doi:10.2533/chimia.2015.627

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Universities of Applied Sciences

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Chemical Modification of Polyhydroxyalkanoates (PHAs) for the Preparation of Hybrid Biomaterials

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Abstract: Polyhydroxyalkanoates (PHAs) are biopolyesters produced by bacteria as intracellular granules under metabolic stress conditions. Many carbon sources such as alkanes, alkenes, alcohols, sugars, fatty acids can be used as feedstock and thus a wide variety of polyesters and monomer units can be potentially synthetized. The work presented here describes the process to chemically modify such biopolymers in order to render them readily available for the preparation of bio-molecular conjugates as promising new classes of biocompatible biomaterials. Such hybrid biomaterials belong to the rapidly growing class of biocompatible polymers, which are of great interest for medical and therapeutic applications. In this work, the biosynthesis of a new PHA homopolymer and the chemical modification, an epoxidation reaction, are described.

Keywords: Biosynthesis · Epoxidation · Kinetic monitoring · Nuclear magnetic resonance · Polyhydroxyalkanoates

1. Introduction

Polyhydroxyalkanoates (PHAs) are optically active biopolyesters produced by a large number of bacteria as intracellular granules under metabolic stress conditions. PHAs were firstly described as storage compounds in *Bacillus megaterium* by Lemoigne in 1926.[1] The monomer composition of the polymers is variable and strongly depends on the substrates supplied, the culture conditions and the bacteria metabolism (enzymatic affinity). A large number of carbon sources have been used (alkanes, alkenes, alcohols, sugars, fatty acids, *etc.*) and thereby a wide variety of polyesters and monomer units have been synthetized. Today, more than 150 different monomer units have been identified, opening thus a wide range of potential material properties. Based on the number of carbon atoms in the monomer unit, these polymers can be classified mainly into two groups: short chain length (scl) PHA and medium chain length (mcl) PHA. Scl-PHAs (*e.g* polyhydroxybutyrate, PHB) are crystalline and have a tensile strength similar to polypropylene (40 MPa) although more brittle. Alternatively, mcl-PHAs are amorphous or semi-crystalline elastomers with low T_m (< 50 °C) and weak tensile strength. Monomer content and their distribution within the polymer as well as molecular weight are the main parameters influencing the polymer properties. PHB and mcl-PHA copolymer production has been widely extended over the last decades.[2–4] While mcl-PHA copolymers are amorphous, semi-crystalline elastomers with weak mechanical properties, mcl-PHA homopolymers were recently shown to exhibit enhanced tensile strength and Young modulus. In general terms, mcl-PHA homopolymers display better thermal and mechanical properties than their counterpart copolymers. Despite polyhydroxyalkanoates having been identified as a valid alternative to replace petrochemical plastics and being interesting candidates as starting base material for novel hybrid biomaterials, their physico-chemical and biological properties need to be enhanced by means of chemical derivatization or blending with other polymers to fulfil the necessary requirements for industrial or biomedical applications.[5,6] The aim of this work is to prepare chemically activated polymers species to enable the downstream preparation of biocompatible conjugates as promising new classes of soft materials. Therefore hybrid biomaterials belong to the rapidly growing class of biocompatible polyesters which offer the possibility to be modified with amino acids, peptides and ultimately proteins ready for conjugation (crosslinking) at specific binding sites of the biopolymer. In this work, the biosynthesis of a new mcl-homopolymer and the chemical modification, an epoxidation reaction, is described. Furthermore, kinetic monitoring of the epoxidation reaction was followed by ^{1}H-NMR.

2. Experimental

2.1 *Materials*

Pseudomonas putida KTQQ20 (PpKT20) was kindly provided by Professor Chen (Dept. Biological Sciences and Biotechnology, Tsinghua University, Beijing, China). All salts and reagents were purchased from Sigma-Aldrich.

2.2 *Polymer Biosynthesis and Downstream Processing*

PpKT20 is an engineered bacterial strain that synthetizes mcl-PHA homopolymers. This strain is a knock-out mutant of *Pseudomonas putida* KT2442 deprived of the PhaG gene and six genes encoding different enzymes related to the β-oxidation and PHA synthesis.[7] In order to facilitate the later chemical derivatization it is necessary that the PHA-polymer contains a functional group that can be chemically modified. Vinyl groups are highly reactive functionalities and relatively easy to incorporate into the polymer structure during biosynthesis; however, a high content of double bonds in the polymer may induce an undesired intramolecular crosslinking reaction.

