were found between the monomer composition of PHAs and their molecular-weight and thermal properties and degree of crystallinity. All copolymers had decreased weight average molecular weights, M_w (to 390–600 kDa), and increased values of polydispersity (3.2–4.6) compared to the P(3HB). PHA copolymers showed different thermal behavior: an insignificant decrease in T_{melt} and the presence of the second peak in the melting region and changes in parameters of crystallization and glass transition. At the same time, they retained thermostability, and the difference between T_{melt} and T_{degr} was at least 100–120 °C. Incorporation of 4HB, 3HV, and 3HHx monomer units into the 3-hydroxybutyrate chain caused changes in the amorphous to crystalline ratio and decreased the degree of crystallinity (C_x) to 20–40%. According to the degree to which the monomers reduced crystallinity, they were ranked as follows: 4HB – 3HHx – 3HV. A unique set of films was produced; their surface properties and physical/mechanical properties were studied as dependent on PHA composition; monomers other than 3-hydroxybutyrate were found to enhance hydrophilicity, surface development, and elasticity of polymer films.

Conclusion: An innovative set of PHA copolymers was synthesized and solution-cast films were prepared from them; the copolymers and films were investigated as dependent on polymer chemical composition. Results obtained in the present study contribute to the solution of a critical issue of producing degradable polymer materials.

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1. Introduction

The most promising area of application for biomaterials is medicine, which urgently needs biocompatible materials for producing medical instruments, drug delivery systems, implants, and bio-constructors of organs and tissues in cell and tissue engineering [1]. The interdisciplinary approach used by tissue engineering is aimed at developing tissue and organ replacements – complex structures of functioning cells attached to cell scaffolds. The effectiveness of this approach is largely based on the properties of the material used to construct cell scaffolds [2]. The necessary properties of the scaffold are determined by the properties of the starting material and the technology employed to process it. Therefore, the main challenge for production of effective bioconstructs is the availability of proper biodegradable and biocompatible material.

The discovery of polyhydroxyalkanoates (PHAs) – thermoplastic, biodegradable, and biocompatible microbial polymers – was a notable event for biotechnology of novel materials [3–10].

Being UV resistant, non-hydrolyzed in liquid media, and thermoplastic, PHAs are processable from different phase states (solution, emulsion, powder, melt) by available techniques [11,12].

These useful properties, along with biodegradability and high biological compatibility, make PHAs promising materials of the 21st century for various applications – from municipal engineering and agriculture to pharmacology and biomedicine [13–15].

PHAs hold the greatest promise for developing biomedical products and devices, including nonwoven and disposable products, sutures and wound dressings, controlled drug delivery systems, scaffolds for cell and tissue engineering, components for reconstructive surgery and transplantation [16–21].

PHAs have great potential as materials for regenerating damaged skin, repairing the defects of soft tissues, bone engineering, cardiovascular applications, blood vessels, heart valves, etc. [22–29].

Until recently, PHA research was mainly focused on the homopolymer of poly-3-hydroxybutyric acid. P(3HB) is a highly biocompatible material, as hydroxybutyric acid is a natural metabolite of cells and tissues in higher animals and humans [24]. The main disadvantage of P(3HB) is the high degree of crystallinity; therefore, it cannot crystallize into ordered structure and is not readily processable; products based on this material have low shock resistance and are rigid and prone to "physical ageing" [13,14].

Properties of polymer materials, including P(3HB), can be improved by using chemical, physical, and biological methods [30–33] such as fabrication of P(3HB) composites with other materials [34], chemical modification of the surface of polymer products [35], treatment by physical methods (plasma, laser) [36,37], and biosynthesis of PHA copolymers [38–42].

A definite advantage of PHAs is the possibility of synthesis of polymers comprising monomer units with different lengths of carbon chains. PHA copolymers show greater potential, as their basic properties can be varied rather widely, depending on the proportions of monomer units in them [39,41–46]. Short-chain-length biopolymers are stiff, brittle and possess a high degree of crystallinity in the range of 60–80% where as medium-chain-length PHA is flexible and elastic materials with low crystallinity (20–25%), low tensile strength, high elongation to break, low melting temperatures and T_g below room temperature.

The discovery of P(3HB) was followed by the discoveries of P(3HB-co-3HV) and P(3HB-co-3HHx) copolymers [47]. P(3HB-co-3HV) can be synthesized by various organisms from a number of substrates [43,48], including processes with *Haloarchaea* in seawater and in the presence of high sodium chloride concentrations [7]. P(3HB-co-3HV) copolymers are isodimorphous due to co-crystallization of monomer units [49]. The ratio of monomers in these copolymers can vary from just a few percent to 50–80 mol%, affecting their crystallization kinetics and physicochemical properties. The most well-known copolymer, P(3HB-co-3HV), has lower crystallinity, lower melting point, decreased stiffness, increased elongation at break and is more flexible (with lower Young's modulus) and tougher (with higher impact strength) than P(3HB) [[27,43,50–53], etc.].

Copolymers comprising short-chain-length (scl) and medium-chain-length (mcl) monomers hold a special position among PHAs, as this type of copolymers, such as poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate [P(3HB-co-3HHx)], are elastomeric and have a decreased degree of crystallinity, in contrast to the high-crystallinity P(3HB) and P(3HB-co-3HV) [43,48,54]. Compared to other PHA polymers, this copolymer is known as the "third-generation commercial PHA product" because of its better machinability, physiological compatibility, and physicochemical properties. Although P(3HB-co-3HHx) has good properties, it still cannot escape the limitations of the PHA family due to the complicated production process and expensive raw materials. Such copolymers can be synthesized by the wild-type and genetically engineered microorganisms of various taxa: *Pseudomonas putida*, *Rhodospirillum rubrum*, *Rhodococcus ruber*, *Thiocapsa pfennigii*, *Aeromonas punctata* FA440, *Pseudomonas* sp. 61–3, *Ectothiorhodospira shaposhnikovi*, *Ralstonia eutropha*. There are studies describing synthesis of scl-mcl PHAs from different substrates (sugars, organic and fatty acids) and in the media supplemented with salts of hexanoic acid, which is usually toxic to many microorganisms, used as the precursor [55–60]. The toxicity of this precursor has, until recently, limited production of copolymers containing 3HHx 15–20 mol%.

More impressive although differing in 3HHx fractions of P(3HB-co-3HHx) data were reported in a number of works. For example, recombinant strain *Cupriavidus necator* Re2160/pCB113 with the cloned PHA synthase gene of *R. aetherivorans* and an enol-CoA hydratase gene from *Ps. aeruginosa* cultivated on the medium containing palm oleic acid alone and combined with fructose synthesized copolymer containing 3HHx between 4 mol% and 27 mol% [61,62]. In another study [63], the same strain synthesized copolymer with a larger 3HHx fraction (56–59 mol%). The same strain grown on crude palm kernel oil (CPKO) and soybean oil (SO) produced copolymer with 3HHx reaching 55–70 mol% [64]. Recombinant strain *Aeromonas hydrophila* AKJ1 synthesized P(3HB-co-3HHx) that contained high percentage of 3HHx (94.5–94.6 mol%) [65]. Those studies demonstrated that 3HHx affected considerably the degree of crystallinity of the polymer, decreasing the C_x to 20%, or by a factor of more than three, when the 3HHx content was increased from 10 to 68 mol%. The composition of P(3HB-*co*-3HHx) and the percentage of 3HHx produced a significant effect on the mechanical properties of the polymer products. As the percentage of 3HHx was increased, the films became considerably more elastic, as evidenced by elongation at break, which increased from a few percent to several hundred percent, while mechanical strength was reduced, as evidenced by tensile strength at break and Young's modulus.

The polymer holding *con*siderable promise is P(3HB-co-4HB). This copolymer is biodegraded in vivo with high rates, it is an elastomer, and its elongation at break and tensile strength are superior to those of the known PHAs [66]. With the efforts on incorporating a 4-hydroxybutyrate unit into P3HB, the obtained P(3HB-*co*-4HB) showed tunable hardness and flexibility via controlling the mole fraction of P4HB [67–70]. Due to its variable physicochemical and mechanical properties, P3HB-*co*-P4HB is favored by researchers in many fields [71].

As synthesis of 4HB monomers can occur only in the presence of precursors, which are usually toxic to microorganisms, the greatest research effort was devoted to the search for wild-type strains and construction of modified strains tolerant to these supplementary carbon substrates, which would synthesize copolymers with high percentages of 4HB (10–20 to 50 mol% or higher) and produce large cell biomass and high content of the copolymer. Very many studies addressed this issue, testing various carbon substrates, culture conditions, and doses of precursors to reduce their toxicity. Microorganisms effectively synthesizing P(3HB-co-4HB) included various strains such as *Cupriavidus necator* Re2058/pCB113, *Cupriavidus malaysiensis* USMAA1020, *C. necator* harboring the gene of synthase from *Burkholderia contaminans*, *Halomonas bluephagenesis* containing various constructed plasmids, wild-type strain *Cupriavidus necator* B-10646, etc. [72–76]. The transformant *Cupriavidus malaysiensis* USMAA1020 containing an additional copy of the PHA synthase gene cultivated in the presence of precursors (1,4-butanediol and 1,6-hexanediol) under different cultivation conditions synthesized P(3HB-co-4HB) containing 4HB from 92 to 99 mol%.

Important results were obtained by researchers in China. P(3HB-*co*-4HB) produced by the engineered bacteria had excellent fracture growth rate and elasticity [77]. P(3HB-co-4HB) was successfully produced by engineered *Halomonas bluephagenesis* TD01 grown in glucose and γ-butyrolactone under open non-sterile conditions. Gene orfZ encoding 4HB-CoA transferase of *Clostridium kluyveri* was integrated into the genome to achieve P(3HB-co-4HB) accumulation comparable to that of strains encoding orfZ on plasmids. Fed-batch cultivations resulted in over 70 g/L cell dry weight (CDW) containing 63% P(3HB-co-4HB) with 4HB 12–14 mol%. This method avoids the addition of expensive and toxic γ-butyrolactone, which can effectively decrease the cost of producing PHA. The processes were further scaled up in a 1000-L pilot fermentor on the mineral medium under nonsterile fermentation to reach 83 g/L CDW containing 61% P(3HB-co- 4HB) with 4HB 16 mol%. The elastic P(3HB-co-4HB) showed an elongation at break of 1022 ± 43%.

