Highly Selective Enzymatic Ring-Opening Polymerization: Syntheses and Characterizations of Thermoplastic Di-Block Co-Polyesters Containing Poly[(R)-3-Hydroxybutyrate] and Poly(ε-Caprolactone) Blocks

*Shiyao Dai and Zhi Li**

Department of Chemical & Biomolecular Engineering

National University of Singapore, 4 Engineering Drive 4, Singapore 117576

* Corresponding Author, E-mail: chelz@nus.edu.sg, Tel: +65-65168416, Fax: +65-67791936

ABSTRACT: Enzymatic modification of a microbial polyester was achieved by the ring-opening polymerization of *ε*-caprolactone (CL) with low-molecular weight telechelic hydroxylated poly[(*R*)-3-hydroxybutyrate] (PHB-diol) as initiator and Novozym 435 (immobilized *Candida antarctica* Lipase B) as catalyst in anhydrous 1,4-dioxane or toluene. The ring-opening polymerization was investigated at different conditions with two different types of PHB-diols: PHB-diol(P) containing a primary OH and a secondary OH end groups; and PHB-diol(M) consisting of 91% PHB-diol(P) and 9% PHB-diol containing two secondary OH end groups. The reactions were followed by GPC analyses of the resulting polymers at different time points, and the optimal conditions were established to be 70°C at a weight ratio of CL/enzyme/solvent of 8:1:24. The ring-opening polymerization of CL with PHB-diol(M) (M_n of 2380, NMR) at the molar ratio of 50:1 under the optimal conditions in 1,4-dioxane gave the corresponding poly[HB(56wt%)-*co*-CL(44wt%)] with M_n (NMR) of 3900 in 66% yield. Polymerization of CL and PHB-diol(P) (M_n of 2010, NMR) at the same condition in toluene gave the corresponding poly[HB(28wt%)-*co*-CL(72wt%)] with M_n (NMR) of 7100 in 86% yield. Both polymers were characterized by ^{1}H- and ^{13}C-NMR and IR analyses as *di*-block co-polyesters containing a PHB block with a secondary OH end group and a poly(*ε*-caprolactone) (PCL) block with a primary OH end group. NMR analyses and control experiments suggested no formation of random co-polymers and no change of the PHB block during the reaction. The enzymatic ring-opening polymerization was selectively initiated by the primary OH group of PHB-diol, whereas the secondary OH group remained as an end group in the final polymers. The thermal properties of the *di*-block poly(HB-*co*-CL)s were analyzed by DSC, with excellent T_g values for the elastomer domain: poly[HB(56wt%)-*co*-CL(44wt%)] with M_n (NMR) of 3900 demonstrated a T_g of -57°C, T_m of 145, 123, and 53 °C; and poly[HB(28wt%)-*co*-CL(72wt%)] with M_n (NMR) of 7100 gave a T_g of -60°C, T_m of 147 and 50 °C. Thus, the selective enzymatic ring-opening polymerization with PHB-diol as macro-initiator provides a new method for the preparation of PHB-based block copolymers as biomaterials with good thermoplastic properties and novel structures containing functional end groups.

Introduction

Poly[(*R*)-3-hydroxyalkanoates] (PHAs) are biodegradable and biocompatible polymers produced by a wide variety of microorganisms, with potential application in environmental, agricultural, marine, and biomedical fields.[1-6] Microbial poly[(*R*)-3-hydroxybutyrate] (PHB) is the most prominent polyester in the PHA family and can be easily produced in large quantity. PHB may be useful for drug delivery or tissue engineering, but its application as thermoplastic material is rather limited partially due to the high melting temperature (T_m) of 175-177°C and high glass transition temperature (T_g) of 4°C.[6] Many methods have been used to prepare PHB-based co-polymer to improve the thermoplastic properties. Bacterial syntheses by

feeding with different substrates allowed for the preparation of random co-polyesters containing PHB and other PHA such as poly[(*R*)-3-hydroxyvalerate(HV)],[7] poly[(*R*)-3-hydroxypropionate],[8] poly[(*R*)-3-hydroxyhexanoate],[9] or poly[(*R*)-3-hydroxydecanoate].[10] Bacterial production of poly(HB-*co*-HV) containing 30% block units was also reported.[11] Chemical modification of PHB led to the preparation of block-co-polyesterurethane[12-15] and block-co-polyesters[16-17] containing the hard PHB blocks and other soft blocks such as poly(*ε*-caprolactone) (PCL),[12-13,17] poly[(*R*)-3-hydroxyoctanoate],[14,16] or poly(ethylene glycol)[15] blocks. A random co-polyester poly(HB-co-CL)[18] were also prepared by chemical modification of PHB. Some of these co-polymers demonstrated good thermoplastic properties. Nevertheless, microbial syntheses generally give random co-polyesters with relatively high production costs; and chemical modifications involve the use of toxic chemicals or catalysts.

We are interested in the enzymatic modification of microbial polyesters such as PHB to prepare block-co-polymers with good thermoplastic properties for biomedical application. Enzyme catalysis is highly chemo-, regio-, and stereo-selective, thus being a useful tool for the preparation of polymers with novel structures. Enzyme catalysis is *non*-toxic, which is very attractive for the preparation of polymeric biomaterials. Many enzymes have been used for the preparation of natural or unnatural materials,[19] and lipase-catalyzed ring-opening polymerizations (ROP) represent prominent examples.[20-22] Enzymes such as *Porcine pancreatic* lipase,[20,23-24] *Pseudomonas sp.* lipase,[25] and *Candida antarctica* lipase B (CALB)[26-30] have been successfully used for the ROP of lactones with alcohols,[20,23-25] diols,[29] and polyols[30] as initiators to prepare polyesters. Nevertheless, enzymatic ROP has not yet been applied for the modification of microbial polyesters.