PpKT20 was cultivated in modified E2 medium in a 19 L bioreactor, with a 10 L working volume with the following culture conditions: 30 ºC, starting stirring at 400 rpm, flow rate of 0.5 L/min and pO2 fixed at 30% using cascade control. Several bacterial fermentations were carried out using 10-undecenoic acid (FA-C11:1) and undecanoic acid (FA-C11:0) as single carbon source or in combination using different ratios FA-C11:1/FA-C11:0 (Fig. 1).

After 12–14 h of cultivation, cells were harvested by centrifugation at 7500xg, for 20 min at 4 ºC. The wet pellet was frozen and freeze dried. Polymer was first extracted with methylene chloride for 2 h with agitation and then filtered to remove cell debris. The polymer solution was precipitated into ice-cold methanol. The purified PHA was dried under vacuum and stored at –20 ºC for further analysis.[8] After purification, polymer films were prepared by film casting (Fig. 1). Poly(3-

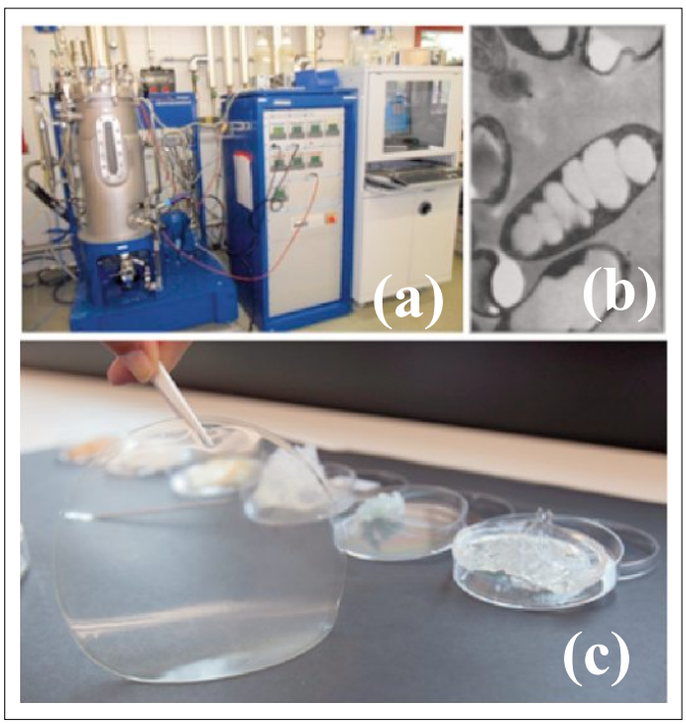

Fig. 1. Polymer production: (a) up-scale fermentation to 42 L reactor, (b) cell containing PHA granules (TEM micrography) and (c) polymer film.

hydroxyundecanoate-co-3-hydroxyundec-10-enoate) polymers containing different vinyl group moieties (100 mol%, 50 mol% and 30 mol%) were synthetized. In polymer code PHA-C11$_x$, the X indicates the mol% of the unsaturated units, which are derived from the 10-undecenoic acid substrate.

2.3 *Polymer Characterization*

The polymer chemical structure was determined by NMR. Molecular weight distribution and thermal properties were determined by gel permeation chromatography (GPC) and differential scanning calorimetry (DSC), respectively.

^{1}H and ^{13}C spectra were recorded with a Bruker-400 (400 MHz) NMR spectrometer. 10–20 mg of polymer was dissolved in 0.7 mL $CDCl_3$. Chemical shifts are given in ppm relative to the solvent as internal reference (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77 ppm). Molecular weight distributions were determined by gel permeation chromatography (HPLC 1200 with RI detector, Agilent) with a column PLgel 5 µm MiniMIX-C 250 × 4.6 mm with a pre-column PLgel 5 µm MiniMIX-C 50 × 4.6 mm. THF was used as eluent at a flow rate of 0.3 mL/min with sample concentration of 5 mg/mL and an injection volume of 10 µL. The calibration curve was obtained using a polystyrene standards kit (Agilent) in the Mw range of 500–3000000 Da. The thermal properties were determined by differential scanning calorimetry (DSC) and the analyses were performed with a DSC-30 (Mettler Toledo instruments, NY, USA). All data were acquired by STARe System acquisition and processing software (Mettler Toledo).