Interest in the synthesis of PHA ter- and quaterpolymers has been growing recently. PHA terpolymers and quaterpolymers are known to

be better materials compared to copolymers. Introduction of more than one secondary monomer is assumed to result in improved properties. On the one hand, synthesis of PHA ter- and quaterpolymers is a complex process, with several precursors added to the culture medium, which increases the risk of inhibiting growth and development of PHA producing microorganisms. On the other hand, when several monomers, rather than one, are incorporated into the 3HB chain, even at low concentrations, the properties of the polymer may change to an extent that is comparable or superior to the effect of the incorporation of a second monomer in PHA bipolymers. Synthesis of two types of terpolymers has been reported: P(3HB-co-4HB-co-3HV) and P(3HB-co-3HV-co-3HHx).

The wild-type strain *Cupriavidus eutrophus* B10646 cultivated in the glucose- or fructose-containing medium with γ-butyrolactone added in one to four controlled doses synthesized P(3HB-co-4HB-co-3HV), in which 4HB content varied between 10.4 and 75.0 mol% and 3HV – between 10.1 and 25.5 mol%. Polymer specimens were produced that had a considerably decreased degree of crystallinity (to 26–35%) but almost unchanged melting point and thermal degradation temperature [73].

Processes of production of similar terpolymers with various percentages of monomers in cultures of different strains (*Alcaligenes* sp. A-04, *Haloferax mediterranei*, *Cupriavidus* sp. USMAA2–4) were reported in a number of studies [78–84]. *Cupriavidus sp*. USMAA2–4 was cultivated on oleic acid supplemented with pentanol and butyrolactone [78]; *Cupriavidus necator* DSM545 was grown on glycerol supplemented with γ-butyrolactone and propionic acid [79]; *Cupriavidus malaysiensis* USMAA2-4 (DSM 19379) was cultivated on glycerol and γ-butyrolactone or 1,4-butanediol [80]; all strains produced terpolymers with different percentages of monomers. For example, the addition of precursors (1,4-butanediol, 1,6-hexanediol, caprolactone, propionic and valeric acids) to the *Cupriavidus* sp. DSM 19379 culture medium resulted in the synthesis of P(3HB-co-4HB-co-3HV) containing 4HB from 16.76 to 27.87 mol% and 3HV from 8.32 to 29.46 mol%; weight average molecular weight of the terpolymers decreased to 137–258 kDa, depending on the monomer percentages, but their melting temperatures were similar to each other (161–164 °C) [80]. The highest result was obtained in the experiment with *Alcaligenes* sp. A-04 cultivated on fructose supplemented with valeric acid and sodium salt of γ-butyric acid or 1,4-butanediol; the authors managed to increase 4HB fraction to 93 mol% with 3HB content between a few percent and 30–40 mol%, but total polymer content was reduced to 30–50% [81].

Another PHA terpolymer, P(3HB-co-3HV-co-3HHx), containing monomers of the scl 3HB and 3HV and the mcl 3HHx, was first synthesized more than 10 years ago [3,85–87] and has been extensively studied since then [88–92]. Wild-type and genetically modified producers (recombinant *Aeromonas hydrophila* 4AK4, *Escherichia coli*, and wild-type *Ralstonia eutropha*) were used to synthesize terpolymers with different 3HV and 3HHx contents (from just a few percent to several dozen mol.%). Studies of this terpolymer produced rather contradictory data. Even the same study reported either considerable (to 60–80 °C) or inconsiderable (to 133–152 °C) decrease in the melting temperature associated with the insignificant variation in monomer percentages [92].

There are a few available studies describing synthesis of PHA quaterpolymers. *P. putida* W619 cultivated on mannitol rich ensiled grass press juice synthesized P(3HHx-co-3HO-co-3HD-co-3HDd) containing the following proportions of the mcl monomers: 1.0:14.0:63.0:21.0 [93]. *Gordonia polyisoprenivorans* VH2 was cultivated to produce P(3HHx-*co*-3HO-co-3HD-co-3HDd) with the following proportions of the mcl monomers: 17.6:40.3:28.5:13.6 mol% [94]. The wild-type strain *C. necator* B10646 was grown in the medium with varied concentrations of precursors (valerate, γ-butyrolactone, hexanoate) to synthesize P(3HB-co-3HV-co-4HB-co-3HHx) quaterpolymers with different proportions of the monomers: 3HB from 63.5 to 89.7; 3HV 4.5 to 19.6; 4HB 2.4 to 12.3, and 3HHx 0.4 to 4.8 mol%. Comparison of physicochemical properties of PHA quaterpolymers with the major fraction of 3HB and with the total fraction of the other three monomers varying

from 10.3 mol% to 36.5 mol% showed that incorporation of 3HV, 4HB, and 3HHx monomers into the 3HB chain resulted in a considerable change in the crystalline to amorphous phase ratio and a decrease in the degree of crystallinity to 30–36% [95].

Thus, there are various literature data on the synthesis of PHAs composed of different monomers in different proportions. However, the data on the effects of the chemical composition of PHA monomers on the physicochemical properties of the polymer are far less abundant. Although it is important to reveal the relationship between the chemical composition of PHA and its basic properties, the relevant literature data are far from being exhaustive. On the one hand, it is difficult to create the specified and reproducible conditions of the synthesis of multicomponent PHAs. On the other hand, the reported data are confusing and contradictory.

Synthesis of PHAs with different compositions is the basis for producing polymer materials with target properties. This is an important objective of materials science of high-molecular-weight compounds. However, data on the properties of PHAs differ considerably, even if the samples have similar chemical compositions. For example, the data on molecular weight of P(3HB) reported by different authors varied by a factor of ten [96]. In different studies, the melting point (T_{melt}) of P(3HB) varied between 162 and 197 °C; the T_{melt} of P(3HB-co-3HV) with similar fractions of 3HV – between 56 and 186 °C; and the T_{melt} of P(3HB/4HB) with 4HB constituting from 2 to 7 mol% varied between 114 and 172 °C [45,46]. As reported by Noda et al. [96], the fraction of 20–22 mol% 3HV in P(3HB-co-3HV) did not affect the degree of crystallinity, but in a study by Dai et al. [97], that copolymer had a significantly lower C_x (5 and 9%, respectively). In studies of the degree of crystallinity of P(3HB-co-3HHx) the C_x was 38–40% for the copolymer containing about 12–18 mol% 3HHx [47], but the same degree of crystallinity was reported in another study [98] for the copolymer with only 1.5 mol% 3HHx.

The reason for such differences could be that the authors used different strains, carbon sources, methods of recovery and purification of polymers, etc. Other factors affecting PHA parameters may be the duration of fermentation (as in the late stationary phase of cultivation, intracellular PHA degradation caused by endogenous PHA-depolymerases may dramatically decrease the molecular weight of the polymer) and factors unrelated to biosynthesis such as the technique employed to extract polymer from the cell biomass, the type of the extraction agent used, technology of polymer processing, etc.

The effect of the composition of a PHA on the properties of the products has been studied even less thoroughly. Only a few studies address the effect of the basic properties of PHAs on the characteristics of the PHA-based products. The properties of nonwoven mats of the P(3HB) were found to be largely determined by the properties of the polymer, which, in turn, differed depending on the type of carbon substrate (different types of sugars) [99,100]. The physical/mechanical and biological properties of P(3HB) solution-cast films varied considerably depending on the degree of crystallinity of the polymer, which was in turn determined by the PHA composition and the carbon source used (sugars or glycerol) [101].

At the same time, many of the studies characterizing the properties of PHA-based products do not provide the data on the processes used to fabricate them and their geometric dimensions, thus preventing analysis of the data.

Therefore, the purpose of this study was to investigate the structure and properties of the polymer films fabricated from the unique set of PHA bipolymers, terpolymers, and quaterpolymers composed of various monomer fractions.

2. Material and methods

2.1. Microorganisms

Polymers were synthesized using the *Cupriavidus necator* B-10646 strain, registered in the Russian National Collection of Industrial

Microorganisms (VKPM) [102]. The strain is capable of synthesizing valuable PHA copolymers at high yields and is tolerant to precursor substrates. The strain has a broad organotrophic potential: it can utilize such carbon sources as sugars, amino acids, organic acids, alcohols, CO_2, and CO. As a nitrogen source, the strain uses nitrates, ammonium salts, urea, and amino acids. The strain synthesizes PHA copolymers composed of short- and medium-chain-length monomers.

2.2. Culture medium and cultivation conditions

The collection culture was grown in the mineral Schlegel medium [103] – a strong phosphate-buffered solution. The main carbon source was glucose (China, purity 98%), which had been sterilized by membrane filtration (Opticap XL300 Millipore Express SHC filters, U.S.). To synthesize PHA copolymers, the cell culture was supplemented with precursors of different monomers: 1,4-butanediol and salts of valeric and hexanoic acids at concentrations of 1–2 g/L, which did not limit cell growth ("Sigma", U.S.). Precursor substrates were added portion-wise to reduce their toxic effects on cell culture. The pH of the culture medium was maintained at 7.0–7.2 by adding the KOH titrant; pH was measured using a Professional Meter PP-15 ("Sartorius", Germany).

Cells were grown in the batch culture, in the mode previously developed for PHA synthesis [83]. Inoculum was produced using an Innova® 44 constant temperature incubator shaker (New Brunswick Scientific, U.S.). Inoculum was prepared by resuspending the stock culture maintained on agar medium. The stock culture was grown in 1-L to 2-L glass flasks half-filled with mineral solution, with the initial concentration of glucose between 5 and 10 g/L.

Cells were grown in a two-phase mode: in the Schlegel medium [103] and limited nitrogen supply (50% of the strain's physiological requirement) for 30–35 h (Phase 1) and in the nitrogen-free medium for 30–35 h (Phase 2). PHA copolymers containing major fractions of short- and medium-chain-length monomers were synthesized using *C. eutrophus* B10646, based on the previously obtained data on the limits of physiological effect of the precursor substrates toxic to the cells (γ-butyrolactone; valerate, hexanoate) and on the effects of concentrations of those precursors on the specific growth rate of the cells, cell biomass concentration, and total PHA yield [31,32]. Cultivation of the cells was performed taking into account that bacterial culture synthesizing PHA copolymers was a multifactorial system, with the carbon source present in excess, nitrogen source limiting cell growth and inducing PHA accumulation, concentrations of toxic precursor substrates enabling synthesis of other than 3HB target monomers without dramatically inhibiting cell growth.