Previously Suter,[12,13] we,[14,16] and others[15] successfully utilized low-molecular-weight telechelic hydroxylated PHB (PHB-diol)[12] as hard segment for chemical preparation of thermoplastic block-co-polymers. PHB-diol containing a primary and a secondary OH end group might also be a suitable initiator for enzymatic ROP. While both OH end groups were reacted in chemical polymerization,[12-16] the primary and the secondary OH end groups may show different reactivity in a lipase-catalyzed ROP. Thus, it could be possible that only the primary OH of the PHB-diol initiates the ROP giving a *di*-block copolymer with the unreacted secondary OH group as an end group. On the other hand, PCL is a biodegradable and biocompatible soft material with a T_m of 60°C and T_g of -60°C.[31] Although random co-polyester poly(HB-co-CL) prepared from PHB and PCL *via* enzyme-catalyzed transesterification[32-33] did not show improved elastic properties, incorporation of PCL block into PHB-based block co-polymers should significantly improve the elastic properties, which has been demonstrated in the chemically prepared block co-polyesterurethanes.[12-13] Recently, we have explored, for the first time, the lipase-catalyzed ROP of *ε*-caprolactone with PHB-diol to prepare block co-polyester poly(HB-co-CL). Here we report our results on this new and selective enzymatic ROP, the preparation of the corresponding block copolymers, the structural analysis of the novel *di*-block co-polyesters, and the characterization of physical properties of the polymers.

Experimental Section

Materials. Novozym 435 (immobilized *Candida antarctica* lipase B, 10000 PLU/g) was purchased from Novozymes. Telechelic hydroxylated poly-[(*R*)-3-hydroxybutrate], PHB-diol(M) (M_n of 3000, GPC, single peak), was a gift from Dr. P. Neuenschwander at ETH Zurich. Microbial PHB (>99%), dibutyltin dilaurate (95%), diglyme (99.5%), *ε*-caprolactone (99%), 1,4-dioxane (99.8%), and toluene (99.8%) were purchased from Aldrich. Ethylene glycol (99%), chloroform (GC, >99%), and *n*-hexane (HPLC, 99%) were obtained from Merck. Novozym 435 and PHB-diol were dried under vacuum at 50°C for 12 hours before use. *ε*-Caprolactone was

freshly distillated over CaH_2 (83°C, 1.7 mmHg). 1,4-Dioxane and toluene were dried by refluxing over Na/benzophenone under argon.

Telechelic hydroxylated poly-[(*R*)-3-hydroxybutrate], PHB-diol(P): PHB-diol (P) was prepared by using the known procedure for the preparation of PHB-diol (M)[12] with large excess of ethylene glycol to PHB. Transesterfication of PHB (8.0 g) and ethylene glycol (40 mL) in the presence of dibutyltin dilaurate (118 mg) in diglyme (32 mL) was performed at 135 ^{o}C for 2 h. The mixture was then poured into cold water, and the precipitate was collected and washed by water (50 ml x 3) to remove the excess ethylene glycol. The crude product was dissolved in chloroform followed by precipitation at 4 ^{o}C by the addition of *n*-hexane. The product was separated by filtration and dried at 50^{o}C under vacuum for 12 h to give 6.2 g of PHB-diol (P) in 76% yield. The molecular weight (M_n) was determined by GPC as 3700 g/mol with M_w/M_n of 1.38. The physical properties were determined by DSC with T_m of 149 and 134°C and T_g of -5.0°C. The chemical structure was identified by ^{1}H-NMR as a PHB-diol with a primary OH group on one end and a secondary OH group on the other end.

General procedure of enzymatic ring-opening polymerization of *ε*-caprolactone with PHB-diol: Novozym 435 (20-160 mg immobilized enzyme) and PHB-diol(M) (M_n of 3000, GPC; 88-212 mg) or PHB-diol(P) (M_n of 3700, GPC; 33-186 mg) were added to a dry schlenk tube containing a magnetic stirring bar and activated 4Å molecular sieves, and then dried at 50°C under vacuum for 12 h. Under argon atmosphere, the freshly distillated *ε*-caprolactone (400-600 mg) and freshly dried 1,4-dioxane or toluene (1.2 g-3.6 g) were added into the schlenk tube using a dry syringe. The mixtures were stirred under argon atmosphere at room temperature, 50°C, or 70°C and samples (50 *μ*L) were taken at regular time intervals and analyzed by GPC. The reaction was stopped at 8-48 h, 10 mL chloroform was added, and the enzyme was removed by filtration. The solvent in the filtrate was removed under reduced pressure, and the product was dissolved in chloroform and then precipitated by adding *n*-hexane or methanol. The results are summarized in Table 1-2.

Preparation of poly[(*R*)-3-hydroxylbutyrate(56wt%)-*co*-*ε*-caprolactone(44wt%)]: According to the procedure described above, reaction of PHB-diol(M) (M_n of 3000, GPC; 212 mg) and *ε*-caprolactone (409 mg) with Novozym 435 (40 mg immobilized enzyme) as catalyst was carried out in 1,4-dioxane (1.6 g) at 70°C, and samples (50 *μ*L) were taken at regular time intervals and analyzed by GPC. After 48 h polymerization, the reaction was terminated by the addition of 10 mL chloroform followed by the removal of Novozym 435 through filtration. 1,4-dioxane and chloroform were removed by evaporation under reduced pressure. The raw product was dissolved in 2 mL chloroform, treated with 18 mL methanol, and then precipitated at 4°C for 12 h. After removal of the solvent by filtration, the precipitates were dried at first by evaporation under vacuum and then in a vacuum oven at 50°C for 24 h. This gave 410 mg (66% yield) of the polymer. The molecular weight M_n was determined by GPC as 5400 (M_w/M_n of 1.63), and the structure was analyzed by NMR and IR as *di*-block co-polyester poly(HB-*co*-CL) with two different OH end groups. The ratio of PHB and PCL block was established as 56/44 (wt/wt) based on M_n (NMR) of PHB-diol(M) and the polymer. The physical properties were determined by DSC and WAXD with T_m of 145°C, 123°C, and 53°C, T_g of -57°C, and crystallinality of 19%.

Preparation of poly[(*R*)-3-hydroxylbutyrate(28wt%)-*co*-*ε*-caprolactone(72wt%)]: According to the procedure described above, reaction of PHB-diol(P) (M_n of 3700, 185 mg) and *ε*-caprolactone (632 mg) with Novozym 435 (76 mg immobilized enzyme) as catalyst was carried out in toluene (1.8 g) at 70°C, and samples (50 *μ*L) were taken at regular time intervals and analyzed by GPC. After 16 h polymerization, the reaction was terminated by the addition

of 10 mL chloroform followed by the removal of Novozym 435 through filtration. Toluene and chloroform were removed under reduced pressure with a rotary evaporator. The raw product was dissolved in 2 mL chloroform, treated with 18 mL methanol, and then precipitated at 4°C for 12 h. After removal of the solvent, the precipitates were dried at first by evaporation under vacuum and then in a vacuum oven at 50°C for 24 h. This gave 702 mg (86% yield) of the polymer. The molecular weight was determined by GPC as 7900 (M_w/M_n of 1.90), the structure was analyzed by NMR and IR as *di*-block co-polyester poly(HB-*co*-CL) with two different OH end groups. The ratio of PHB and PCL block was established as 28/72 (wt/wt) based on M_n (NMR) of PHB-diol(P) and the polymer. The physical properties were determined by DSC with T_m of 149°C and 54°C, and T_g of -61°C.

