

PRODUCTION AND CHARACTERIZATION OF POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE)-POLY(ETHYLENE GLYCOL) HYBRID COPOLYMER WITH ADJUSTABLE MOLECULAR WEIGHT*

Ya-li Zhang, Xiao-yun Lu**, Qian-qian Liu, Ming-chuan Li, Zhi-qian Yang and Jian-gang Ma
Department of Biological Science and Bioengineering, Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710049, China

Abstract A novel natural-synthetic hybrid block copolymer was synthesized by *Aeromonas hydrophila* 4AK4 in poly(ethylene glycol) (PEG, $M_n = 200$) modified fermentation. This hybrid biomaterial consists of the natural hydrophobic polymer poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) end-capped with hydrophilic PEG, which has the increased flexibility as well as the improved thermal stability. Addition of diethylene glycol (DEG) and ethylene glycol could not result in the accumulation of hybrid block copolymer. DEG and ethylene glycol, together with PEG-200, could cause a reduction of molar mass of PHBHHx, resulting in a series of low molecular weight polymer and the reduction of the polymer yield as well as the cellular productivity. *In vitro* degradation of PHBHHx and PHBHHx-PEG with different molecular weight showed that the decrease of molecular weight accelerated the degradation of copolymers, but PEG modification has little effect on its degradation rate. The results in this study provided a convenient and direct method to produce a series of PHBHHx and PHBHHx-PEG materials with adjustable molecular weight and broad molecular weight distribution which will be very useful for the biomedical applications.

Keywords: Biopolyester; Biodegradable; Molecular weight; Polyhydroxyalkanoates; Polyethylene glycol.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters composed of different 3-hydroxy fatty acid monomers^[1], which are accumulated in many bacteria as intracellular carbon and energy storage materials^[2]. PHAs have attracted increasing interest for tissue engineering and drug release applications due to their general biodegradability, good biocompatibility and some other mechanical properties^[3]. Among them, the random copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) was reported to have improved properties because of the incorporation of 3-hydroxyhexanoate (3HHx) units^[4, 5]. PHBHHx is more suitable for cell growth than polyhydroxybutyrate (PHB) and poly(lactic acid) (PLA)^[3, 6] and has good biocompatibility with various cultured cells including fibroblasts, osteoblasts, cartilage-derived chondrocytes, human umbilical vein endothelial cells and marrow stromal cells in terms of promoting cell growth and differentiation^[6–12]. It indicated that PHBHHx was a promising biomaterial that could be widely used for tissue engineering or other medical applications.

While *in vitro* and *in vivo* experiments showed that the biodegradation of PHBHHx is too slow for the biomedical applications, which influenced its ability to successfully serve as a biomaterial for tissue

* This work was financially supported by the National Natural Science Foundation of China (No. 30801059) and Doctoral Fund of Ministry of Education of China (No. 200806981053).

** Corresponding author: Xiao-yun Lu (卢晓云), E-mail: luxy05@mail.xjtu.edu.cn

Received February 14, 2011; Revised April 16, 2011; Accepted April 25, 2011

doi: 10.1007/s10118-012-1095-5

engineering^[13–15]. The degradation rate and the mechanical properties of PHBHHx could be controlled by adjusting the molecular weight. Thus, production of PHBHHx with adjustable molecular weight is of great importance and highly interesting.

UV or radiation treatment can be used to adjust the molecular weight of PHA polyesters, but there is the potential risk to induce byproduct contaminations. Therefore, to produce PHBHHx with adjustable molecular weight by controlling the fermentation process directly is a more convenient and economical method. Ashby *et al.* reported that the molecular weight of PHB could be modulated by adding poly(ethylene glycol) (PEG), and a decrease in the molecular weight of the resulting polymer was observed^[16–19]. Thus, PEG might also be served as an effective additive to control the molecular weight of PHBHHx without affecting its biocompatibility. Furthermore, the natural-synthetic hybrid copolymer of PHB end-capped with diethylene glycol (DEG) and polyhydroxyoctanoate (PHO) terminated with units of DEG were produced by PEG-modified fermentations of *Azotobacter vinelandii* UWD and *Pseudomonas oleovorans*, respectively^[20, 21]. Because PEG is also a polymer that is recognized for its biocompatibility, the PEGylation of biomaterials was reported to have many applications in different fields. Especially, in nanomedicine area, the PEGylation process of nanocarriers gave rise to the stealth nanoparticles which exhibited decreased levels of uptake by the mononuclear phagocyte system and, consequently, an increased circulation time in the blood, allowing passive targeting of the special tissues such as tumors^[22–24]. Therefore, the PEG-modulated PHA could also be extensively used for a variety of biomedical applications^[20]. This also inspires us to develop the techniques for producing PEGylated PHBHHx in a direct, convenient and economic way.

In this study, PEG-200, DEG and ethylene glycol were added to the culture media respectively to investigate their effect on PHBHHx production, the composition and the molecular weight of the resulting copolymer. The molecular weight distribution of PHBHHx associated with the adding-time of additives is also discussed. The goals of this study were to develop a convenient method to produce PHBHHx with adjustable molecular weights as well as to synthesize PEG-terminated PHBHHx which might be served as a novel candidate for the biomedical application. The PHBHHx with wide molecular weight distribution has certain advantage because the low M_w PHBHHx can accelerate the *in vivo* biodegradation of the material, while the high M_w PHBHHx can maintain the mechanical strength. And the PHBHHx-PEG copolymer might be a novel candidate for the biomedical application.