2.3. PHA recovery from cell biomass

Polymer recovery was performed in two stages. First, lipids and fatty acids were removed using ethanol and, then, polymer was extracted with dichloromethane. The dichloromethane extracts were pooled and evaporated twice using an R/210 V rotary evaporator (Büchi, Switzerland). Then, polymer was precipitated with hexane, 1:2. Polymer content in the residual mass was determined using a 7890A gas chromatograph equipped with a 5975C chromatograph-mass spectrometer (Agilent Technologies, U.S.). Polymer was re-dissolved in chloroform several times and precipitated using isopropanol or hexane to purify it. The resulting polymer was dried at 40 °C [104].

2.4. PHA chemical composition

Intracellular content of the polymer and its composition were determined by chromatography of methyl esters of fatty acids after methanolysis of cell biomass using a 7890A chromatograph-mass spectrometer (7890A Agilent Technologies, U.S.) equipped with a 5975C mass detector. Purity of the polymer and its composition were determined by chromatography of methyl esters of fatty acids after

methanolysis of purified polymer samples using a chromatograph-mass spectrometer equipped with a 5975C mass detector. Methanolysis of the samples was conducted as follows: 1 mL chloroform, 0.85 mL methanol, and 0.15 mL concentrated sulfuric acid were added to a 4.0–4.5 mg polymer sample and boiled under reflux condensers for 160 min. At the end of methanolysis reaction, 1 mL distilled water was added to the flask. The bottom chloroform layer was used for analysis by chromatography [33,34].

2.5. FTIR spectroscopy

All initial materials and blends were analyzed using FTIR spectroscopy. IR spectra were taken in the 400–4000 cm^{-1} range using a "NICOLET 6700" FT-IR spectrometer (Thermo Scientific, U.S.) and a Smart Orbit accessory, by the attenuated total reflection (ATR) technique.

2.6. Physicochemical properties of PHAs

Physicochemical properties of PHAs were examined using high performance liquid chromatography, X-Ray structure analysis, and differential scanning calorimetry; methods and instruments have been described in detail elsewhere [25,29,30].

Molecular-weight properties of PHAs were examined with a gel permeation chromatograph, a DB-35MS column (Agilent Technologies 1260 Infinity, U.S.) was used. Weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity (Đ) were measured using polystyrene standards (PS) (Agilent Technologies, U.S.).

Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). The specimens were heated at a rate of 10 °C/min to 180 °C, allowed to stay for 2 min, cooled to −25 °C, allowed to stay for 3 min, and reheated to 200 °C. All experiments were conducted in the nitrogen flow to prevent oxidation of the specimens by air oxygen. As the starting specimens were films, the first heating was needed to erase the thermal history of the specimen and eliminate artefacts on thermograms. Crystallization temperature (T_c) was determined from exothermic peaks, and glass transition temperature (T_g), melting point (T_{melt}), and thermal degradation temperature (T_{degr}) were determined from endothermic peaks in thermograms. Thermograms were analyzed using the "STARe v11.0." software (METTLER TOLEDO, Switzerland).

X-Ray structure analysis was performed to determine crystallinity of copolymers employing a D8 ADVANCE X-Ray powder diffractometer equipped with a VANTEC fast linear detector (Bruker, AXS, Germany). The degree of crystallinity (C_x) was calculated as a ratio of the total area of crystalline peaks to the total area of the radiogram (the crystalline + amorphous components). Calculations were done by using the Eva program of the diffractometer software.

2.7. Production and investigation of polymer films

Films of PHAs were prepared by casting chloroform solutions (1–2%) heated to 35 °C on degreased glass and subsequent drying for 48 h in a dust-free box (Labconco, U.S.).

The thickness of the films was measured with a LEGIONER EDM-25-0.001 electronic digital micrometer (Legioner, China).

The microstructure of the surface of PHA films was analyzed using scanning electron microscopy (FE-SEM S 5500, Hitachi, Japan). Prior to microscopy, the samples were sputter coated with platinum (at 10 mA, for 40 s), using an Emitech K575XD Turbo Sputter Coater (Quorum Technologies Limited, UK).

Surface properties were studied with a Drop Shape Analyzer – DSA-25E (Krüss, Germany) using the DSA-4 software for Windows. The Owens, Wendt, Rabel and Kaelble method was used to calculate surface free energy and its dispersion and polar components (mN/m).

The roughness of film surface was determined using atomic-force microscopy (AFM) in semicontact mode (Integra Aura, ZAO NT-NDT,

Russia). The arithmetic means surface roughness (R_a) and the root mean square roughness (R_q) were determined based on 10 points, as the arithmetic averages of the absolute values of the vertical deviations of the five highest peaks and lowest valleys from the mean line of the surface profile, using conventional equations(Surface parameters, ISO 4287/1). Sites of areas 20 × 20 μm were examined, and 2 × 2 μm sites with local maxima and minima were selected; they were analyzed at a higher resolution, and the roughness of each sample was calculated as the average of three measurements [35].

Physical/mechanical properties of the films were investigated using an Instron 5565 electromechanical tensile testing machine (UK). Dumbbell-shaped samples 50 mm long, 6.1 mm wide and 20–25 μm thick were prepared for studying physical and mechanical properties of the films. The thickness of films was measured prior to testing, using a LEGIONER EDM-25-0.001 electronic digital micrometer (Germany). Samples were maintained under ambient conditions at 22 °C and 54% humidity for at least two weeks to reach equilibrium crystallization. At least five samples were tested for each type of films. Measurements were conducted at ambient temperature; the clamping length of the samples was 30 mm. The speed of the crosshead was 3 mm/min. Young's modulus (E, MPa), tensile strength (σ, MPa), and elongation at break (ε, %) were calculated by the Bluehill 2 software (Elancourt, France). To obtain Young's modulus, the software calculated the slope of each stress-strain curve in its elastic deformation region. Measurement error did not exceed 10%.

2.8. Statistics

Statistical analysis of the results was performed by conventional methods, using the standard software package of Microsoft Excel. Each experiment was performed in triplicate. Arithmetic means and standard deviations were found. The statistical significance of results was determined using Student's t-test (significance level: $p \leq 0.05$).

3. Results and discussion

Synthesis of PHAs with different compositions is the basis for producing polymer materials with target properties. This is an important objective of materials science of high-molecular-weight compounds. However, data on the properties of PHAs differ considerably, even if the samples have similar chemical compositions. For example, the data on molecular weight of P(3HB) reported by different authors varied by a factor of ten [36]. In different studies, the melting point (T_{melt}) of P(3HB) varied between 162 and 197 °C; the T_{melt} of P(3HB-co-3HV) with similar fractions of 3HV – between 56 and 186 °C; and the T_{melt} of P(3HB-co-4HB) with 4HB constituting from 2 to 7 mol% varied between 114 and 172 °C [13]. As reported by Noda et al. [33], the fraction of 20–22 mol% 3HV in P(3HB-co-3HV) did not affect the degree of crystallinity, but in a study by Dai et al. [37], that copolymer had a significantly lower C_x (5 and 9%, respectively). In studies of the degree of crystallinity of P(3HB-co-3HHx), the C_x was 38–40% for the copolymer containing about 12–18 mol% 3HHx [36], but the same degree of crystallinity was reported in another study [38] for the copolymer with only 1.5 mol% 3HHx. The reason for such differences could be that the authors used different strains, carbon sources, methods of recovery and purification of polymers, etc. Other factors affecting PHA parameters may be the duration of fermentation (as in the late stationary phase of cultivation, intracellular PHA degradation caused by endogenous PHA-depolymerases may dramatically decrease the molecular weight of the polymer) and factors unrelated to biosynthesis such as the technique employed to extract polymer from the cell biomass, the type of the extraction agent used, technology of polymer processing, etc.

As the studies cited above reported considerably different data even on the properties of the same PHA types, in the present study, PHAs with different compositions were synthesized under similar conditions. The same inoculum with the same physiological activity was used, and glucose was the main carbon source for all cultures. Cultivation duration was the same as well. The same polymer recovery and purification technology was employed; polymer films were prepared from solutions with the same PHA concentration (2%) using the technique of solution casting followed by solvent evaporation at the stabilized ambient temperature; all analyses of polymer and film specimens were performed using the same instruments.

Controlled feeding of precursor substrates to the cell culture synthesizing PHAs and strictly regulated duration of the process after the addition of precursor substrates resulted in the synthesis of polymers with different compositions. Those were a homopolymer of P(3HB) and copolymers that contained monomers other than 3HB, which differed in the structure and the length of the carbon chain, such as 4HB, 3HV, and 3HHx, and terpolymers and quaterpolymers – the PHAs that are difficult to synthesize and that remain insufficiently studied. The composition of the polymers and monomer proportions in them were determined using gas chromatography–mass spectrometry (Fig. 1). The quaterpolymers contained 50–60 mol% 3HB, and three other monomers (4HB + 3HV + 3HHx) constituted the rest. PHA bipolymers contained major fractions of monomers other than 3HB at concentrations of at least 20–35 mol%, reaching 50 mol% or even more (Table 1).

IR spectroscopy – a tool to study the structure of different macromolecules – was used to reveal possible structural differences between PHA specimens. The intensity of the bands in the low frequency range provided information about the crystalline to amorphous ratio. Fig. 2 shows IR spectra of P(3HB), bipolymers, terpolymers, and quaterpolymers obtained in the 400–4000 cm^{-1} range. The IR absorption spectra of P(3HB) contained absorption bands corresponding to vibrations of the main structural components of the polymers except absorption bands of vibrations of the terminal C-OH and COOH groups. The bands of the ordered optical densities (crystalline phase) were in the 1261 cm^{-1} range while the bands of the amorphous phase were shifted to 1182 cm^{-1}. The spectra showed distinct absorption bands of asymmetric stretching of CH$_3$- and CH$_2$- groups (2978 and 2960 cm^{-1}); symmetric stretching of CH- and CH$_2$ groups (2994 and 2937 cm^{-1}); stretching of conjugated (1687 cm^{-1}) and unconjugated (1720 cm^{-1}) carbonyl groups C = O; skeletal CH vibrations (599 cm^{-1}); and CH- bending (622 cm^{-1}).