2.4 *Chemical Modification: Epoxidation*

The epoxidation reaction was optimized based on the protocol described by Sparks and Scholz[9] using mCPBA (in excess) as oxidant reagent and dry dichloromethane as solvent. The solution was stirred overnight at room temperature under argon.[9] The epoxidation reaction was successfully carried out in three PHA-polymers containing different vinyl moieties: PHA-C11$_{100}$ (100 mol% vinyl groups), PHA-C11$_{50}$ (50 mol% vinyl groups) and PHA-C11$_{30}$ (30 mol% vinyl groups).

In order to optimize the reaction time, the epoxidation reaction was later monitored by ^{1}H-NMR. For the NMR study, 37 mg of PHA-C11$_{50}$ (0.42 µmol), containing 0.083 mmol of the vinyl group, was dissolved in deuterated chloroform ($CDCl_3$). When the polymer was completely dissolved, it was transferred to the NMR tube and a two-fold molar excess of mCPBA was added under argon atmosphere. The NMR spectrum was immediately recorded (t_0) and subsequently every 10–30 minutes.

3. Results and Discussion

3.1 *Polymer Production*

Different PHA polymers, namely PHA-C11$_{100}$, PHA-C11$_{50}$ PHA-C11$_{30}$, containing different vinyl moieties in their side

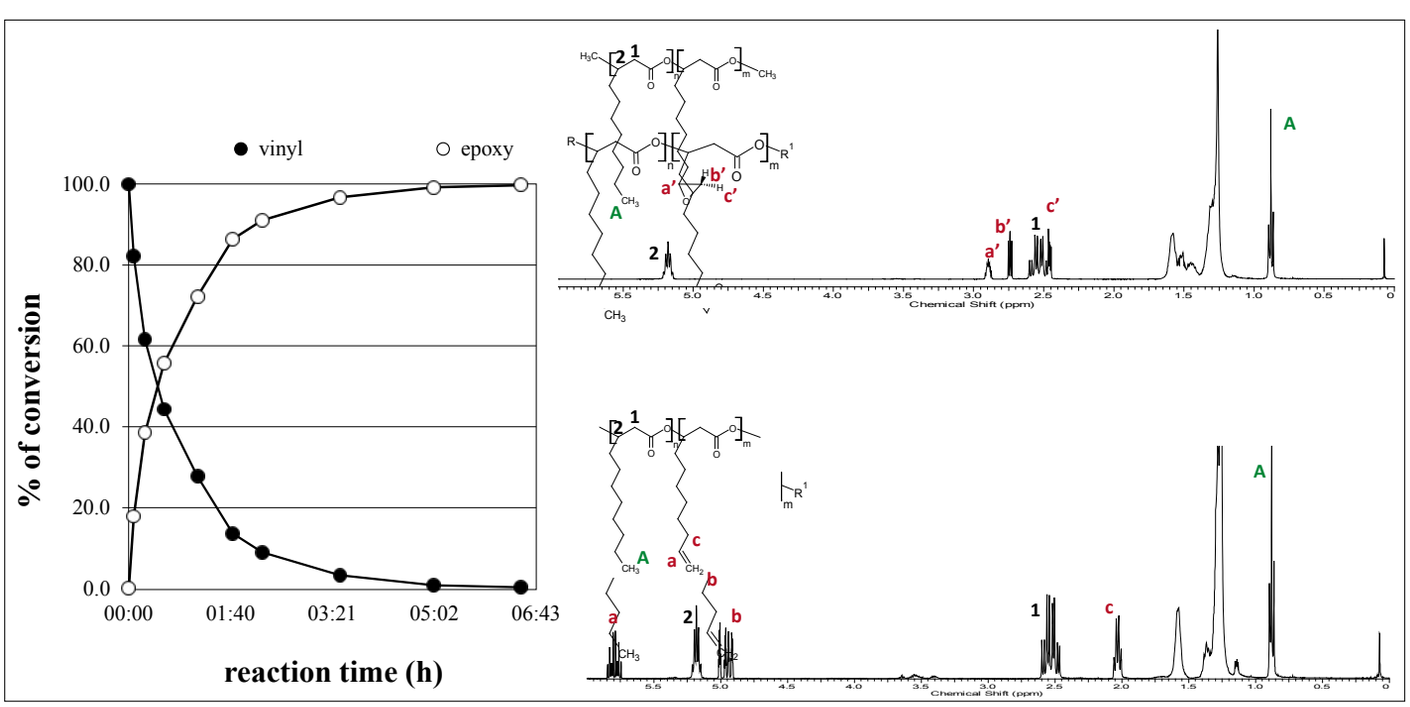

Fig. 2. Kinetic curve of the epoxidation reaction in the polymer PHA-C11$_{50}$ (50 mol% vinyl groups) and the corresponding ^{1}H-NMR spectra of the PHA-C11$_{50}$ before and after epoxidation.