EXPERIMENTAL

Bacterial Strain and Growth Condition

A. hydrophila 4AK4 was cultivated at 30°C in mineral medium which contained (g L^{-1}) 9 $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.5 KH_2PO_4 , 1 $(\text{NH}_4)_2\text{SO}_4$, 0.41 MgSO_4 , 0.03 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.02 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 1 mL of trace elemental solution. Trace elemental stock solution contained (mg per liter of 1 mol L^{-1} HCl) 100 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 23 MnSO_4 , 150 H_3BO_3 , 200 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 20 $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ and 24 NH_4MoO_4 . Lauric acid was used as carbon source with concentration of 8 g L^{-1} . PEG-200, DEG and ethylene glycol (with concentration of 10–50 g L^{-1}) were added to separate cultures, respectively, at the beginning or at 24th hour of cultivation. The cultures were incubated in 500 mL conical flasks containing 100 mL culture broth shaken at 200 r/min and 30°C (PYB, Wuhan Scientific Instrument Factory, Hunan, China). Cells were harvested by centrifugation after 48 h followed by lyophilization.

Polymer Extraction and Purification

The intracellular PHBHHx was extracted from the lyophilized cell mass with excess of chloroform at 90°C for 4 h. The polymer was preliminarily purified by repeat methanol precipitation^[25]. The crude PHBHHx material was dissolved in chloroform and washed by water for several times to remove the PEG contamination^[26].

Gas Chromatography Analysis of Intracellular PHA

The polymer content and PHA composition were determined by gas chromatography after the methanolysis of the biomass^[27]. Thirty milligrams lyophilized cells were subjected to methanolysis (4 h at 100°C) in an equal

volume (2 mL) of chloroform containing methylbenzoate as an internal standard. After phase separation, the methyl esters were analyzed on a gas chromatography system (PerkinElmer Clarus 500, USA) equipped with a PE-1 column. For peak identification, a PHA standard (kindly donated by Tsinghua University, Beijing, China) was used. The average results of three parallel experiments were recorded.

Polymer Structural Analysis by ^1H -NMR

Polymers were characterized for the chemical structure by ^1H -NMR at 500 MHz on a Varian Unity Inova 500NB spectrometer as described by Kato *et al.*^[27]. Polymer samples were dissolved in deuterated chloroform (4 g L^{-1}), and tetramethylsilane (TMS) was used as an internal reference. Polymer samples were operated at 500 MHz, and ^1H spectra were recorded with a spectral width of 8003 Hz, an acquisition time of 1.892 s and referenced internally to chloroform ($\delta = 7.26$ with respect to TMS).

Molecular Weight Determinations

The number- and weight-average molecular weights (M_n and M_w , respectively) and polydispersity (M_w/M_n) of the PHBHHx samples were determined by high performance liquid chromatography-gel permeation chromatography (HPLC-GPC) with a Spectra-System P2000 pump, a AS3000 autosampler with a 50 mL, fixed loop injector and a Shimadzu HSG-GO column (Shimadzu Corp., Kyoto, Japan). Chloroform was used as eluent at a flow rate of 1.0 mL min^{-1} . The sample concentration was 2 mg mL^{-1} and the injection volume was 50 mL. The calibration curve was generated with polystyrene standards (Shodex, Japan)^[28].

Differential Scanning Calorimetry (DSC) and Thermogravimetric (TGA) Analysis

Thermal transitions of terpolyesters were characterized by differential scanning calorimetry (Mettler Toledo DSC822e, Switzerland) with an auto-cooling accessory calibrated by indium over a temperature range from -50°C to 200°C at a heating rate of 10 K/min under nitrogen atmosphere (50 mL min^{-1}). Samples of cast films weighted approximately 10 mg were packed in an aluminium pan and then heated from -50°C to 200°C at a scanning rate of 10 K/min (first run). The second heating round of the DSC curves was used to analyze the thermal properties. The glass transition temperature (T_g) was taken as the midpoint of the heat capacity change. The melting temperature (T_m) was determined from DSC endothermal peaks. In the presence of multiple endothermal peaks, the maximum peak temperature was taken as T_m .

Thermogravimetry analysis was performed on a TGA/SDTA 851 instrument (Mettler Toledo, Switzerland). The samples were tested at a heating rate of 10 K/min under nitrogen to a temperature well above the degradation temperature of the polymers (315°C).

Mechanical Tests

Mechanical property studies were conducted on PHA films. The films were prepared by a conventional solvent-casting method. The dumbbell-shaped samples of PHA films (thickness 0.33 mm, base width 5 mm, base length 10 mm) were used for mechanical testing. The elongation at break, Young's modulus and tensile strength of the tested samples were determined using a Universal Testing Machine MinMAT2000 (TA Instruments Inc, USA) at room temperature. The speed of the cross-head was 1 mm/min ^[29].

In vitro Hydrolytic Degradation of Copolymer

Films made from the resulting copolymers (each weighted about 100 mg) were submerged in simulated body fluid (SBF) at 37°C . Samples were periodically removed, gently washed in distilled water, and then dried in a vacuum by lyophilization prior to analysis. Weight losses after incubation were calculated and compared with those of the films prior to incubation^[30].

Statistical Analysis

All data were presented as the mean value $\pm$ standard deviation of each group. Variation between groups was evaluated using the Students *t*-test, with a confidence level of 95% ($p < 0.05$) considered statistically significant.