The IR absorption spectra of copolymers overlapped with a slight shift and contained absorption bands corresponding to vibrations of the main structural components of the polymers except absorption bands of vibrations of the terminal C-OH and COOH groups. The spectra showed absorption bands of asymmetric stretching of CH$_3$- and CH$_2$- groups (2994, 2974, and 2936 cm^{-1}); symmetric stretching of CH- and CH$_2$ groups (2874 and 2878 cm^{-1}); and stretching of carbonyl groups C = O (1700–1760 cm^{-1}). Examination of the fine structure of absorption bands using expanded scales, in the 1235–1335 cm^{-1} range, showed some differences between P(3HB) and P(3HB-co-3HV) specimens: in the right-hand shoulder of the absorption band of the P(3HB) specimen, there was a narrow local peak with the maximum at 1300 cm^{-1}, while in the P(3HB-co-3HV) specimens, the intensity of the corresponding peak was lower and its maximum was shifted to 1296–1297 cm^{-1}. The most characteristic difference of the IR spectra of P(3HB-co-3HHx) specimens from those of P(3HB) ones was the higher intensity of absorption bands in the short-wave region of the spectrum (2800–3000 cm^{-1}). The reason may be a greater contribution of the stretching vibrations of -C-H and-CH$_2$-groups in P(3HB-co-3HHx) copolymers. IR spectra of P(3HB-co-4HB) specimens showed that the intensity of the absorption band in the 1235–1335 cm^{-1} range decreased significantly due to asymmetric stretching vibrations of the -C-O-C- group. The decrease in the intensity of this absorption band could be attributed to a decrease in the mass percent of the -C-O-C- group and an increase in the percent of the CH$_2$ group. The IR spectra described above were not informative enough to study PHAs with different chemical structure, and special research of the use of IR spectroscopy for this purpose is needed.
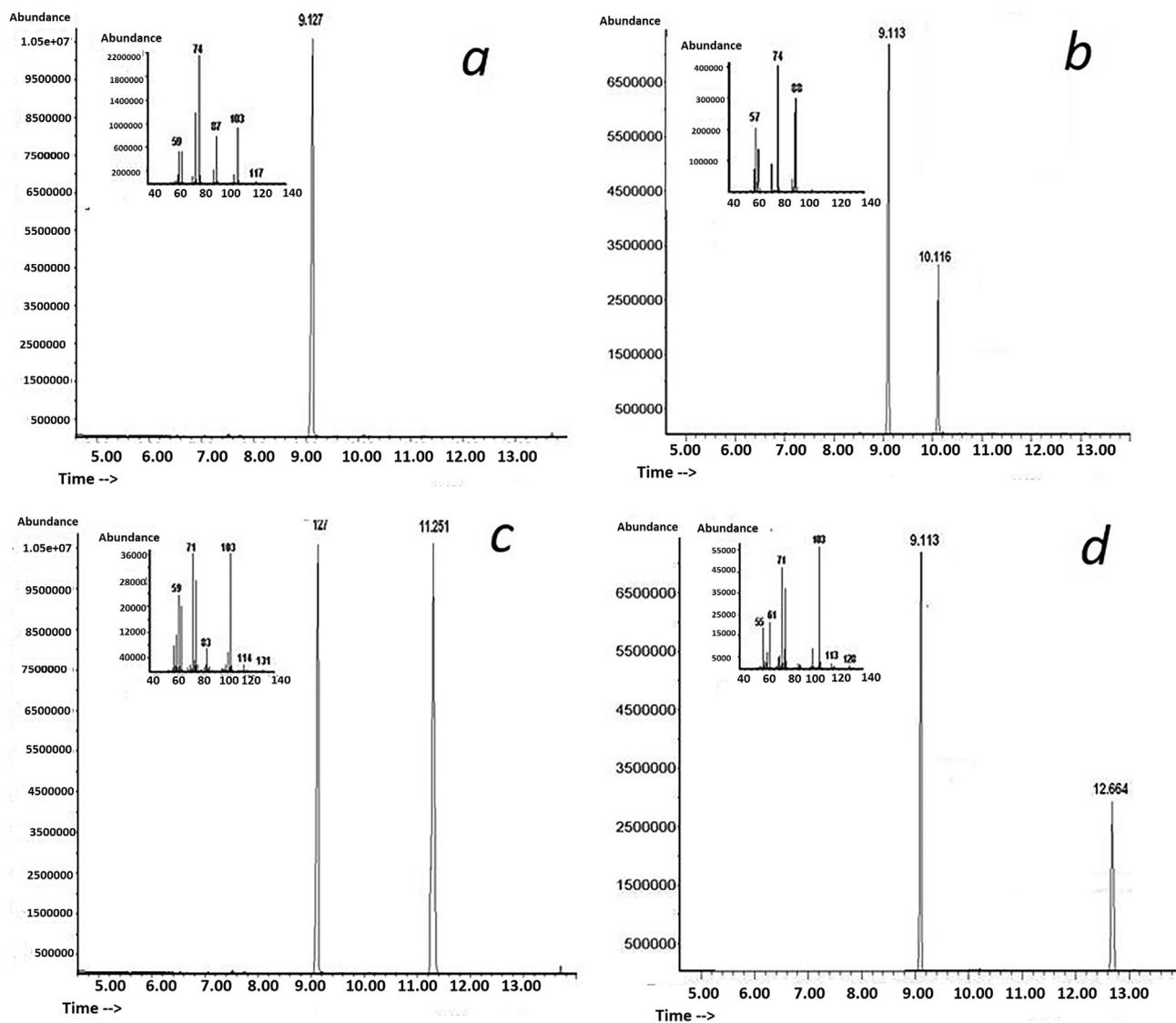
Fig. 1. Ion chromatograms of PHA copolymers with different compositions: a – P(3HB), b – P(3HB-*co*-3HV), c – P(3HB-*co*-4HB), d–P(3HB-*co*-3HHx), and mass spectra of methyl esters of different monomers constituting PHAs, with retention times: methyl-3-hydroxybutyrate (3HB) – 9.113; methyl-3-hydroxyvalerate (3HV) – 10.116; methyl-4-hydroxybutyrate (4HB) – 11.251; methyl-3-hydroxyhexanoate (3HHx) – 12.664 mini mass spectra of methyl esters.

3.1. Chemical structure and properties of PHAs with different compositions of monomer units

The chemical structure and properties of PHAs with different compositions were studied as dependent on their monomer composition and monomer proportions, while the possibility of the effects of supplementary and secondary factors was excluded to the fullest extent. The study of physicochemical properties of PHA copolymers (degree of crystallinity, temperature and molecular-weight parameters) showed considerable differences between specimens with different compositions (Table 1). In addition, Table 1 contains the data on the properties of some synthetic polymers for comparison purposes.

One of the basic characteristics of high-molecular-weight compounds is molecular-weight distribution, which affects polymer processing and properties of polymer-based products. All copolymers, regardless of their monomer composition, had lower molecular weights and higher polydispersity than the P(3HB) homopolymer (Table 1). A direct relationship was found for P(3HB-*co*-3HV) copolymers: as 3HV increased from 10 to 65 mol%, the M_w decreased from 836 to 398 kDa. The weight average molecular weight of P(3HB-*co*-4HB) and P(3HB-*co*-3HHx) decreased as well, but there was no clear relationship with

the molar fractions of 4HB and 3HHx. The M_w values of all ter- and quaterpolymers, which contained 50–55 mol% 3HB and 45–50 mol% of the three other monomers (4HB + 3HV + 3HHx), were similar to each other and decreased (470–620 kDa). In addition, PHA copolymers had increased polydispersity (between 3.2 and 4.2–4.6), suggesting higher heterogeneity of the polymer and an increase in the number of carbon chain fragments with different degrees of polymerization.

Thermal properties of PHAs and their ability to crystallize in their native state are their most significant parameters, as they determine the thermomechanical properties of the polymers and, hence, their ability to be processed from the melts. PHAs, like many other polymers, have a heat distortion temperature somewhat lower than the thermal degradation temperature. Thus, polymers cannot exist in the gaseous state, and the main type of phase equilibrium in them is a condensed state – crystalline, glassy, viscoelastic, and liquid. The significant difference between the melting point and the thermal degradation temperature is an important processing property of the polymer: owing to this property, the polymer can be processed into different products from melts, using generally accepted methods (solvent molding, extrusion, injection molding). P(3HB) has softening point about 110 °C and crystallization temperature (T_c) between 85 and 110 °C (Table 1, Fig. 3). The

Table 1

Composition and physicochemical properties of different PHAs: 3-hydroxybutyrate homopolymer; bi-, ter-, and quaterpolymers (For comparison purposes, the table contains the data on a number of synthetic polymers (PP [105], PET [106], PCL [107])).

Sample	PHA composition (mol.%)				M_w (kDa)	Đ	C_x (%)	T_g (°C)	T_c (°C)	T_{melt} (°C)	T_{degr} (°C)
3-hydroxybutyrate homopolymer											
P(3HB)											
1	100.0				920	2.5	78	–	85–117	170–182	280–300
Copolymers											
P(3HB-co-3HV)											
2	89.8	10.2			826	1.8	76	−0.9	52.5 57.5	163.9	277.8
3	85.0	15.0			690	2.8	65	−1.0	64.2	133.4 144.4	272.1
4	72.8	27.2			576	3.2	54	−1.9	78.1	122.5 162.5	275.9
5	35.0	65.0			398	3.44	58	−4.4	57.0	130.0 148.6	278.6
P(3HB-co-4HB)											
6	64.5	35.5			660	3.6	22	−9.5	58.5	165.5	278.4
7	24.8	75.2			840	3.8	16	−3.6	78.1 57.7	164.3	265.3
8	86.0	14.0			715	2.9	44	−11.7	68.2	129.8 161.4	272.3
P(3HB-co-3HHx)											
9	62.0	38.0			486	3.7	52	−1.6	71.2	169.2	260.1
10	83.6	16.4			390	4.3	49	−0.6	57.2	168.7	281.5
11	91.0	9.0			520	3.9	60	−0.2	63.2	170.2	262.7
Terpolymers											
P(3HB-co-3HV-co-3HHx)											
12	55.0	29.3	15.7		459	3.4	32	−0.1	60.2	166.4	264.6
13	57.6	21.9	20.5		548	4.2	21	0.5	54.5 59.5	146.9 165.3	267.1
14	49.4	27.2	23.4		360	3.8	28	−7.0	55.7	142.4 165.0	286.4
P(3HB-co-3HV-co-4HB)											
15	55.0	28.6	16.4		570	3.0	35	−1.7	53.2	167.0	284.0
16	56.2	13.4	30.4		620	4.1	28	−5.7	59.8	170.4	280.0
Quaterpolymers											
P(3HB-co-3HV-co-4HB-co-3HHx)											
17	61.4	19.4	14.0	5.2	542	3.7	37	1.2	59.9 50.4	167.2	282.6
18	73.5	17.6	6.4	2.5	479	4.6	40	−4.6	70.2	170.6	284.4
Synthetic polymers											
PET							75–90	−130	–	–	120–130
PP							45–47	–	104.5	99.8	158
PCL						50	58–65	–	30	–	67

«-» - no data available.

melting peak of P(3HB) is sharp and narrow, with a short low-temperature "tail".