Gel permeation chromatography (GPC). Molecular weight analysis (M_n and polydispersity index M_w/M_n) was performed by using a Waters instrument, with Waters 510 pump, Waters 410 refractive index detector, and Waters HR4E, HR5E and HR6 columns placed in series. THF was used as the eluent at a flow rate of 1.0 mL/min at 30°C. Sample concentration was about 0.1% (w/v) and the injection volume was 100 μL. Polystyrene standards with molecular weights of 1310, 2970, 13900, 30200, 197000 and 696000 g/mol were used to generate a calibration curve.

Nuclear magnetic resonance (NMR). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded with a Bruker AMX500 NMR instrument in $DMSO_{d6}$ at 333K. Chemical shifts were referred to TMS at 0 ppm.

Differential scanning calorimetry (DSC). The thermal properties of polymers were measured on a Mettler Toledo DSC 822 system. Nitrogen was used as purge gas with a flow rate of 30 ml/min. Samples of 10 mg were prepared in aluminum foils, where the aluminum weights of the sample and reference were closely matched. The samples were heated from room temperature to 180 °C with a heating rate of 20 °C/min, cooled down to -100 °C with a cooling rate of -20 °C/min, and heated again from -100 °C to 180 °C at a heating rate of 20°C/min. T_m and T_g of the samples were obtained from the second heating curves.

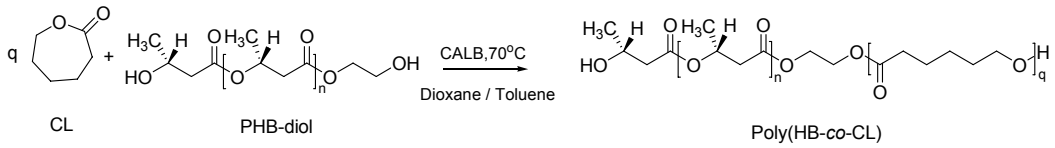
Wide angle X-ray diffraction (WAXD). The crystallinity analysis was preformed with a SHIMADZU 6000 X-ray diffractometer with Cu Kα radiation at 40 kV and 30 mA in a 2*θ* range of 5-40 ^{o} at scanning speed of 1.2 ^{o}/min.

Fourier transform infrared spectrophotometer (FTIR). IR spectra of the polymers were analyzed with a SHIMADZU FTIR-8400 system using potassium bromide (KBr) pressing.

Results and Discussion

Enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol(M)

Reaction conditions. The enzymatic ring-opening polymerization of ε-caprolactone (CL) was initially investigated with PHB-diol (M) as an initiator (Scheme 1). PHB-diol(M) is a telechelic hydroxylated PHB with a M_n of 3000 g/mol (GPC) prepared by transesterification of PHB and ethylene glycol.[12] Novozym 435 [immobilized *Candida antarctica* lipase B (CALB)] was chosen as catalyst, as it is well known with high catalytic activity and good solvent resistance for the ROP of lactones.[21-22] The polymerization temperatures were examined from room temperature to 70°C, since Novozym 435 has the highest catalytic activity at around 70°C. [21-22]



Scheme 1 Enzyme-catalyzed ring-opening polymerization of ε-caprolactone with PHB-diol

1,4-Dioxane and toluene were known solvents for enzymatic ROP,[21-22] and they have a boiling point of 103°C and 110°C, respectively, and good solubility for PHB-diol, thus being selected as solvents in our experiments. To avoid water-initiated ring-opening polymerization, solvent was dried before use and the reaction was carried out at anhydrous conditions under Argon atmosphere. In order to study the effect of different reaction conditions on the polymerizations, a set of experiments were designed with different weight ratios of CL/solvent, CL/enzyme, CL/PHB-diol and different temperature (Table 1). The reactions were performed in 1,4-dioxane and followed by taking samples at different time points to determine the molecular weight by GPC. As an example, the course of polymerization in experiment 11 in Table 1 was shown in Figure 1: the number average molecular weight (M_n) of the samples taken at 4 h, 8 h, 24 h and 48 h reached 3000, 4700, 4900, 6700, and 8400 g/mol, respectively. The molecular weight and yield of polymers under different reaction conditions are summarized in Table 1.

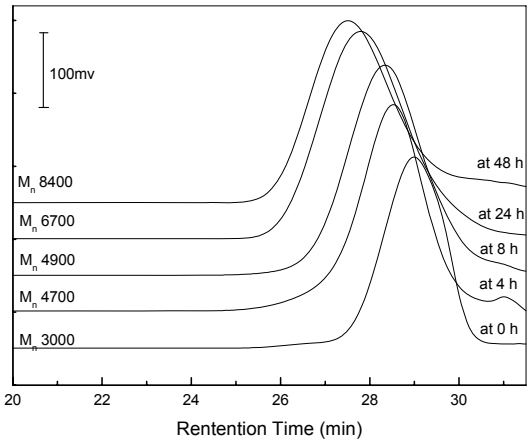


Figure 1. GPC chromatograms of block poly(HB-*co*-CL) formed at different time from experiment 11 in Table 1.