RESULTS AND DISCUSSION

Synthesis of PHBHHx and PHBHHx-PEG Hybrid Copolymer

A. hydrophila 4AK4 was cultivated at 30°C in mineral medium plus 8 g L⁻¹ lauric acid for 48 h. Fermentation of *A. hydrophila* 4AK4 without any additive produced a maximum cell dry weight (CDW) of approximately 3.17 g L⁻¹. The addition of either PEG-200, DEG or ethylene glycol at the beginning of cultivation reduced the cellular biomass. The higher the additives' concentration, the lower the CDW achieved (Table 1). Unlike DEG and ethylene glycol, addition of PEG-200 did not decrease the cell residuum along with the increase of PEG-200 concentration, which indicated that PEG-200 only reduced the polymer synthesis but did not affect the cell growth significantly. The cell residuum remains at 1.35–1.51 g L⁻¹ when 10–50 g L⁻¹ PEG-200 was used, which was even higher than that of 1.26 g L⁻¹ cell residuum of the control group. In contrast, the addition of DEG or ethylene glycol has negative effect on cell growth. The addition of 50 g L⁻¹ DEG or ethylene glycol to the culture medium strongly restrained cell growth and no biomass could be collected.

Table 1. Cell growth and PHBHHx production in PEG modified fermentation of *A. hydrophila* 4AK4

Additive	Conc. (g L ⁻¹)	CDW (g L ⁻¹)	PHA content (wt%)	HHx ^a content (mol%)	PHA yield (g L ⁻¹)	Cell residuum (g L ⁻¹)	Productivity (mg mg ⁻¹)
Control	0	3.17 ± 0.33	60.44 ± 1.95	16.51 ± 0.81	1.91 ± 0.14	1.26 ± 0.19	1.53 ± 0.13
PEG-200	10	3.13 ± 0.18	54.97 ± 3.03	16.41 ± 1.19	1.72 ± 0.17	1.41 ± 0.08	1.23 ± 0.15
	20	2.59 ± 0.90	48.06 ± 3.77	15.02 ± 0.77	1.23 ± 0.36	1.36 ± 0.56	0.93 ± 0.14
	30	2.37 ± 0.13	43.16 ± 0.78	14.97 ± 1.19	1.02 ± 0.09	1.35 ± 0.05	0.76 ± 0.06
	40	2.31 ± 0.25	29.14 ± 3.71	14.67 ± 0.97	0.68 ± 0.16	1.63 ± 0.10	0.41 ± 0.08
	50	1.95 ± 0.61	22.31 ± 1.58	14.38 ± 1.29	0.44 ± 0.17	1.51 ± 0.45	0.29 ± 0.03
	5	3.12 ± 0.03	36.56 ± 3.50	12.63 ± 1.14	1.14 ± 0.11	1.98 ± 0.12	0.58 ± 0.09
DEG	10	2.94 ± 0.12	29.77 ± 3.59	12.37 ± 1.51	0.88 ± 0.13	2.06 ± 0.08	0.43 ± 0.07
	15	2.53 ± 0.36	24.11 ± 1.42	11.76 ± 0.73	0.61 ± 0.08	1.92 ± 0.29	0.32 ± 0.02
	20	2.11 ± 0.15	22.32 ± 2.38	9.51 ± 0.12	0.47 ± 0.03	1.64 ± 0.16	0.29 ± 0.04
	25	1.84 ± 0.07	20.16 ± 1.79	7.59 ± 0.34	0.37 ± 0.02	1.47 ± 0.09	0.25 ± 0.03
	30	1.27 ± 0.17	17.01 ± 1.93	4.42 ± 0.52	0.22 ± 0.03	1.06 ± 0.15	0.21 ± 0.03
	40	0.75 ± 0.19	11.60 ± 1.21	ND ^b	0.09 ± 0.03	0.66 ± 0.16	0.13 ± 0.02
EG ^d	50	NG ^c	—	—	—	—	—
	10	2.99 ± 0.37	58.20 ± 1.68	17.54 ± 1.24	1.75 ± 0.26	1.25 ± 0.12	1.40 ± 0.10
	20	2.75 ± 0.06	53.85 ± 3.51	16.90 ± 1.83	1.60 ± 0.01	1.15 ± 0.06	1.39 ± 0.08
	30	2.25 ± 0.12	24.64 ± 1.14	12.49 ± 1.67	1.21 ± 0.14	1.04 ± 0.05	1.18 ± 0.17
	40	1.13 ± 0.19	10.52 ± 1.45	6.06 ± 0.48	0.12 ± 0.04	1.01 ± 0.15	0.12 ± 0.02
	50	NG	—	—	—	—	—

^a HHx, 3-hydroxyhexanoate; ^b ND, not detected; ^c NG, no growth; ^d EG, ethylene glycol;

Strains were grown on MM medium plus lauric acid (8 g L⁻¹) at 30°C for 48 h; PEG-200, DEG or EG (10–50 g L⁻¹) were added at the beginning of inoculation.

The addition of PEG-200, DEG or ethylene glycol all has the negative effect on polymer production, leading to a decrease in the polymer content and its yield. In control experiments without any additives, *A. hydrophila* 4AK4 accumulated 60.44 wt% PHBHHx and the polymer yield was 1.91 g L⁻¹. When 50 g L⁻¹ PEG-200 was used, the polymer content decreased to 22.31 wt%, with the polymer yield of only 0.44 g L⁻¹. The effects of DEG and ethylene glycol on polymer production were more obviously compared with PEG-200. When 40 g L⁻¹ of DEG or ethylene glycol was added, the PHBHHx content were just 11.60 wt% and 10.52 wt%, respectively. Comparing with PEG-200 and ethylene glycol, DEG has the most notable effect on the polymer accumulation, which caused a significant decrease of PHBHHx accumulation even at low concentration (10–30 g L⁻¹) (Table 1). Consequently, the cellular productivity of PHBHHx decreased from 1.53 mg mg⁻¹ to 0.29 mg mg⁻¹, 0.13 mg mg⁻¹ and 0.12 mg mg⁻¹ in the presence of PEG-200 (50 g L⁻¹), DEG (40 g L⁻¹) and ethylene glycol (40 g L⁻¹), respectively.