PHA copolymers differed from P(3HB) in their thermal properties (Table 1, Fig. 3). P(3HB-co-3HV) copolymers had the widest melting ranges, from 107 to 168 °C. Polymers with larger 3HV fractions had lower T_{melt}, which decreased from 163.9 °C for the polymer with 3HV higher than 10 mol% to 130 °C for the polymer with 3HV 65 mol%. Copolymers with 3HV higher than 10 mol% had two melting peaks, and no crystallization was observed at these cooling rates; crystallization occurred when the specimen was reheated. All copolymers containing 3HV were characterized by glass transition followed by relaxation in the temperature range between −0.9 and −4.4 °C; the glass transition temperature decreased as the molar fraction of 3HV increased. P(3HB-co-4 HV) copolymers had broader peaks compared to P(3HB). As the molar fraction of 4HB increased, thermal analysis showed two melting peaks and two crystallization peaks. This type of copolymers had the lowest glass transition temperatures, which increased with an increase in 4HB. For copolymers containing 3HHx, melting points lay between 168 and 170 °C. The melting peaks were sharper than those of other bipolymers, and no multiple melting peaks were observed. Only the specimen containing 3HHx 16.4% had a broad crystallization peak

during cooling (from 102.24 °C to 42.2 °C). The glass transition temperature ranged between −1.6 and −0.2 °C, decreasing with an increase in 3HHx concentration. The melting points of terpolymers were insignificantly lower compared to P(3HB). Terpolymer shad two melting peaks, one in the 142–150 °C range and the other in the 165–170 °C range, and two crystallization peaks. Glass transition temperature of these copolymers was between −0.1 and 0.5 °C. The T_{melt} of quaterpolymers was 167.2 and 170 °C, respectively. The difference between the melting point and the degradation temperature was found for all specimens. The earliest onset of thermal degradation was noted for the copolymers containing monomers of the medium-chain-length 3HHx (260 °C), while terpolymers containing 4HB as the third monomer and quaterpolymers had the highest degradation temperatures (about 280 °C). Despite slight variations, all types of PHA copolymers showed the difference between T_{melt} and T_{degr} of at least 100 °C, i.e., remained thermostable.

The most important parameter of polymer materials is the degree of crystallinity: the ratio of the crystalline to amorphous phase. The ability of a PHA to crystallize is determined by the inner properties of its chains and is characterized by crystallization temperature, T_c. In a number of polymers, crystallization develops only partly, and most of the
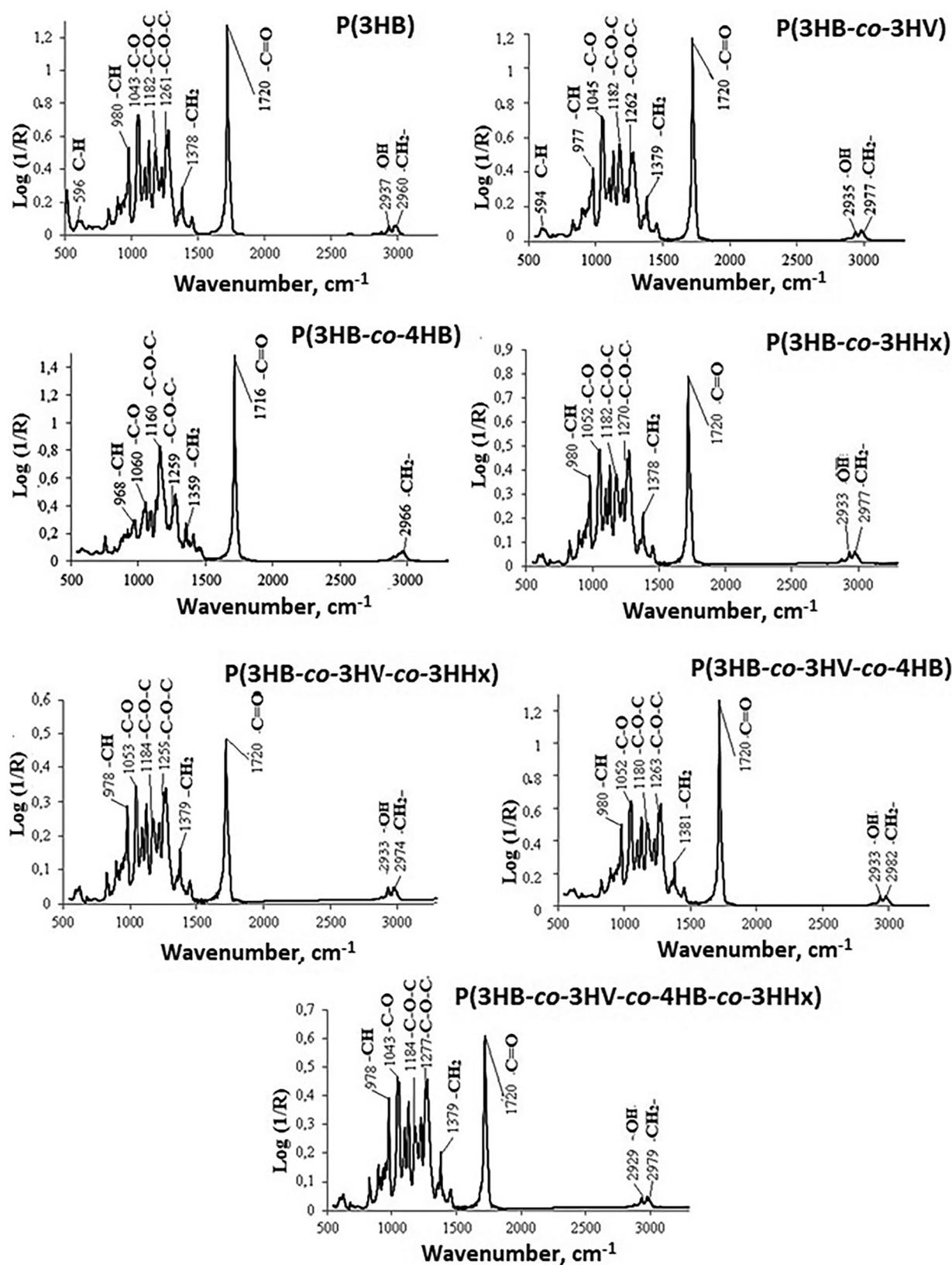
Fig. 2. IR spectra of PHAs with different compositions: P(3HB), bi-, ter-, and quaterpolymers.

polymers, including PHAs, are semi-crystalline materials. The degree of crystallinity is the least-studied aspect of PHAs.

X-Ray structure analysis showed that in the homopolymer of 3-hydroxybutyric acid, the crystalline phase prevailed over the amorphous one (C_x was 70% or higher). All types of copolymers exhibited lower crystallinity compared to P(3HB) (Table 1). P(3HB-co-3HV) copolymers are isodimorphous due to co-crystallization of the monomer units. When the 3HB to 3HV ratio changes, the crystal lattice of
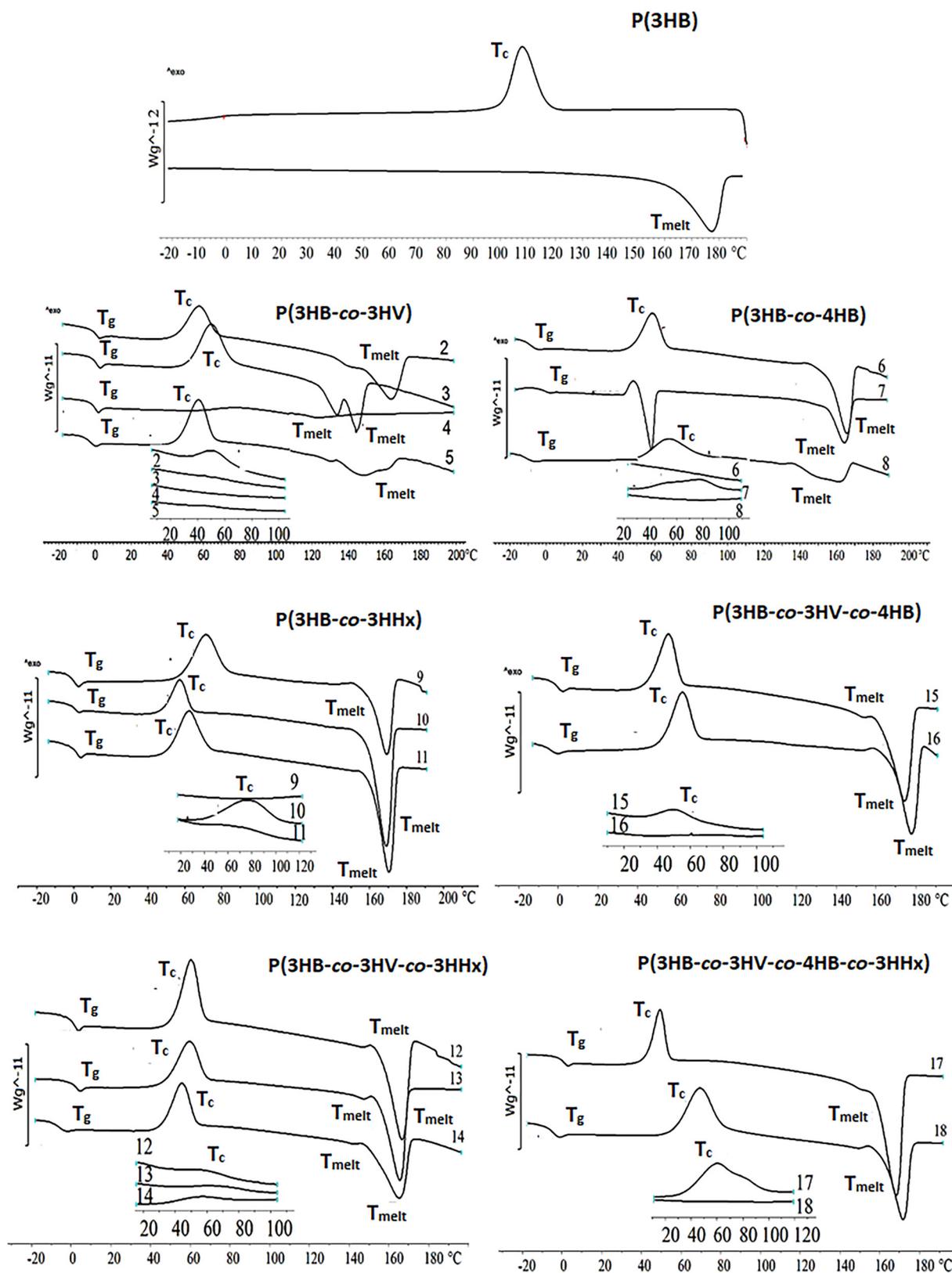
Fig. 3. Results of thermal analysis of PHAs with different compositions: P(3HB), bi-, ter-, and quaterpolymers (numbering as in Table 1).