Table 1. Ring-opening polymerization of ε-caprolactone with PHB-diol(M) catalyzed by Novozym 435 in dioxane

No.	CL:**E** [a]	CL:diol	CL:**Sol** [b]	Temp.	Time	M_n (GPC)[c]	M_w/M_n	Yield	CL Conv.	Code
	wt:wt	mol:mol	wt:wt	°C	h	g/mol		%	%	
1	5:1	100:1	1:4	70	48	5500	1.65	63	53	
2	10:1	100:1	1:4	70	48	4500	1.56	57	47	
3	20:1	100:1	1:4	70	48	4000	1.48	51	40	
4	8:1	100:1	1:3	70	48	6700	1.68	63	54	
5	8:1	100:1	1:6	70	48	4300	1.53	64	55	
6	8:1	100:1	1:9	70	48	3400	1.38	64	55	
7	8:1	100:1	1:3	70	48	6700	1.79	64	53	A
8	8:1	100:1	1:3	50	48	4100	1.66	65	56	
9	8:1	100:1	1:3	RT	48	3600	1.55	69	59	
10	8:1	50:1	1:3	70	32	5400	1.63	66	49	B
11	8:1	75:1	1:3	70	48	8400	1.66	58	45	C

a: **E** for immobilized enzyme, Novozym 435. b: **Sol** for solvent, Dioxane c: Polystyrene was used as standards in GPC measurement

In experiment 1 to 3 (Table 1), three different ratios of CL/enzyme (E) were studied at 70°C with fixed ratios of CL/PHB-diol and CL/Solvent, and polymerization were shown in Figure 2(i). The highest molecular weight of the resulting product was obtained at CL/E in a weight ratio of 5:1. Within the range of 20-5:1, the molecular weight of the resulting polymers increased with catalyst amount. To reduce the amount of enzyme involved while achieving a high molecular

weight of the polymer, the ratio of CL/E of 8/1 was used in the rest of experiments. In experiment 4-6 (Table 1), CL/solvent was examined in the weight ratio of 1:3, 1:6 and 1:9 at 70°C with fixed ratios of CL/PHB-diol and CL/E. As shown in Figure 2(ii), the use of less solvent resulted in higher molecular weight of the polymers, probably due to higher concentration of reacting species. On the other hand, the ratio of CL/solvent should not be too small, since sufficient amount of the solvent is required to dissolve PHB-diol. The effect of the reaction temperature on the polymerization was investigated in the experiment 7-9 (Table 1) at rt, 50°C and 70°C, respectively. Figure 2(iii) clearly demonstrated that higher temperature resulted in higher molecular weight of the polymers. These results are in close correlations to the reported best temperatures of 65-70 °C for ROP with Novozym 435. Finally the effect of the molar ratio of CL/PHB-diol was investigated in experiment 7, 10, and 11 (Table 1) at 70°C with fixed ratios of CL/E (8:1) and CL/Solvent (1:3). As shown in Figure 2(iv), the highest molecular weight of the resulting polymer was obtained with a molar ratio of CL/PHB-diol in 75:1. The use of more PHB-diol could create more opportunity for the opening of CL. However, this could be in competition with the chain growth by CL, resulting possible decrease of the molecular weight of final products. From these experiments, the best conditions examined so far for achieving highest molecular weight was 70°C, a weight ratio of CL/enzyme/solvent of 8:1:24, and a molar ratio of CL/PHB-diol of 75:1.

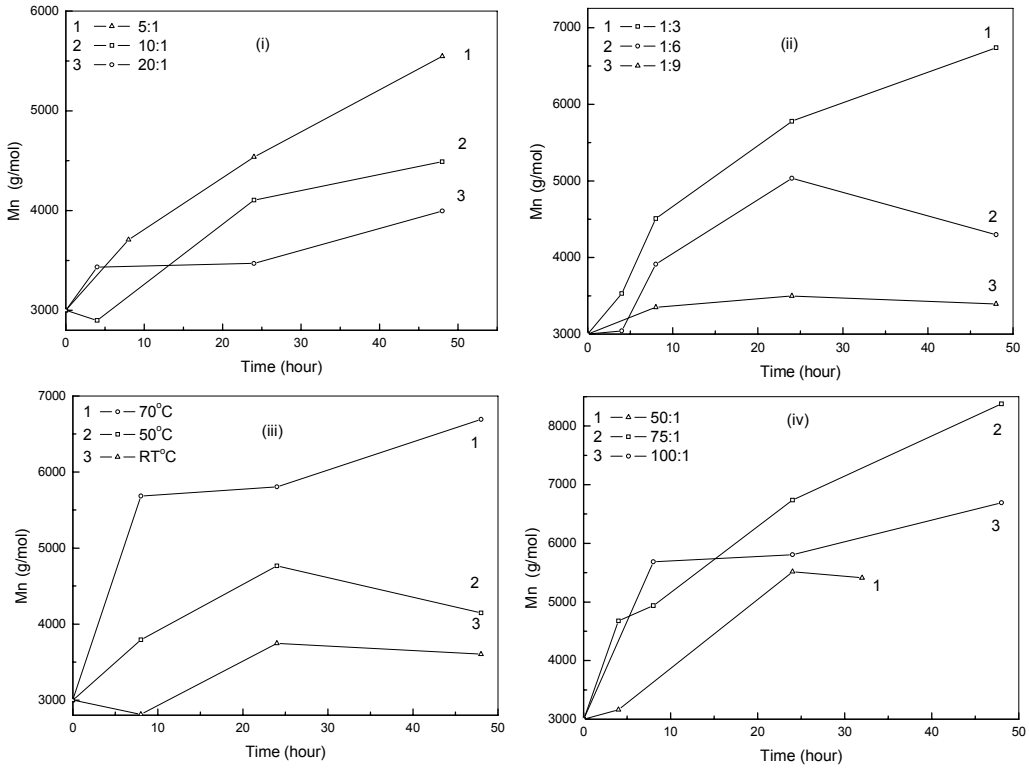


Figure 2. Enzyme-catalyzed ring-opening polymerization of ε-caprolactone with PHB-diol(M) under different reaction conditions: (i) different ratio of CL/Enzyme; (ii) different ratio of CL/Solvent; (iii) different reaction temperature; (iv) different ratio of CL/ PHB-diol(M).

Polymer isolation. The polymerization products from all experiments in Table 1 were isolated by adding chloroform to the reaction mixture, removing enzyme through filtration, evaporating 1,4-dioxane and chloroform at reduced pressure, and precipitating in chloroform/*n*-hexane or chloroform/methanol (1:9). Drying the precipitates under high vacuum at 50°C for 24 h gave

the corresponding block co-polyesters with M_n (GPC) of 3600-8,400g/mol in 51-69% yields and 40-59% CL conversion, respectively.