The addition of PEG-200, DEG and ethylene glycol also had influence on the composition of the PHBHHx copolymer. The HHx monomer content decreased slightly from 16.51 mol% to about 14 mol% with addition of PEG-200. But the reduction of HHx content was much more obvious in DEG and ethylene glycol modified fermentation. The HHx content was just 6.06 mol% when 40 g L⁻¹ ethylene glycol was used and it could not even be detected when 40 g L⁻¹ DEG was used.

The adding-time of PEG-200 and DEG also affected the polymer production. On the whole, the decrease of CDW and PHA content was not that obvious when PEG-200 or DEG was added at the 24th hour (Figs. 1 and 2), especially for DEG, because cells grow normally during the first 24 hours and a certain amount of biomass could be accumulated before PEG-200 or DEG treatment. The PHBHHx content and HHx fraction of the resulting copolymer were both higher than those when the same concentrations of additives were used at the beginning. It might be because that the accumulated polymers were a blend of high-molecular-weight polymer and low-molecular-weight polymer synthesized before and after PEG-200 or DGE treatment, respectively, which could be partly proved by the higher PDI values of the resulting polymers.

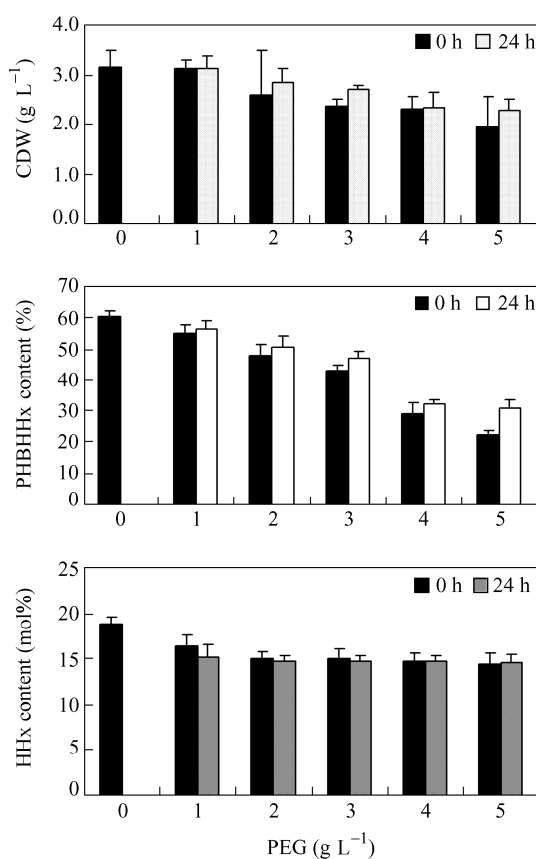


Fig. 1 Effect of PEG-200 on production of PHBHHx by *A. hydrophila* 4AK4

Strains were grown on MM medium plus lauric acid (8 g L⁻¹) at 30°C for 48 h; PEG-200 (10–50 g L⁻¹) was added at the beginning (dark columns) or at the 24th hour (light columns) of inoculation.

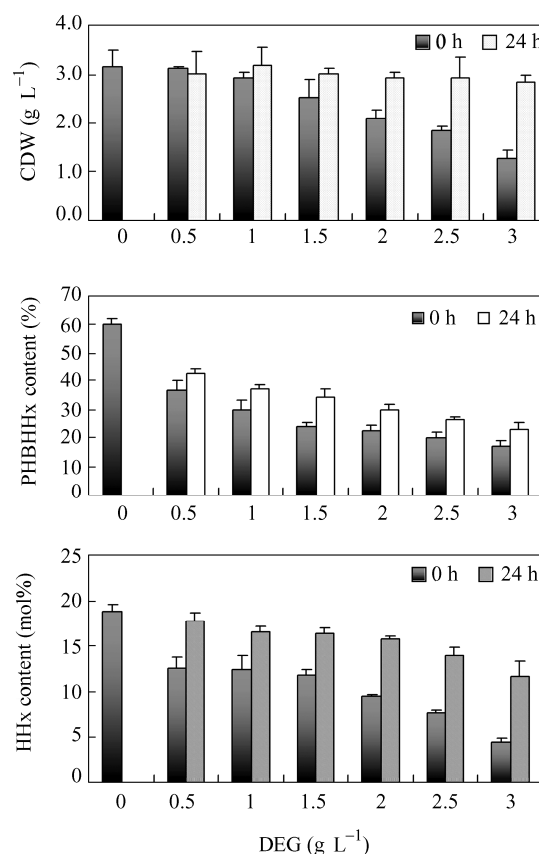


Fig. 2 Effect of DEG on production of PHBHHx by *A. hydrophila* 4AK4

Strains were grown on MM medium plus lauric acid (8 g L⁻¹) at 30°C for 48 h; DEG (5–30 g L⁻¹) was added at the beginning (dark columns) or at the 24th hour (light columns) of inoculation.

Considering the inhibition effects on both cell growth and PHA accumulation, it's reasonable to use the fed-batch feeding technique during the cultivation to avoid the significant decrease of biomass and polymer accumulation. As for the production of PHBHHx-PEG hybrid copolymer, based on the synthesis behavior

feature of *A. hydrophila* 4AK4 who accumulate PHBHHx along with its growth, it will be very important to optimize the initial PEG-200 concentration and the feeding speed to achieve the pure synthesis of the PHBHHx-PEG copolymer, because the insufficient supply of PEG-200 will result in a mixture product of PHBHHx and PHBHHx-PEG. This will be further studied in our follow-up experiments.