the copolymer changes as well. Both monomer units (3-hydroxybutyrate and 3-hydroxyvalerate) have similar forms and take up equal amounts of space. The conformations of the polymer chains of both monomers are compatible with both types of crystal lattice. Isodimorphism affects the degree of crystallinity of the copolymer. In spite of isodimorphism, however, copolymers of this type retained the

chemical properties of the high-crystallinity P(3HB). A linear relationship of the C_x of 3HV was noted in the 3HV range between 25 and 35 mol%.

The C_x of P(3HB-*co*-4HB) and P(3HB-*co*-3HHx), which contained 4HB and the medium-chain-length 3HHx, respectively, changed in a similar way (Table 1). The degree of crystallinity of these copolymers was decreased as well (49–60%). The copolymers containing large molar fractions of 4HB had a poly-4-hydroxybutyrate-type crystal lattice, as the high concentration of 4HB caused the crystal growth rate to decrease, and the 4HB monomer units were not incorporated into the 3-hydroxybutyrate lattice, which resulted in considerable amorphization of the copolymer. The degree of crystallinity of the copolymers containing 4HB 35.5 and 75 mol% dropped to 16 and 22%, respectively, suggesting that 4HB caused the strongest amorphization of PHAs. These findings support the results that our team obtained in previous studies, in which PHA copolymers with smaller fractions of monomer units other than 3HB were used, indicating that PHA copolymers have decreased molecular weights and increased polydispersity values and that the most significant decrease in the degree of crystallinity is observed in copolymers containing 4-hydroxybutyrate [25,29,30].

3.2. Surface microstructure of polymer films prepared from PHAs with different compositions

Films prepared from PHAs with different chemical composition were used to study the effects of the monomer composition and percentages on the surface properties of the films and their physical/mechanical parameters.

The significance of this research is determined by the interdisciplinary approach used in tissue engineering to create equivalents of tissues and organs – complex structures of functioning cells attached to cell scaffolds. The properties of the scaffold result from the properties of the starting material and the technology employed to process it. Biological properties of biomedical materials and products are regulated in order to alter physicochemical properties of their surface (chemical composition, porosity, hydrophilic properties, charge, roughness, morphology, etc.). This is the way to affect properties of polymer products, increase biodegradation rates, enhance flexibility and mechanical strength, and make the surface more hydrophilic and porous, which facilitates cell attachment, improves gas dynamic properties of the scaffolds, and increases their permeability to substrates and metabolic products of cells and tissues. Thus, an integrated study was performed to characterize the films prepared from PHAs of different structures.

After the polymers were examined and characterized, they were used to produce films. Films were prepared by casting 2% PHA solutions onto Teflon surface followed by solvent evaporation. The effect of PHA composition on the parameters of polymer films was conducted using SEM, AFM, measurements of contact angles for liquids, and measurements of physical/mechanical parameters (Figs. 4, 5, Table 2–4). The study using polymer films prepared from PHA bi-, ter-, and quaterpolymers was performed for the first time.

The films were similar in thickness (298.94 ± 12.79 μm) but considerably different in morphology. The ImageJ image processing program was used to analyze SEM images of the films with different chemical composition, taking into account the total number of pores and their sizes (Fig. 4). On the surface of the films, there were both crater-like hollows of various shapes and true pores (holes), which could be associated with differences in the crystallization kinetics during solvent evaporation and formation of films from polymers with different compositions. All copolymer films were more porous than the dense and virtually pore-free film prepared from the P(3HB) homopolymer (on which there were a few pores 1.0 μm in diameter or smaller). The number and sizes of pores on copolymer films varied considerably, regardless of the PHA composition. Numerous large pores, 2 to 4 μm in diameter, were observed on films prepared from different PHA types (bi-, ter-, and quaterpolymers). At the same time, on the surface of P(3HB-*co*-4HB) and P(3HB-*co*-3HV) films, the pores tended to grow larger as the fractions of the monomer other than 3HB (4HB and 3HV) increased. On the P(3HB-*co*-4HB) and P(3HB-*co*-3HV) films with the maximal fractions of 4HB and 3HV (75 and 65 mol%, respectively), there were a few pores of the largest diameter (5–6 μm). The surface of bipolymers containing 3HHx was covered with numerous smaller pores – 1.5 to 1.0 μm or even less. The number of pores on the films prepared from PHAs with different compositions varied noticeably: between several pores per unit area and a few dozen pores or more. Except the most porous film prepared from P(3HB-*co*-4HB), on which there were very many pores of the largest diameter, on other films, the number of pores usually increased with a decrease in their size. Thus, PHAs with different chemical compositions and differing in their molecular weight and degree of crystallinity can be used to prepare solution-cast films with pores varying in number and size, which could affect attachment and development of eukaryotic cells.

Another important parameter of polymer product is physicochemical reactivity of the surface. Nanometer roughness determines protein adhesion, cell attachment, growth, and synthesis of specific proteins. Examination of polymer films using atomic force microscopy showed the effect of the chemical composition of PHAs on surface roughness (Fig. 5, Table 2). The polymer specimen prepared from the P(3HB) homopolymer had the lowest arithmetic mean surface roughness (S_a) and root mean square roughness (S_q) – 154 and 180 nm, respectively. Most of the films prepared from PHA copolymers had higher S_a and S_q values. Only two specimens, P(3HB-*co*-3HHx) (3HHx 9 mol%) film and one quaterpolymers film, had lower roughness parameters compared to the P(3HB) film.

The absolute values of the arithmetic mean surface roughness, the root mean square roughness, and the maximal height (S_z), integrating the peak-to-valley heights, differed considerably between copolymer films. The S_a values of P(3HB-*co*-3HV) copolymers with the lowest 3HV and with 3HV 65 mol% were 297 nm and 689 nm, respectively. P(3HB-*co*-4HB) copolymers had similar S_a values, regardless of 4HB concentration; the S_a of P(3HB-*co*-3HHx) varied from 93 to 202 nm. Films of PHA terpolymers of both types had similar S_a values – about 273–352 nm, which was similar to the S_a of one of the quaterpolymer films. Hence, chemical composition of the PHA strongly affected the surface roughness of the films.

The findings obtained in the present study are in good agreement with the data suggesting the effect of the monomer composition of PHAs on the surface microstructure of PHA films. For instance, atomic force microscopic analysis showed that surface roughness values of all films from P(3HB-*co*-3HV) with 26 and 12 mol% HV were 92.5 and 290.8 nm, increasing to 588.8 nm for films made from the polymer blended with polyethylene glycol [39]. A study by Bera, et al. [40] demonstrated that incorporation of 3HV increased the film surface roughness. At the same time, there are data suggesting that an increase in 4HB in another copolymer, P(3HB-*co*-4HB), caused a decrease in the film surface roughness, making the films smoother [41–43]. Even small changes in the surface profile may result in changes in cellular response, which may vary from slight enhancement to considerable inhibition of cellular activity. Variations in surface roughness and topography, however, cause different cellular response, depending on how sensitive the cells are to such variations, and, thus, special research should be performed in cultures of various eukaryotic cells.

3.3. Properties of polymer films prepared from PHAs with different compositions

Hydrophilic/hydrophobic balance of the surface is a major parameter that indirectly characterizes hydrophilicity of the film and affects cell adhesion and viability. The hydrophilic/hydrophobic balance is estimated by measuring contact angle for liquids.