Structure analysis. The chemical structure of PHB-diol(M) was determined by ^{1}H-NMR spectrum in Figure 3(i). Two different structures A and B were found in PHB-diol(M). All signals were assigned to the different protons in structure A and B according to the literatue.[12] Two different proton signals for primary OH and secondary OH were observed at 4.58 ppm (*c* proton) and 4.45ppm (*d* proton), respectively. The molar ratio of structures A and B was estimated as 91%:9% based on the intensities of *h* proton and *u* proton, and the number of repeating unit n was deduced as 23.6 based on the ratio of the intensities of *m* and *u* protons. The M_n of PHB-diol(M) can be thus established as 2380 g/mol which is comparable with the M_n of 3000 g/mol determined by GPC. In fact, the M_n obtained from NMR is more reliable, since the M_n determined from GPC was based on the use of polystyrene as standard.

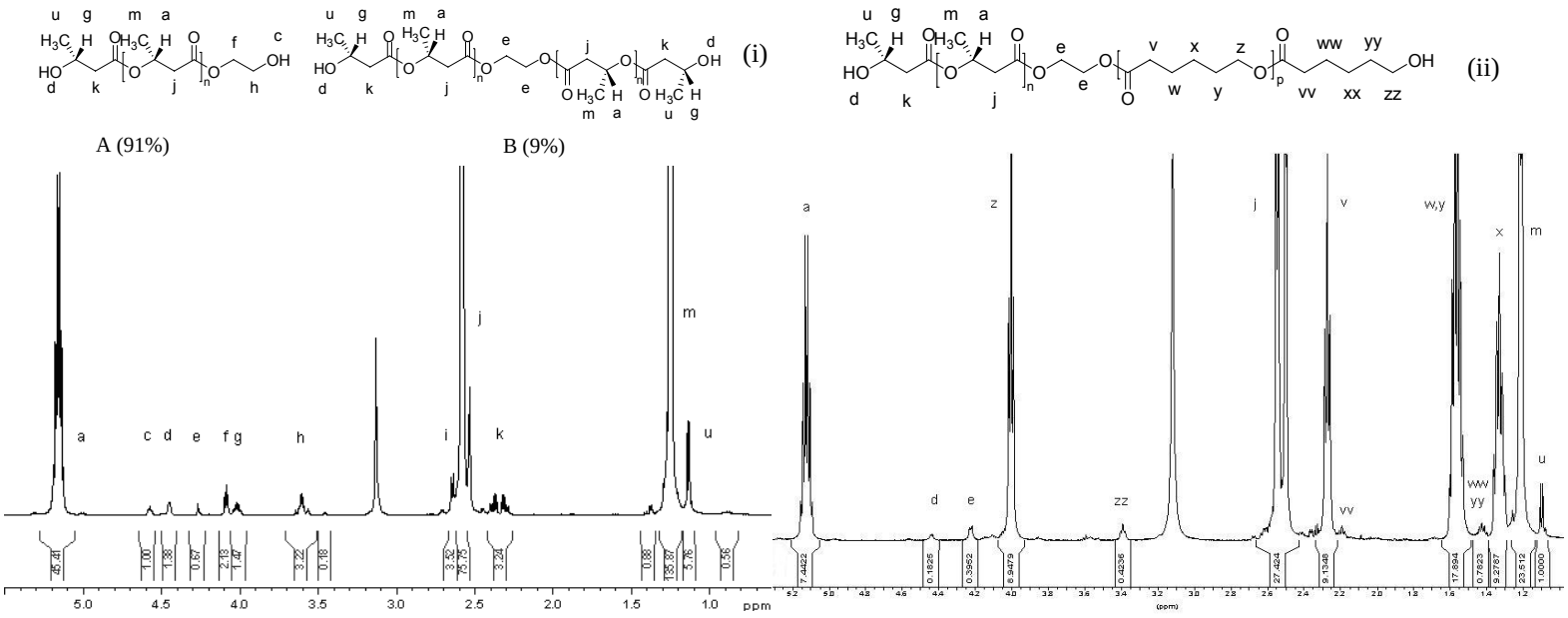


Figure 3. ^{1}H-NMR spectra in DMSO$_{d6}$ at 333K: (i) PHB-diol(M); (ii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 1).

The chemical structures of the polymers were determined by NMR and IR spectra. The ^{1}H-NMR spectrum of sample B (Table 1) was shown in Figure 3(ii). The *c* proton (primary OH, δ = 4.58 ppm) of PHB-diol(M) disappeared and the *d* proton (secondary OH, δ = 4.45ppm) was clearly observed. This indicates that the primary OH end group of PHB-diol(M) was more reactive and hence totally reacted, while the secondary OH end group is less reactive thus remaining in the polymer. The signal of *h* proton (δ = 3.56ppm) and *f* proton (δ = 4.10ppm) disappeared, which suggested again that all primary OH groups of PHB-diol(M) was reacted. The polymerization was further evidenced by the increased intensity of *e* proton. While reaction with a primary OH end group of PHB-diol(M) did not change the ratio of *m*/u protons, polymerization with a secondary OH end group would transform a *u* proton into a *m* proton, thus increasing the ratio of *m*/*u* protons. From Figure 3 (ii), the ratio of *m*/*u* in the polymer was calculated as 23.5 which was the same as the *m*/*u* ratio for the starting material PHB-diol(M). Thus it is unlikely that the secondary OH end group was reacted in the ROP. As a result, the polymer is a *di*-block. The signals of PCL block were also assigned in Figure 3 (ii). With the ratio of *z*/*a* of 1.20:1, the number of repeating unit p in PCL block was calculated as 14.2. The molecular weight of poly(HB-*co*-CL) could thus be established as 3900 g/mol. This value is

smaller than the M_n of 5400 g/mol determined by GPC. But, as it was mentioned above, the M_n obtained from NMR is more reliable.

The block copolymer structure was further evidenced by the ^{13}C-NMR spectrum in Figure 4. In the area of 160-180 ppm, only two signals at 168 and 172 ppm were observed and they were assigned to the carbonyl groups in PHB block and PCL block, respectively. From previous report[18] and our control experiment of lipase-catalyzed transesterification of PLC and PHB-diol, random polymer poly(HB-*co*-CL) contains at least two different types of carbonyl groups which absorbed at 169 and 171 ppm between the two signals at 168 and 172 ppm. In the ^{13}C-NMR spectrum of our polymer, no such signals can be detected. This indicated no random polymer formed during the polymerization.