Polymer Structure Characterization

To answer the question that whether the PEG modulated fermentation led to the formation of hybrid block copolymers, purified copolymer samples from the PEG modulated fermentation were examined using ^1H -NMR spectroscopy. Figure 3 shows the ^1H -NMR spectrum of the copolymer obtained in the fermentation modulated by PEG-200. The spectrum shows a strong methine signal at $\delta = 5.2$ and the signal for the main chain methylene protons adjacent to the chiral carbon between $\delta = 2.4$ and 2.6 . The methylene protons of the side chains ($\delta = 1.3$ and 1.6) and the terminal methyl groups in the side chains ($\delta = 0.9$) are also readily observed. Signals at $\delta = 3.6$, corresponding to protons of PEG could also be detected in ^1H -NMR spectrum. A signal at $\delta = 4.2$ correlating to a covalent bond between PEG and PHBHHx was also observed, which strongly suggested the PEG units were linked to the termination of PHBHHx chains. The molar ratio of the natural and PEG-200 blocks is approximately 700:1. It's contrasted with cultivations of *A. hydrophila* 4AK4 in the presence of DEG and ethylene glycol, which failed to show any covalent bonding between PHBHHx and PEG, indicating no PEGylation occurred under the DEG or ethylene glycol treatment.

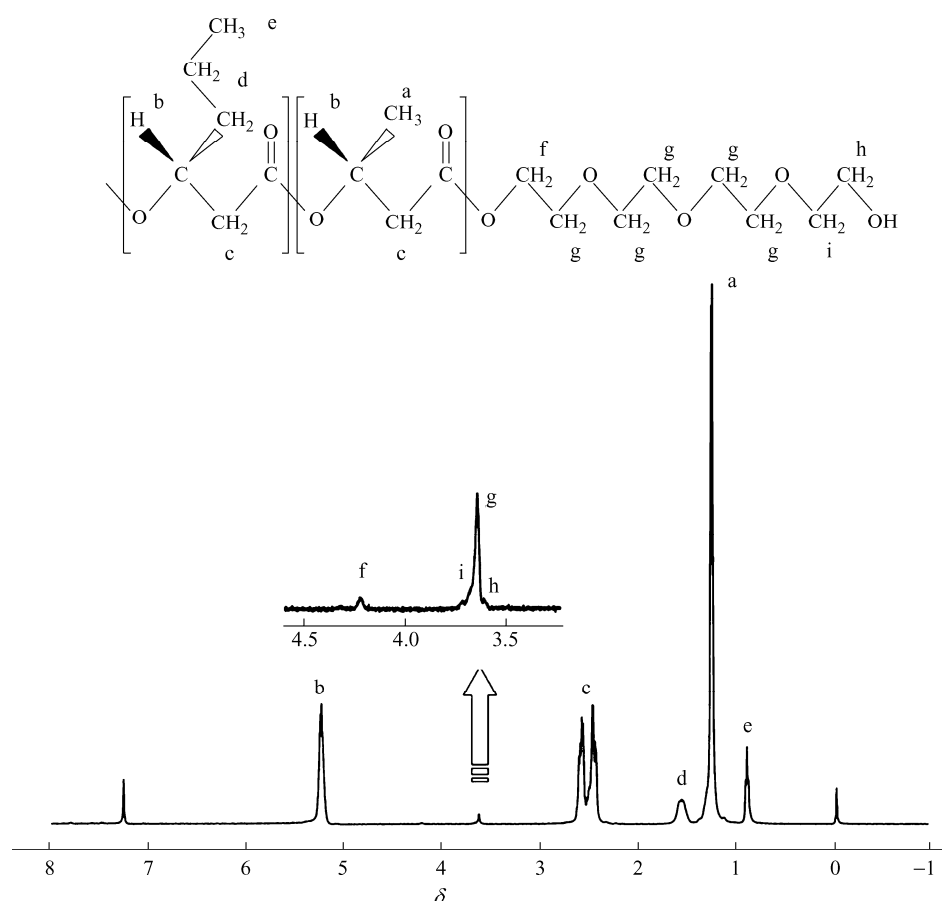


Fig. 3 ^1H -NMR spectrum of the polymer obtained in the fermentation modulated by 20 g L^{-1} PEG-200

It was reported that PEG of small molecular weight could be incorporated into PHA by several kinds of PHA producers. Both *A. vinelandii* UWD and *P. oleovorans* could synthesize the hybrid copolymer with addition of PEG, but only DEG (PEG106) units could be observed at the end of PHA chains^[20, 21]. Cultivations of *A. vinelandii* UWD and *P. oleovorans* in the presence of PEG-200 and other high molar mass PEG failed to form any hybrid copolymer. Nonetheless, PEG-200 could be covalently attached to the end of short-chain-length PHA (scl-PHA) in *Azotobacter chroococcum* MAL-201^[31], *Alcaligenes latus*^[17], *Pseudomonas oleovorans* B-14682 and B-778^[19]. Not every PHA synthase has the ability to incorporate PEG into PHA main chain. The medium-chain-length (mcl-PHA) synthases in *Pseudomonas oleovorans* B-14683 and B-778 failed to form the linkage between PHA and any kinds of PEG. Also in the PEG-200 modified fermentation of *Wautersia eutrophus* no hybrid copolymers were obtained^[18]. The percentage of PEG incorporated into PHA copolymers were variant in different bacteria. *Azotobacter chroococcum* MAL-201 could incorporate 1.76 mol%–15.5 mol% of PEG into PHBV copolymers depending on the molar mass, concentration and adding time of PEG additives^[31]. But the molecular weight ratio of the PHO and DEG blocks was only 1000:1 in *Pseudomonas oleovorans*^[21]. In this study, the molecular weight ratio of the PHBHHx and PEG-200 was about 250:1 according to the integration of the ¹H-NMR spectrum of PHBHHx-PEG copolymer. This indicated the relatively high ability of PHA synthase in *A. hydrophila* 4AK4 to incorporate the PEG units into copolymers, which might be due to the relatively broad substrate spectrum of PHA synthase in *A. hydrophila* 4AK4.