chains (100, 50 and 30 mol%, respectively) were successfully synthetized and purified for further chemical derivatization.

3.2 *Monitoring the Epoxidation Reaction by ^{1}H-NMR*

The epoxidation reaction was carried out initially in the PHA-C11$_{50}$ and the reaction was monitored by ^{1}H-NMR (Fig. 2) by recording a spectrum every 10–30 minutes. After 1 h of reaction, the conversion rate was about 80% as indicated in Fig. 2 and a conversion of > 99.9 mol% was reached after 7 h of reaction.

As shown in Fig. 3, signals corresponding to the vinyl and allylic protons disappeared while simultaneously new signals corresponding to the epoxy formation appeared. The signals corresponding to the vinyl group (4.9 ppm and 5.8 ppm) and the methylene protons in allylic position (2.0 ppm) clearly disappeared as the reaction progressed and at the same time new signals appeared due to the formation of the epoxy group. The new signals corresponding to the formation of the epoxy (2.55 ppm, 2.75 ppm and 2.9 ppm) increased until 100 mol% conversion was reached after 7 h of reaction. NMR data also confirmed that the aliphatic side chains, indicated by the methyl protons (0.90 ppm), of the polymer remained constant suggesting that no secondary reactions occurred. The structure of the new polymer containing the side chains with the epoxy group (PHA-C11$_{50\ epoxy}$) was confirmed *via* ^{13}C-NMR and COSY (data not shown).

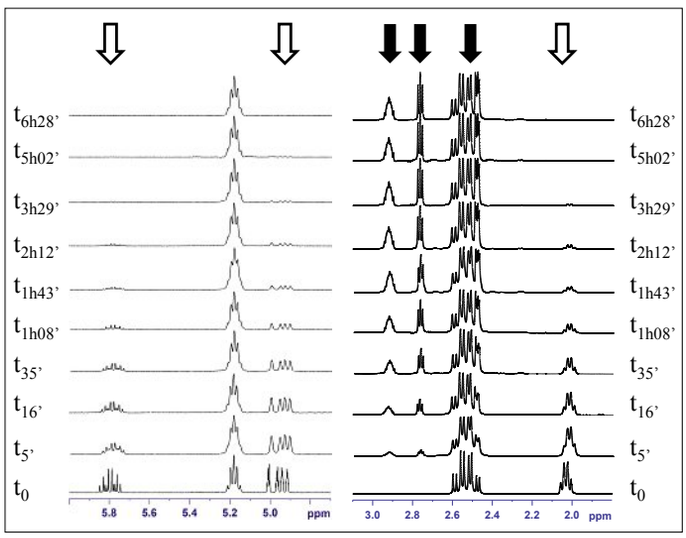

Fig. 3. Monitoring the epoxidation reaction *via* ^{1}H-NMR: reaction conversion was monitored by ^{1}H-NMR. White arrows indicate the signals corresponding to the vinyl group and the methylene protons in allylic position (2.0 ppm) and black arrows indicate the new signals appearing due to the formation of the epoxy group in the polymer side chains.

3.3 *Polymer Characterization*

The epoxidation reaction was successfully carried out on the PHA-C11$_{50}$ and PHA-C11$_{30}$ leading to two new polymers namely PHA-C11$_{50epoxy}$ and PHA-C11$_{30epoxy}$. DSC analyses were performed on these polymers and poly(3-hydroxyundecanoate) and poly(3-hydroxyundec-10-enoate), namely PHA-C11:0 and PHA-C11:1 respectively, were taken as a reference for 100 mol% saturated and 100 mol% unsaturated side-chain polymers. DSC analyses indicate that the content of the vinyl units within the polymeric structure has a direct impact in the polymer crystallinity. PHA-C11:1 homopolymer and PHA-C11$_{50}$ are amorphous polymers with glass transition temperatures (T_g) of –55.4 °C and –48 ºC, respectively. In contrast, the PHA-C11:0 homopolymer shows a T_m of 70 °C and no T_g was detected, indicating higher crystallinity. However, PHA-C11$_{30}$ is a semicrystalline polymer with a higher T_g (–38 ºC) and a T_m of 67 ºC.