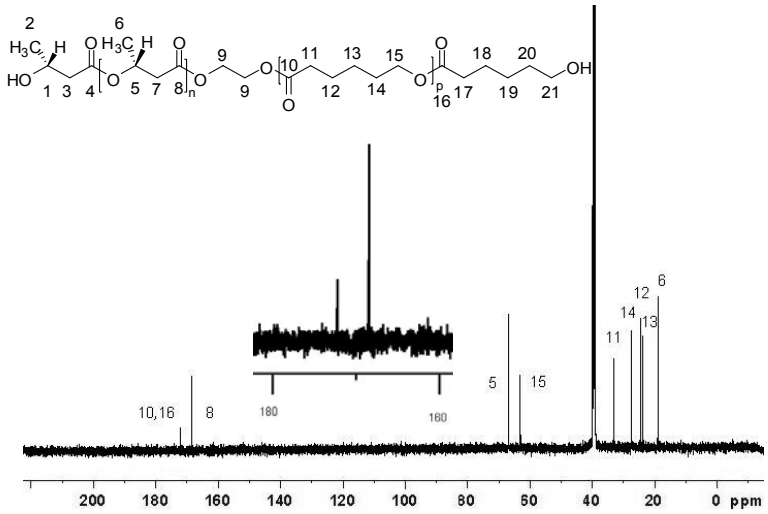


Figure 4. ^{13}C-NMR spectra of Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table1) in $DMSO_{d6}$ at 333K.

In the IR spectra in Figure 5(i) and (iii), the primary and the secondary terminal OH group of the starting material PHB-diol(M) showed two different absorptions at 3437 cm^{-1} and 3538 cm^{-1}. There were also two absorptions at 3438 cm^{-1} and 3533 cm^{-1} for the OH groups in the block co-polymer. This further confirmed that poly(HB-*co*-CL) is a *di*-block polymer containing a primary and a secondary OH group. In the case of A-B-A (PCL-PHB-PCL) *tri*-block structure, the OH end groups would be the same, thus giving only one absorption peak in the IR spectrum.

The weight percentage of PHB and PCL block in the co-polymer was calculated using the repeating unit *n* and *p* for PHB and PCL block, respectively, obtained from NMR analysis. In the case of the polymer prepared above, *n* is 23.5, thus the molecular weight for PHB block is 86 x (23.5+1) + 1 + 60 = 2168; p is 14.2, therefore, the molecular weight for PCL block is 114 x (14.2 +1) + 1 = 1734. Based on these results, it can be deduced that the *di*-block polymer contains 56wt% of PHB block and 44wt% of PCL block. This method was used to calculate the weight ratio of the two blocks for all *di*-block polymers in this study.

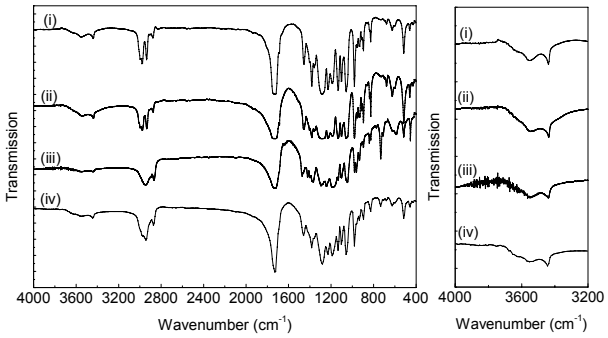


Figure 5. IR spectra of different polymers: (i) PHB-diol (M); (ii) PHB-diol (P); (iii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 1); (iv) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 2).

Enzymatic ring-opening polymerization of *ε*-caprolactone with PHB-diol(P):

PHB-diol(P). For the easy investigation of the selectivities of the primary and secondary OH group of PHB-diol in the enzymatic ROP and for the easy preparation and characterization of the corresponding *di*-block co-polymer with simpler structure, PHB-diol that contains 100% structure A with a primary and a secondary OH end groups was designed as the initiator. This

type of PHB-diols with M_n between 2000-4000 g/mol was prepared by transesterification of PHB and ethylene glycol according to the same procedure for the preparation of PHB-diol(M)[12], but with large excess of ethylene glycol to PHB. Their desired chemical structures were confirmed by ^{1}H-NMR analysis. As an example, the ^{1}H-NMR spectrum of PHB-diol(P) with M_n of 3700g/mol (GPC) was shown in Figure 6(i). The primary OH and secondary OH group absorbed at 4.58 ppm (*c* proton) and 4.45 ppm (*d* proton),[12] respectively, with nearly equal intensities. Similar to the case of PHB-diol(M), the molar ratio of structures A and B in PHB-diol(P) was estimated based on the intensities of *h* proton from the primary OH end and *u* proton from the secondary OH end as 100:0. The number of repeating unit n was calculated as 21.7 according to the ratio of *m*/*u* absorption intensity, which gave a M_n of 2010g/mol for PHB-diol(P). The primary and secondary OH groups of PHB-diol(P) showed two different absorptions at 3437 cm^{-1} and 3538 cm^{-1} in the IR spectrum in Figure 5(ii).