Molecular Weight Characterization of Copolymer

Addition of PEG-200, DEG or ethylene glycol all decreased the molecular weight of the resulting copolymers (Table 2). In the control experiment, the M_w and M_n of PHBHHx were 934 kD and 428 kD, respectively, and the polydispersity index (PDI) was 2.19. Addition of PEG-200, DEG and ethylene glycol caused a reduction of molar mass of PHBHHx, resulting in a series of low molecular weight polymers. Comparing with PEG-200, DEG and ethylene glycol were more efficient in molecular weight reduction. The M_w decreased significantly to 55 kD and 150 kD when only 10 g L⁻¹ of DEG or ethylene glycol was added at the beginning. When 30 g L⁻¹ of DEG was used, the M_w of PHBHHx was even reduced by 97.3% and the M_w of copolymer was only 25 kD. PHBHHx molecular weight distribution was also decreased in DEG or ethylene glycol modified fermentation, which indicated the resulting polymers were more homogeneous.

Table 2. The effect of PEG-200, DEG or EG on molecular weight of PHBHHx

Additive	Conc. (g L ⁻¹)	A (0 h)			B (24 h)		
		M_w (kDa)	M_n (kDa)	PDI	M_w (kDa)	M_n (kDa)	PDI
Control	0	934 ± 14	428 ± 10	2.19			
	10	497 ± 12	150 ± 9	3.31	866 ± 23	168 ± 15	5.19
	20	179 ± 7	85 ± 4	2.12	792 ± 12	135 ± 10	5.89
	30	158 ± 8	77 ± 5	2.05	767 ± 7	130 ± 12	5.95
	40	134 ± 11	67 ± 4	2.01	753 ± 17	127 ± 8	5.96
	50	92 ± 8	37 ± 2	2.49	748 ± 9	123 ± 14	6.11
PEG-200	5	100 ± 11	70 ± 8	1.43	701 ± 12	123 ± 8	5.70
	10	55 ± 6	35 ± 5	1.63	634 ± 6	107 ± 13	6.02
	15	43 ± 5	32 ± 4	1.35	596 ± 8	89 ± 4	6.71
	20	37 ± 4	28 ± 3	1.34	503 ± 5	74 ± 7	6.87
	25	30 ± 4	19 ± 3	1.64	503 ± 14	77 ± 8	6.59
	30	25 ± 4	16 ± 3	1.64	475 ± 11	64 ± 5	7.41
DEG	10	150 ± 6	116 ± 3	1.24			
	20	123 ± 10	100 ± 8	1.23			
	30	41 ± 4	38 ± 3	1.08			
	40	22 ± 4	21 ± 2	1.09			

M_w : weight-average molecular weight; M_n : number average molecular weight; EG: ethylene glycol; For group A and B, PEG-200, DEG or EG were added at the beginning or the 24th hour of the cultivation, respectively.

When the fermentation was modified with either PEG-200 or DEG at the 24th hour, the molecular weight of resulting PHBHHx was decreased slightly, but the molecular weight distribution was increased. This was caused by the switch of accumulated polymers from the high-molecular-weight fraction to the low-molecular-weight fraction after the first 24th hours. Variation in molecular weight is vitally important for controlling the mechanical properties of polymer materials. It will be very useful for the biomedical applications because the low-molecular-weight PHBHHx will accelerate the *in vivo* biodegradation of the polymer, while the high-molecular-weight fraction will provide the mechanical strength. Thus, by controlling the adding time of the additives, PHBHHx with different molecular weight distribution could be achieved.

To date, control of PHA molecular weight at the level of polymer synthesis has been developed by two main methods. The first method is to control the expression level and time of PHA synthase in a PHA-producing bacterium^[32]. In this way, PHA polymers with low molecular weight or high molecular weight could be achieved under high or low expression of PHA synthase, respectively. The second method involves the addition of PEG to the fermentation medium. In previous studies, PEG with M_n molar masses less than 1000 was effective in reducing the molar mass of PHA polymers produced by *Alcaligenes latus*^[16, 17], *Wautersia eutrophus*^[18], *A. vinelandii* UWD^[20] and *P. oleovorans*^[19, 21] of the organisms. PEG was utilized as a molar mass regulator for both scl-PHA and mcl-PHA. As discussed previously, only some kinds of PHA producers could incorporate PEG with specific molar mass into PHA polymers. These results indicated that the mechanism for PEG mediated modulation of PHA component and the molecular weight reduction might be different for different PHA synthases. It was reported that PEG can be absorbed by cellular membranes and subsequently interact and interfere with the membrane of intracellular granules, affecting the PHA synthase activity and specificity^[18]. Two possible mechanisms might explain the PEG mediated reduce of PHA molecular weight^[20]. First, PEG could attack the carbonyl carbon of the PHA chain as a nucleophile, resulting in the PEG-terminated hybrid copolymer. Second, the hydrophilicity of PEG arising from its ability to coordinate with water molecules changed the molecular environment at the active site of PHA synthase. This allowed water molecules to act as nucleophile and terminate the growing PHA chain. In this case, unlike we expected, the molecular weight of PHBHHx could be reduced by DEG but without the formation of PHBHHx-DEG hybrid copolymer. This showed that in *A. hydrophila* 4AK4 DEG might interact with the PHA synthase in such a way as to change the molecular environment of PHA synthase and thus increase the rate of chain termination by water relative to chain propagation reactions. It might also explain the decrease of HHx monomer content. The hydrophilic environment around the active site of PHA synthase was not favorable for the incorporation of hydrophobic HHx monomers. The exact mechanism requires further study. Unlike DEG, addition of PEG-200 resulted in the molecular weight reduction as well as the formation of PHBHHx-PEG hybrid copolymer. This might because that PEG-200 is not as hydrophilic as DEG and it could attack the carbonyl carbon of the PHBHHx chain directly and form the covalent bond with PHBHHx. Since it's not so hydrophilic, PEG-200 slightly changed the molecular environment of PHA synthase and thus didn't affected the HHx content significantly.