Being hydrophobic, poly-3-hydroxybutyrate is not the material of choice for fabricating cell scaffolds, as hydrophobicity negatively affects
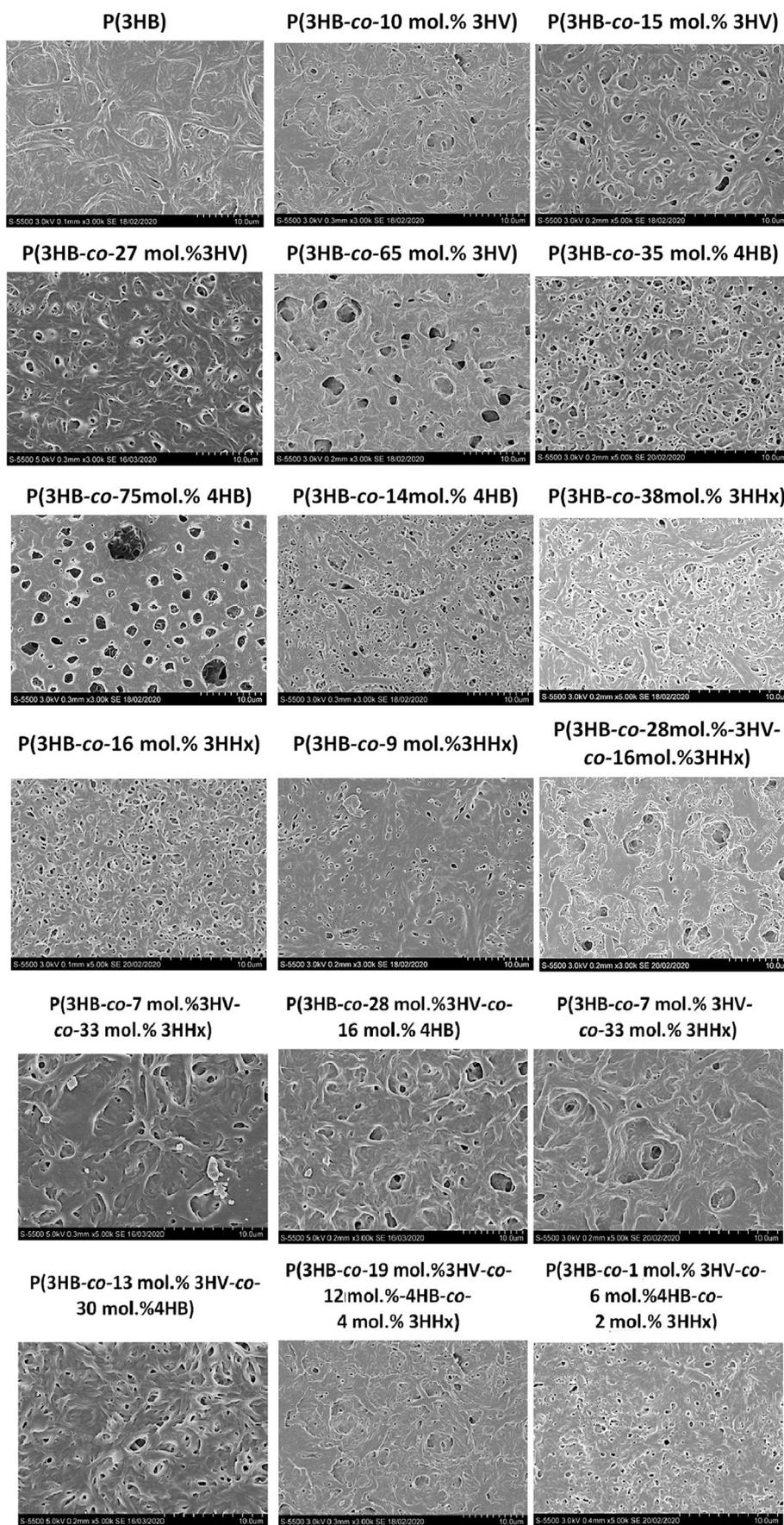
P(3HB) **P(3HB-*co*-10 mol.% 3HV)** **P(3HB-*co*-15 mol.% 3HV)**

P(3HB-*co*-27 mol.%3HV) **P(3HB-*co*-65 mol.% 3HV)** **P(3HB-*co*-35 mol.% 4HB)**

P(3HB-*co*-75mol.% 4HB) **P(3HB-*co*-14mol.% 4HB)** **P(3HB-*co*-38mol.% 3HHx)**

P(3HB-*co*-16 mol.% 3HHx) **P(3HB-*co*-9 mol.%3HHx)** **P(3HB-*co*-28mol.%-3HV-*co*-16mol.%3HHx)**

P(3HB-*co*-7 mol.%3HV-*co*-33 mol.% 3HHx) **P(3HB-*co*-28 mol.%3HV-*co*-16 mol.% 4HB)** **P(3HB-*co*-7 mol.% 3HV-*co*-33 mol.% 3HHx)**

P(3HB-*co*-13 mol.% 3HV-*co*-30 mol.%4HB) **P(3HB-*co*-19 mol.%3HV-*co*-12mol.%-4HB-*co*-4 mol.% 3HHx)** **P(3HB-*co*-1 mol.% 3HV-*co*-6 mol.%4HB-*co*-2 mol.% 3HHx)**

Fig. 4. SEM images of films prepared from PHAs with different compositions. Bar = 10 μm.

P(3HB) **P(3HB-co-10 mol.% 3HV)** **P(3HB-co-15 mol.% 3HV)**

P(3HB-co-27 mol.%3HV) **P(3HB-co-65 mol.% 3HV)** **P(3HB-co-35 mol.% 4HB)**

P(3HB-co-75 mol.% 4HB) **P(3HB-co-14mol.% 4HB)** **P(3HB-co-38mol.% 3HHx)**

P(3HB-co-16 mol.% 3HHx) **P(3HB-co-9 mol.%3HHx)** **P(3HB-co-28mol.%-3HV-co-16mol.%3HHx)**

P(3HB-co-22 mol.%3HV-co-20 mol.%3HHx) **P(3HB-co-7 mol.%3HV-co-33mol.% 3HHx)** **P(3HB-co-28 mol.%3HV-co-16mol.% 4HB)**

P(3HB-co-13 mol.% 3HV-co-30mol.%4HB) **P(3HB-co-19 mol.%3HV-co-12mol.%-4HB-co-4mol.%3HHx)** **P(3HB-co-1 mol.%-3HV-co-6mol.%4HB-co-2mol.%3HHx)**
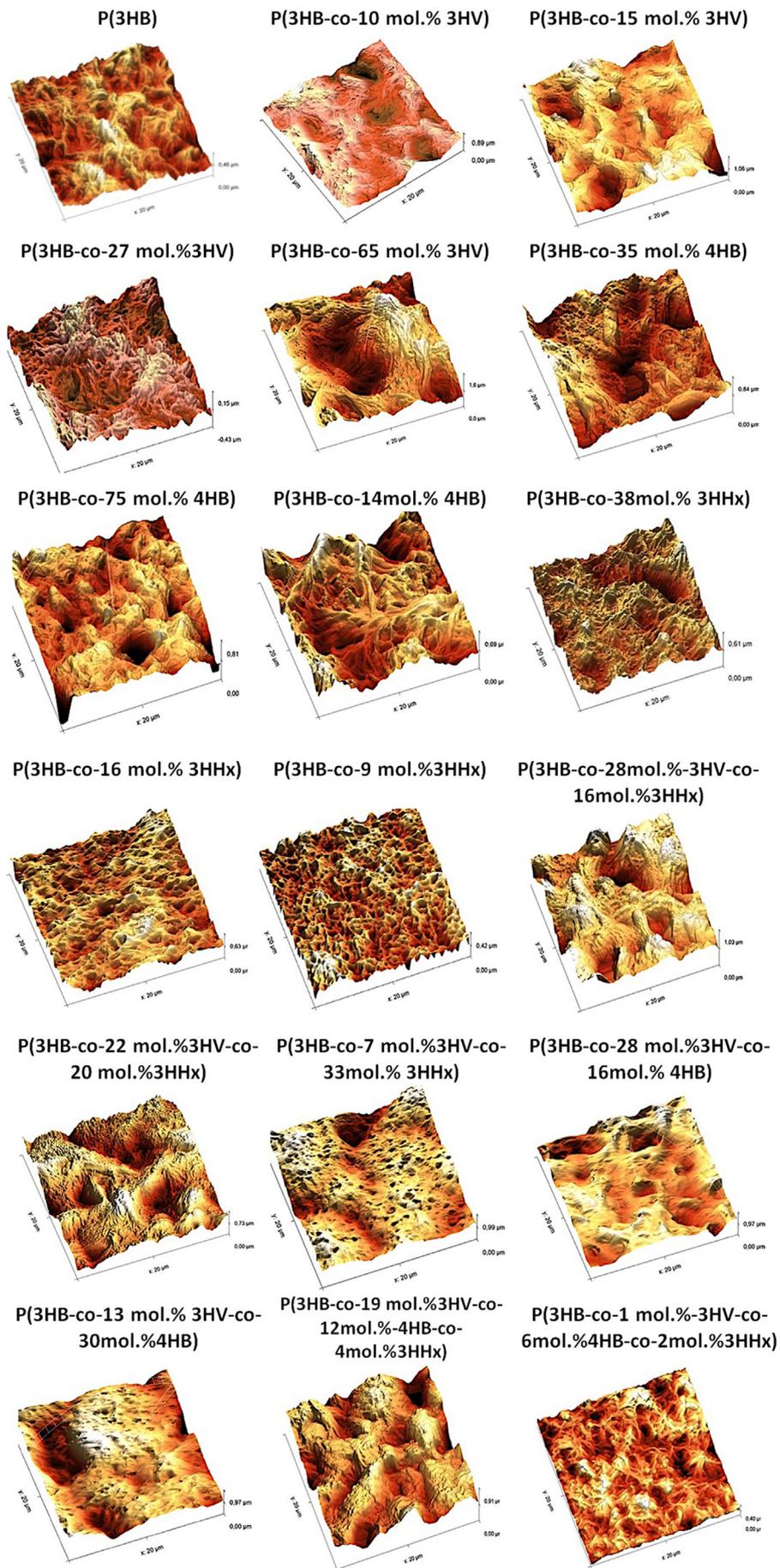
Fig. 5. AFM images of surfaces of the films prepared from PHAs with different compositions.

cell attachment. PHAs are hydrophobic because of long-chain hydroxyl fatty acids with fewer functional groups. Because of their hydrophobic properties, the biocompatibility associated with PHAs is found to be inadequate [44–47].

The surface of PHA films can be rendered more hydrophilic using a number of approaches: blending of the polymer with other materials, chemical modification of the surface, plasma treatment, and biological synthesis of polymers with different compositions, chiefly copolymers. The last-mentioned approach was successfully used in the present study. Water contact angle of the film prepared from the high-crystallinity P(3HB) reached 92°, and the calculated values of surface energy and its dispersion and polar components were 30.8, 28.6, and 2.3 mN/m, respectively (Table 3). At the same time, water contact angles of copolymer films were lower although the decrease ranged between 10 and 100%. As the values of water contact angle differed greatly, it was impossible to establish any definitive relationship between the concentrations of different monomers in copolymers and the value of water contact angle. Films of bi- and terpolymers containing 3HHx had the smallest contact angles (up to 48–56°), and water contact angles of the films made from copolymers containing 3HV and 4HB reached 60–79°. Parameters calculated from the contact angle (surface energy and its dispersion and polar components) showed that the surfaces of copolymer films were more hydrophilic. Thus, incorporation of other monomers into the carbon chain of 3HB causes favorable changes not only in polymer properties (a decrease in crystallinity) but also in the properties of the products based on these polymers, which, being more hydrophilic, will have higher affinity for cells.

The study of physical/mechanical properties of the films (Table 4) showed that copolymer films differed from the P(3HB) films in their mechanical strength. The parameters of strength (tensile strength and Young's modulus) of P(3HB) films were18.9 and 2034 MPa, respectively, while elongation at break (the parameter of elasticity) was very low – 3.1%. All copolymer films exhibited considerably decreased values of tensile strength (between 11 and 12 and 7–6 MPa) and Young's modulus (between 1420 and 1180 MPa). However, films are generally characterized by rather low mechanical strength. An essential effect of PHA composition was enhanced elongation at break, which was higher by one and a half to two orders of magnitude in copolymers. That is, copolymer films had enhanced elasticity – an important property for films. Thus, the targeted change in carbon nutrition conditions resulted in the synthesis of PHAs with different chemical compositions and diverse and enhanced properties.