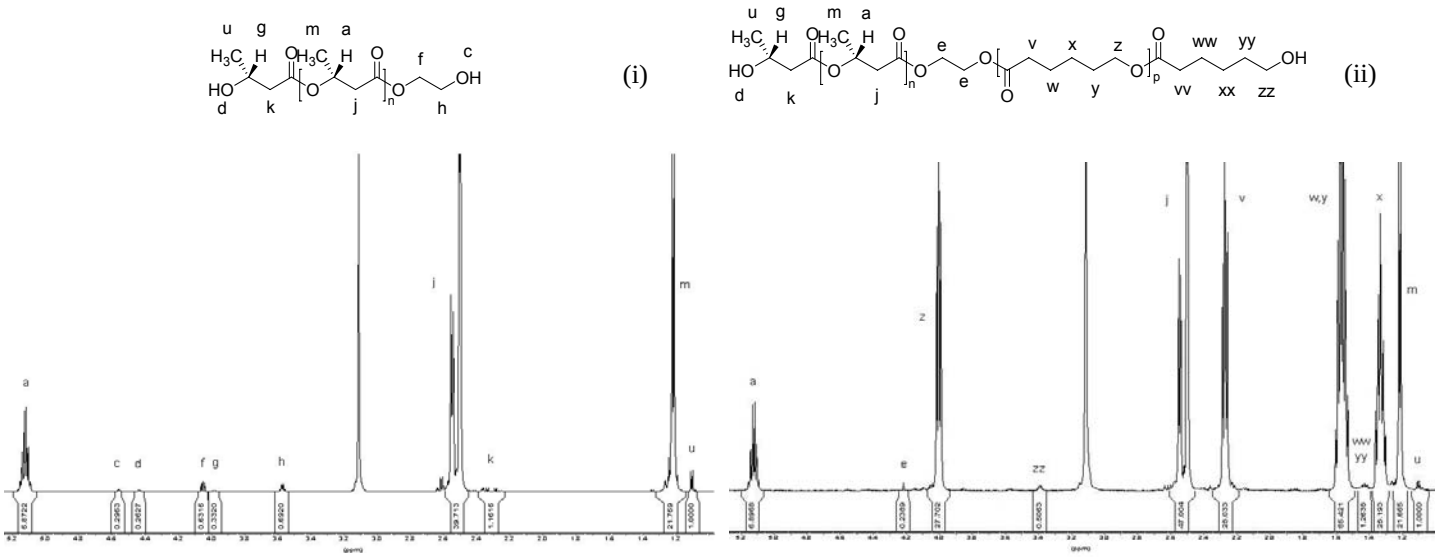


Figure 6. ^{1}H-NMR spectra in DMSO$_{d6}$ at 333K: (i) PHB-diol (P); (ii) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 2).

The physical properties of the new type of PHB-diols were characterized by DSC, and the T_m and T_g were found to be dependent on the M_n: PHB-diol (P) with M_n of 3700g/mol (GPC; M_w/M_n of 1.38) has T_m of 134 and 149^{o}C and T_g of -5^{o}C, while PHB-diol (P_8) with M_n of 2300g/mol (GPC; M_w/M_n of 1.17) has T_m of 115 and 129^{o}C and T_g of -11.9^{o}C. Both of them are good hard segment for block co-polymer syntheses. For the preparation of biomaterials with the possibility of sterilization, T_m of the polymers needs to be above 120^{o}C. Therefore, PHB-diol (P) is an appropriate hard block for the preparation of such type of materials.

Ring-opening polymerization with PHB-diol(P). New batches of lipase-catalyzed ROP of CL with PHB-diol(P) (M_n of 3700, GPC) as the initiator were carried out in dioxane (experiments 12-15 in Table 2). The optimal conditions established for PHB-diol(M) such as 70 ^{o}C, CL:E in 8:1 wt ratio, and CL:Sol in 1:3 wt ratio were used for the ROP. Reaction for 16 h with a molar ratio of CL:PHB-diol(P) in 50:1 gave a block co-polymer with M_n (GPC) of 5500 g/mol. The molecular weight achieved here is similar to that obtained in the ROP with PHB-diol(M). Further increase of the molar ratio of CL:PHB-diol(P) to 100:1 did not increase the M_n of the final polymer. Changing the CL:E ratio from 8:1 to 4:1 did not result in big change of the polymer molecular weight, either. In experiment 16, ROP of CL and PHB-diol(P_8) with M_n(GPC) of 2300g/mol was carried out at similar conditions, affording a polymer with M_n (GPC) of 5500g/mol. To further increase the molecular weight of the polymer, toluene was examined as solvent (experiments 17-21). The ROP of CL with PHB-diol(P) were initially performed at the

optimal conditions established for dioxane: reaction with CL and PHB-diol(P) in 100:1 and 50:1 molar ratio, respectively, gave the corresponding polymers with M_n of 8900 and 7900 g/mol, respectively. The reaction conditions were also varied in other experiments, which did not improve the molecular weight of the polymer. Nevertheless, the polymers prepared in toluene have higher M_n than those prepared in dioxane. This is possibly due to the different log P value of the solvents. Toluene with log P of 2.5 is possibly less toxic to the enzyme than 1,4-dioxane with log P of -0.42. All the polymers were isolated in 69%-90% yield with 64-88% CL conversion by using the same methods descript for the ROP with PHB-diol (M).

Table 2. Ring-opening polymerization of *ε*-caprolactone with PHB-diol(P) in dioxane or toluene

No.	Sol[a]	CL:**E**[b]	PHB-diol	CL:PHB-diol	CL:**Sol**	Temp.	Time	M_n (GPC)	M_w/M_n	Yield	CL Conv.	Diblock	T_m	T_g	Cod
		wt:wt		mol:mol	wt:wt	°C	h	g/mol		%	%	%	°C	°C	
12	D[c]	8:1	P	100:1	1:3	70	16	5300	1.56	73	69		51.3 / 132.3 / 148.3	-57.5	
13	D	8:1	P	50:1	1:3	70	16	5500	1.55	79	73		52.0 / 135.7 / 150.0	-57.7	
14	D	4:1	P	100:1	1:3	70	12	5200	1.75	88	86		50.0 / 128.7 / 145.7	-60.0	
15	D	4:1	P	50:1	1:3	70	48	4200	1.57	69	64	100	48.3 / 123.3 / 141.0	-59.1	
16	D	4:1	P8[e]	75:1	1:3	70	48	5500	1.75	76	73	97	48.7 / 99.0 / 116.0	-55.4	
17	T[d]	8:1	P	100:1	1:3	70	16	8900	1.93	89	87		56.7 / 154.3	-57.3	
18	T	8:1	P	50:1	1:3	70	16	7900	1.90	86	82	100	50.0 / 147.0	-60.0	D
19	T	4:1	P	100:1	1:3	70	12	7900	1.86	90	88		50.0 / 153.3	-58.8	
20	T	16:3	P	100:1	1:4	70	8	7900	1.98	87	85		52.0 / 153.3	-60.3	
21	T	16:3	P	100:1	1:6	70	8	5600	1.95	79	76	98	54.0 / 149.0	-61.0	E

a: **Sol** for solvent, Dioxane. b: **E** for immobilized enzyme, Novozym 435. c: D for dioxane. d: T for toluene. e: P8 for PHB-diol (P8) with M_n (GPC) of 2300 g/mol.