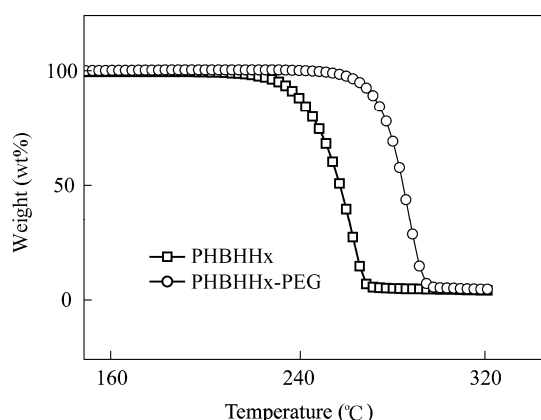
Physical Properties of PHBHHx-PEG Hybrid Copolymer

DSC revealed that T_g of PHBHHx-PEG samples was about -2.0°C which was lower than that of 0.5°C of PHBHHx (Table 3). The T_m of PHBHHx-PEG and PHBHHx were 111.8°C and 117.0°C , respectively. This indicated that the incorporation of PEG into polyester PHBHHx influenced the thermal properties of the copolymer, decreasing the crystallization processes as confirmed by their changes in ΔH_f . The temperature at 5% weight loss termed T_d (5%) was employed to evaluate polymer thermal stability which was important for their melt processing. PHBHHx had the lower T_d (5%) of 235°C , yet PHBHHx-PEG had the higher T_d (5%) of 270°C which reflecting the improved thermal stability of PHBHHx-PEG copolymer (Fig. 4, Table 3).

Table 3. Thermal properties and mechanical properties of PHBHHx and PHBHHx-PEG

Copolymer	PHBHHx ($M_w = 150$ kDa)	PHBHHx-PEG ($M_w = 179$ kDa)
T_d (5%) ^a (°C)	235.6	270.4
T_g ^b (°C)	0.5	-2.0
T_m ^c (°C)	117.0	111.8
ΔH_f	36.2	10.1
Tensile strength (MPa)	10.3	9.5
Elongation at break (%)	112	160
Young's modulus (MPa)	147.3	128.5

^a Temperature at 5% weight loss determined by TGA; ^b T_g , glass-transition temperature; ^c T_m , melting temperature

**Fig. 4** Thermogravimetry analysis curves (TGA) of PHBHHx and PHBHHx-PEG

The mechanical properties of PHBHHx and PHBHHx-PEG samples were studied using stress-strain measurements (Table 3). The Young's modulus and tensile strength of PHBHHx-PEG were 128.5 MPa and 9.5 MPa, respectively, which were slightly lower than that of 147.3 MPa and 10.3 MPa of PHBHHx. This indicated the decreased mechanical strength of the copolymer with the incorporation of PEG fragment. Nonetheless, the presence of PEG fraction increased the elongation at break to 160% which indicated the improved flexibility comparing with the PHBHHx polyesters. This might be attributed to the soft and non-crystal properties of PEG termination.

In vitro Degradation of PHBHHx and PHBHHx-PEG with Different Molecular Weight

Films made from the resulting copolymer (each weighted about 100 mg) were submerged in SBF at 37°C. After 80 days of degradation in SBF, the weight average M_w of all films changed little (data not show). Weight loss was observed for all films but it was more apparent with films made from low M_w copolymers (Fig. 5). About 85% of original weight remained after the degradation of PHBHHx film of M_w 22. In comparison, films prepared with PHBHHx of M_w 934 maintained over 96% of their original weight. PEGylation of PHBHHx has little effect on the polymer degradation rate. PHBHHx-PEG film with M_w 93 retained 90.33% of its original weight, while the PHBHHx film with M_w 100 retained 90.77% of its original weight. The native and hybrid PHBHHxs of M_w 150 and M_w 159, respectively, also have almost the same degradation rate. Undoubtedly, the variation of polymer molecular weights will result in the various biodegradation rates which will be very important for the actual applications. Considering the big difference in their molecular weight between the 934 kDa PHBHHx and the 22 kDa PHBHHx, there was no proportional increase in their degradation rate. This might be because the decreased molecular weight always results in the increased crystallization which does not benefit the degradation process.

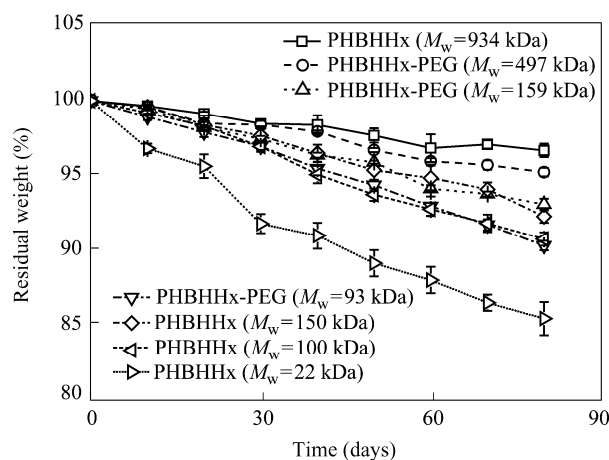


Fig. 5 *In vitro* degradation of PHBHHx films and films prepared from PEG-terminated PHBHHx with different molecular weight
The films were submerged in SBF at 37°C ($n = 5$); Samples were periodically removed and dried under lyophilization prior to analysis.