Similar behavior was observed in the epoxidized counterparts. Although in this case, both epoxy polymers are more crystalline

Table 1. Monomer composition and thermal properties of the different polymers synthetized in this study

Polymer	3OHC11:0 [mol%]	3OHC11:1 [mol%]	Epoxy [mol%]	T_g [°C]	T_m [°C]	T_c [°C]	ΔH_c [J/g]	T_m (2) [°C]	ΔH_m (2) [J/g]	T_d [°C]
PHA-C11:0	100	/	/	/	34.7	40.0	16.22	70.0	15.4	288.3
PHA-C11$_{50}$ (50/50)	55	45	/	–48.2	27.2	38.6	4.48	nd	nd	300.1
PHA-C11$_{30}$ (30/70)	76	24	/	–38.2	30.1	39.7	14.31	66.89	7.78	292.9
PHA-C11$_{50epoxy}$ (50/50)	55	/	45	–47.3		24.8	3.86	49.8	4.1	299.5
PHA-C11$_{30epoxy}$ (30/70)	76	/	24	–47.5		26.1	18.54	55.6	17.9	295.1
PHA-C11:1	/	100	/	–55.4	27.3	/	/	/	/	297

Table 2. Monomer composition and molecular weight distribution

Polymer	3OHC11:0 [mol%]	3OHC11:1 [mol%]	Epoxy [mol%]	Mn [kDa]	Mw [kDa]	PDI
PHA-C11:0	100	/	/	88	194	2.2
PHA-C11$_{50}$ (50/50)	55	45	/	83	117	2.1
PHA-C11$_{30}$ (30/70)	76	24	/	84	186	2.2
PHA-C11$_{50epoxy}$ (50/50)	55	/	45	63	228	3.6
PHA-C11$_{30epoxy}$ (30/70)	76	/	24	77	179	2.3
PHA-C11:1	/	100	/	62	166	2.7

than the PHA-C11$_{50}$. PHA-C11$_{50epoxy}$ and PHA-C11$_{30epoxy}$ have a T_m of 49.8 ºC and 55.6 ºC, respectively, but according to the enthalpy of fusion (ΔH_m) PHA-C11$_{30epoxy}$ is much more crystalline (Table 1).

No significant differences were observed in the molecular weight distribution among the synthetized and derivatized polymers (Table 2). However, PHA-C11$_{50epoxy}$ showed an unusual higher polydispersity index (PDI) for this type of random copolymer reaching a PDI of 3.6. In addition, a significant decrease in the Mn was also observed (Mn 63 kDa) that might indicate a possible cleavage of the polymer chain during the epoxidation reaction. Nevertheless, the two new polymers described in this study, PHA-C11$_{50epoxy}$ and PHA-C11$_{30epoxy}$, present a great potential as building blocks for further peptide conjugation.

4. Conclusion

Different PHA polymers, namely PHA-C11$_x$, containing different vinyl moieties in their side chains (30, 50 and 100 mol%, respectively) were successfully synthetized. An epoxidation reaction was successfully carried out on the PHA-C11$_{50}$ and PHA-C11$_{30}$ leading to two new polymers. The structure of the new polymers containing the side chains with the epoxy group (PHA-C11$_{50epoxy}$ and PHA-C11$_{30epoxy}$) was confirmed *via* ^{13}C-NMR and COSY. The epoxidation reaction was easily monitored by ^{1}H-NMR for the PHA-C11$_{50}$ polymer showing that after 1 h of reaction, the conversion rate was close to 80%. Complementary DSC analyses indicate that the content of the vinyl units within the polymeric structure has a direct impact in the polymer crystallinity. This work is an illustration where classical chemistry meets a class of promising biocompatible biopolymers to generate new hybrid polymers of high potential for biomedical applications. We are currently working on the conjugation of biological molecules.

Acknowledgements

This work was financially supported by HES-SO RESEARCH Funds for Interdisciplinary Projects, CH-2800 Délémont.

Received: September 6, 2015

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