Similar results were obtained by other researchers. A number of studies showed that surfaces of the films prepared from copolymers containing 3-hydroxyhexanoate and 4-hydroxybutyrate facilitated attachment and proliferation of eukaryotic cells [48–50]. Previously, our team studied properties of two types of PHAs (the P(3HB) homopolymer and P(3HB-co-3HV) copolymers) and found that the presence of 3-hydroxyvalerate monomer units enhanced biological compatibility of polymer films with various eukaryotic cells (fibroblasts, hepatocytes, endothelial cells) [17] and increased hemocompatibility of the copolymer material on contact with blood [51]. In more recent studies, when synthesis of a wider range of PHAs was achieved, our team examined surface microstructure and properties of the films prepared from P(3HB) and copolymers of 3-hydroxybutyrate with 3-hydroxyvalerate, 3-hydroxyhexanoate, and 4-hydroxybutyrate and found considerable differences between them. However, concentrations of the monomers other than 3HB in those copolymers were rather low, about 10 mol%. According to the degree to which the monomers caused a decrease in water contact angle and enhanced surface hydrophilicity and elasticity of the films, they were ranked as follows: 4HB – 3HHx – 3HV [25,26,52].

4. Conclusions

A unique set of PHA bi-, ter- and quaterpolymers that differed in their monomer composition was synthesized by the *Cupriavidus necator*

Table 2
Surface roughness parameters of the films prepared from PHAs with different compositions based on results of atomic force microscopy (AFM).

Sample	PHA composition (mol.%)			Arithmetic mean surface roughness (S_a) (nm)	Root mean square roughness (S_q) (nm)	Peak-to-valley height (S_z) (nm)
P(3HB)						
1	100.0			154.0	180.1	1255.9
Copolymers						
P(3HB-co-3HV)						
2	89.8	10.2		297.0	367.4	2355.4
3	85.0	15.0		372.9	470.8	3259.6
4	72.8	27.2		206.8	254.1	1594.7
5	35.0	65.0		489.1	444.8	4295.0
P(3HB-co-4HB)						
6	64.5	35.5		290.1	370.4	2321.3
7	24.8	75.2		211.2	286.8	2219.8
8	86.0	14.0		243.1	302.5	1900.8
P(3HB-co-3HHx)						
9	62.0	38.0		172.4	222.4	1677.8
10	83.6	16.4		202.4	250.5	1271.6
11	91.0	9.0		93.7	118.2	880.6
P(3HB-co-3HV-co-3HHx)						
12	55.0	29.3	15.7	352.8	449.3	2841.9
13	57.6	21.9	20.5	273.3	346.7	1999.8
14	49.4	27.2	23.4	313.7	404.5	2714.5
P(3HB-co-3HV-co-4HB)						
15	55.0	28.6	16.4	330.0	401.7	2679.6
16	56.2	13.4	30.4	306.3	387.7	2668.1
P(3HB-co-3HV-co-4HB-co-3HHx)						
17	61.4 19.4	14.0	5.2	308.0	380.8	2514.9
18	73.5 17.6	6.4	2.5	119.9	151.8	1089.3

Arithmetic mean surface roughness (S_a) is similar to root mean square roughness (Sq), and the difference is that it is calculated as total difference modules between the data value and the mean rather than squared difference.

Peak-to-valley height (S_z) comprises the full range of values; this is total difference between the profile valleys and peaks (between the lowest valley (S_v) and S_p (the highest peak)).

B-10646 bacterial strain cultured in the medium supplemented with measured portions of butyrolactone, potassium valerate, and potassium hexanoate – precursors of 4-hydroxybutyrate (4HB), 3-hydroxyvalerate (3HV), and 3-hexanoate (3HHx). The highly purified specimens were examined using HPLC, DSC, and X-Ray techniques, and considerable differences were found in their crystallinity, molecular weight, and thermal properties. All copolymers, regardless of their monomer composition, had decreased weight average molecular weights and increased values of polydispersity compared to the high-crystallinity P(3HB). PHA copolymers showed different thermal behavior: an insignificant decrease in T_{melt} and the presence of the second peak in the melting region and changes in parameters of crystallization and glass transition. At the same time, they retained thermostability and the difference between T_{melt} and T_{degr} of at least 100–120 °C. Incorporation of 4HB, 3HV, and 3HHx monomer units into the 3-hydroxybutyrate chain affected crystallization kinetics, causing changes in the amorphous to crystalline ratio and decreasing the degree of crystallinity (C_x), which made PHAs more readily processable and improved their quality. The present study was the first to investigate surface microstructure and physical/mechanical properties of the films made from PHA copolymers with widely diverse chemical compositions as dependent on their monomer composition. Copolymer films exhibited enhanced elasticity and an increase in elongation at break by two to three orders of magnitude (to 100–300% compared with 2–3% in P(3HB)), but their mechanical strength, measured as tensile strength and Young's modulus, decreased. Monomer units other than 3-hydroxybutyrate were found to enhance film surface hydrophilicity and development. Results obtained in the present study contribute to the solution of a critical issue of producing degradable functional polymer materials.

Table 3

Surface properties of the films prepared from PHAs with different compositions.

Sample	PHA composition (mol.%)			Water contact angle (°)	Surface energy (mN/m)	Dispersion component (mN/m)	Polar component (mN/m)
P(3HB)							
1	100.0			92.1 ± 6.3	30.8 ± 0.5	28.6 ± 0.3	2.3 ± 0.21
Copolymers							
P(3HB-*co*-3HV)							
2	89.8	10.2		64.6 ± 4.1	49.6 ± 1.3	38.8 ± 0.6	10.8 ± 0.7
3	85.0	15.0		68.4 ± 5.2	49.7 ± 1.5	41.5 ± 1.1	8.2 ± 0.4
4	72.8	27.2		69.4 ± 9.4	50.8 ± 2.6	43.7 ± 1.5	7.1 ± 1.2
5	35.0	65.0		76.3 ± 3.5	46.0 ± 1.3	41.1 ± 1.1	4.9 ± 0.3
P(3HB-*co*-4HB)							
6	64.5	35.5		81.7 ± 3.2	41.4 ± 0.9	37.8 ± 0.7	3.7 ± 0.2
7	24.8	75.2		70.5 ± 6.4	47.5 ± 2.0	40 ± 1.5	7.6 ± 0.5
8	86.0	14.0		82.4 ± 5.4	43.8 ± 0.5	40.9 ± 0.3	2.9 ± 0.2
P(3HB-*co*-3HHx)							
9	62.0	38.0		56.3 ± 6.2	57.1 ± 2.9	43.5 ± 2.0	13.6 ± 0.8
10	83.6	16.4		41.9 ± 3.4	65.7 ± 2.2	44.8 ± 1.5	20.9 ± 0.7
11	91.0	9.0		71.5 ± 1.9	48.8 ± 2.4	42.3 ± 1.9	6.5 ± 0.5
P(3HB-*co*-3HV-*co*-3HHx)							
12	55.0	29.3	15.7	65.9 ± 7.4	47 ± 0.9	35.9 ± 0.3	11.1 ± 0.5
13	57.6	21.9	20.5	68.9 ± 7.7	47.3 ± 2.9	38.6 ± 2.2	8.7 ± 0.8
14	49.4	27.2	23.4	67.9 ± 10.8	50 ± 1.6	41.7 ± 1.1	8.3 ± 0.5
P(3HB-*co*-3HV-*co*-4HB)							
15	55.0	28.6	16.4	48.2 ± 6.7	63.4 ± 1.2	46.6 ± 0.6	16.8 ± 0.6
16	56.2	13.4	30.4	68.8 ± 7.6	44.8 ± 2.5	34.9 ± 1.1	9.9 ± 1.4
P(3HB-*co*-3HV-*co*-4HB-*co*-3HHx)							
17	61.4	19.4	14.0	5.2	70.8 ± 6.6	51.1 ± 1.3	44.9 ± 0.9	6.2 ± 0.4
18	73.5	17.6	6.4	2.5	87.4 ± 9	41.9 ± 0.5	40.3 ± 0.2	1.7 ± 0.3

Funding

Table 4

Physical/mechanical properties of the films prepared from PHAs with different chemical compositions (For comparison purposes, the table contains the data on a number of synthetic polymers (PP [108], PET [109], PCL)).

Sample	PHA composition (mol.%)				Tensile strength (MPa)	Young's modulus (MPa)	Elongation at break (%)
P(3HB)							
1	100.0				18.9 ± 1.3	2034.5 ± 157.7	3.1 ± 0.4
Copolymers							
P(3HB-*co*-3HV)							
3	85.0	15.0			8.1 ± 0.7	1198.2 ± 110.0	107.8 ± 6.2
4	72.8	27.2			7.9 ± 0.9	1109.6 ± 98.9	170.4 ± 7.9
P(3HB-*co*-4HB)							
6	64.5	35.5			18.1 ± 1.4	1180.1 ± 121.4	510.2 ± 10.2
8	86.0	14.0			7.93 ± 1.0	1290.7 ± 117.3	390.6 ± 21.1
P(3HB-*co*-3HHx)							
10	83.6	16.4			11.8 ± 1.1	1198.6 ± 97.7	320.0 ± 32.6
11	91.0	9.0			6.9 ± 0.5	1189.2 ± 85.9	167.3 ± 12.8
P(3HB-*co*-3HV-*co*-HHx)							
12	55.0	29.3	15.7		13.4 ± 0.9	1278.2 ± 112.2	390.46 ± 23.5
P(3HB-*co*-3HV-*co*-4HB)							
15	55.0	28.6	16.4		9.8 ± 0.7	1230.5 ± 130.0	360.76 ± 19.1
P(3HB-*co*-3HV-*co*-4HB-*co*-3HHx)							
17	61.4	19.4	14.0	5.2	12.1 ± 1.1	1420.6 ± 128.9	210.2 ± 17.2
Synthetic polymers							
PET					20–30	1100	11–13
PP					20–40	1100–1600	150–600
PCL					10–16	150–200	400

CRediT authorship contribution statement

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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