CONCLUSIONS

In this study, a natural-synthetic hybrid copolymer consisting of PHBHHx terminated with PEG was successfully produced by *A. hydrophila* 4AK4. The PHBHHx-PEG hybrid copolymer exhibited improved thermal stability and flexibility properties. Furthermore, the presence of PEG-200, DEG and ethylene glycol in the fermentation resulted in the synthesis of a series of low-molecular-weight PHBHHx, accompanied with the reduction of polymer yield and HHx monomer content. In this way, PHBHHx of different molecular weight and of different molecular weight distribution could be achieved by controlling the concentration and adding time of the additives. The variation of PHBHHx molecular weights resulted in the different biodegradation rates. PEGylation has little effect on the degradation of PHBHHx-PEG hybrid. Thus, we provide a convenient method to obtain PHBHHx and PHBHHx-PEG with adjustable molecular weight by PEG-200, DEG or ethylene glycol modified fermentations of *A. hydrophila* 4AK4.

REFERENCES

- 1 Anderson, A.J. and Dawes, E.A., *Microbiol. Rev.*, 1990, 54: 450
- 2 Lee, S.Y., *Biotech. Bioeng.*, 1996, 49: 1
- 3 Chen, G.Q. and Wu, Q., *Biomaterials*, 2005, 26: 6565
- 4 Doi, Y., Kitamura, S. and Abe, H., *Macromolecules*, 1995, 28: 4822
- 5 Yoshie, N., Saito, M. and Inoue, Y., *Macromolecules*, 2001, 34: 8953
- 6 Qu, X.H., Wu, Q., Liang, J., Qu, X., Wang, S.G. and Chen, G.Q., *Biomaterials*, 2005, 26: 6991
- 7 Deng, Y., Zhao, K., Zhang, X.F., Hu, P. and Chen, G.Q., *Biomaterials*, 2002, 23: 4049
- 8 Zhao, K., Yang, X., Chen, G.Q. and Chen, J.C., *J. Materials Sci.-Materials Med.*, 2002, 13: 849
- 9 Yang, X.S., Zhao, K. and Chen, G.Q., *Biomaterials*, 2002, 23: 1391
- 10 Wang, Y.W., Wu, Q. and Chen, G.Q., *Biomaterials*, 2003, 24: 4621
- 11 Wang, Y.W., Wu, Q. and Chen, G.Q., *Biomaterials*, 2004, 25: 669
- 12 Yang, M., Zhu, S.S., Chen, Y., Chang, Z.J., Chen, G.Q., Gong, Y.D., Zhao, N.M. and Zhang, X.F., *Biomaterials*, 2004, 25: 1365
- 13 Wang, Y.W., Mo, W.K., Yao, H.L., Wu, Q., Chen, J.C. and Chen, G.Q., *Polym. Degrad. Stab.*, 2004, 85: 815

- 14 Qu, X.H., Wu, Q., Zhang, K.Y. and Chen, G.Q., *Biomaterials*, 2004, 27: 3540
- 15 Cheng, S., Wu, Q., Zhao, Y., Zou, B. and Chen, G.Q., *Polym. Degrad. Stab.*, 2006, 91: 3191
- 16 Ashby, R.D., Shi, F.Y. and Gross, R.A., *Tetrahedron*, 1997, 53: 15209
- 17 Ashby, R.D., Shi, F.Y. and Gross, R.A., *Biotech. Bioeng.*, 1999, 62: 106
- 18 Shi, F.Y., Ashby, R.D. and Gross, R.A., *Macromolecules*, 1996, 29: 7753
- 19 Ashby, R.D., Solaiman, D.K.Y. and Foglia, T.A., *Appl. Microbiol. Biotechnol.*, 2002, 60: 154
- 20 Zanzig, J. and Scholz, C., *J. Polym. Environ.*, 2003, 11: 145
- 21 Foster, L.J.R., Sanguanchaipaiwong, V., Gabelish, C.L., Hook, J. and Stenzel, M., *Polymer*, 2005, 46: 6587
- 22 Moghimi, S.M., Hunter, A.C. and Murray, J.C., *Pharmacol. Rev.*, 2001, 53(2): 283
- 23 van Vlerken, L.E., Vyas, T.K. and Amiji, M.M., *Pharm. Res.*, 2007, 24(8): 1405
- 24 Huynh, N.T., Roger, E., Lautram, N., Benoit, J.P. and Passirani, C., *Nanomedicine*, 2010, 5(9): 1415
- 25 Ramsay, J.A., Berger, E., Voyer, R., Chavarie, C. and Ramsay, B.A., *Biotechnol. Tech.*, 1994, 8: 589
- 26 Gan, D.J., Lu, S.Q. and Cao, W.W., *Eur. Polym. J.*, 2004, 40: 2481
- 27 Kato, M., Bao, H.J., Kang, C.K., Fukui, T. and Doi, Y., *Appl. Microbiol. Biotechnol.*, 1996, 45: 363
- 28 Fernandez-Castillo, R., Rodriguez-Valera, F., Gonzales-Ramos, J. and Ruiz-Berraquero, F., *Appl. Environ. Microbiol.*, 1986, 51: 214
- 29 Xie, W.P. and Chen, G.Q., *Biochem. Eng. J.*, 2008, 38: 384
- 30 Shangguan, Y.Y., Wang, Y.W., Wu, Q. and Chen, G.Q., *Biomaterials*, 2006, 27: 2349
- 31 Saha, S.P., Patra, A. and Paul, A.K., *J. Ind. Microbiol. Biotechnol.*, 2006, 33: 377
- 32 Snell, K.D., Hogan, S.A., Sim, S.J., Sinskey, A.J. and Rha, C., 1998, U.S. Pat., 5,811,272