Structure Analysis. Polymers from experiments 15, 16, 18, and 21 were analyzed by ^{1}H-NMR and they all contain *di*-block poly(HB-*co*-CL) in 97%-100%. The ^{1}H-NMR spectrum of sample D (experiment 18) from Table 2 was given in Figure 6(ii). The *di*-block structure was clearly evidenced by the absorption of u proton at the end of PHB block and the *zz*, *ww*, *yy* protons at the end of PCL block. The intensity ratio of *ww* and *yy* signals (4 protons,1.45 ppm) and *u* signal (3 protons, 1.10 ppm) was 1.26:1, which corresponds to a ratio of the two ends of the polymers about 0.97:1. New signal of *e* proton appeared at 4.22 ppm, indicating the formation of polymer. From the ^{1}H-NMR spectrum, the ratio of *m/u* signal intensities of the polymer was determined as 21.7 which is exactly the same as that for PHB-diol(P). As previously described, the formation of *tri*-block polymer would increase the ratio of *m/u*. Therefore, it can be concluded that there is no *tri*-block structures in the polymer sample D. The ratio of the absorption intensities of *z* proton in PCL block and *a* proton in the PHB block was determined as 4.02:1, thus the *p* in the

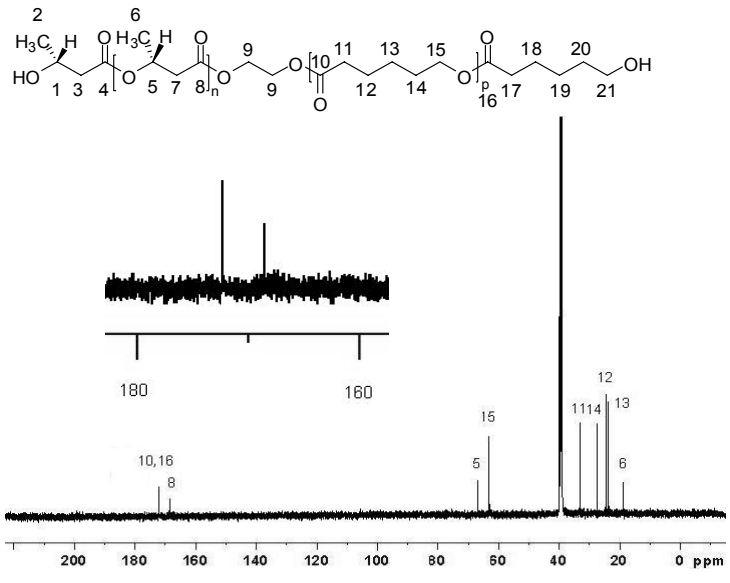


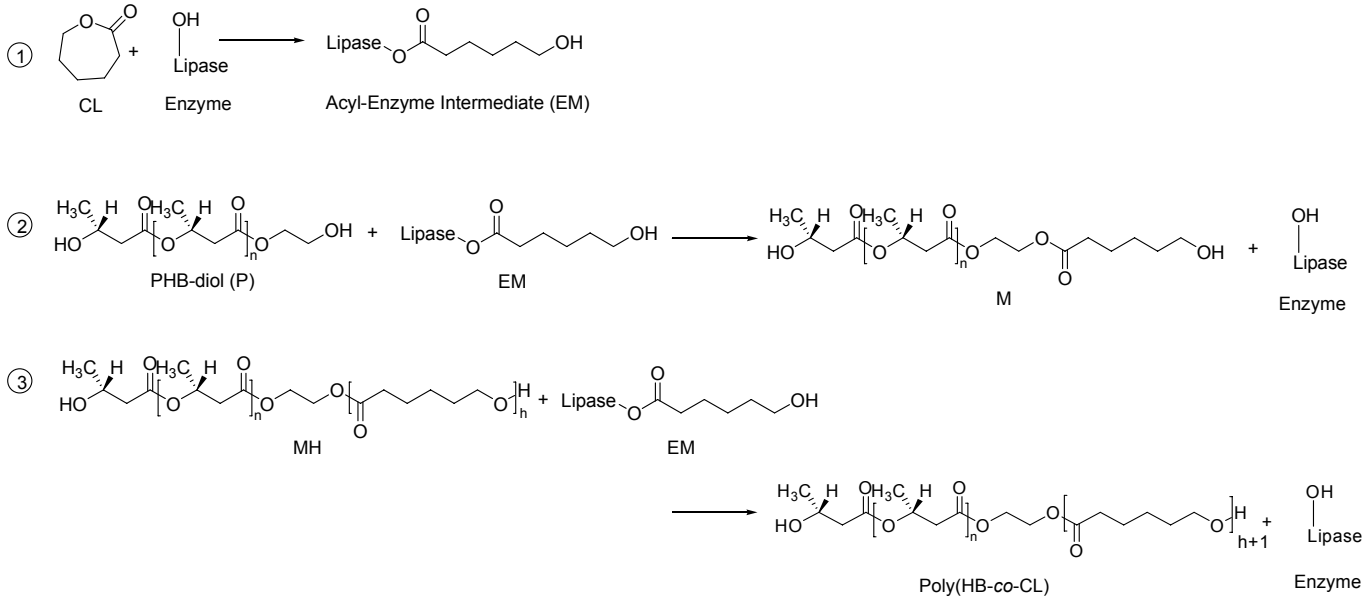
Figure 7. ^{13}C-NMR spectrum of poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 2) in $DMSO_{d6}$ at 333K.

PCL bock can be deduced as 43.6. This led to the establishment of the polymer M_n as 7100g/mol. Calculation based on the intensities of *m* and *x* protons gave a M_n of 6900g/mol for the polymer. These values are in good correlation to the M_n measured by GPC (7900g/mol).

The ^{13}C NMR spectrum of sample D in Table 2 was shown in Figure 7. In the area of 160-180 ppm, the two signals observed are the carbonyl groups of the PHB and PCL block. No other carbonyl signals were detected, suggesting no random copolymers formed during ROP. The *di*-block structure was further confirmed by two different absorption of the primary and the secondary end OH groups at 3438 cm^{-1} and 3533 cm^{-1} in the IR spectrum of sample D shown in Figure 5(iv). Based on NMR analysis, n = 21.7 and p = 43.6, thus the ratio of PHB and PCL block was established as 28/72 (wt/wt) for the polymer.

Mechanism of PHB-diol initiated ring-opening polymerization of *ε*-caprolactone.

Is the polymerization really initiated by the PHB-diol? Is it possible that PCL is first formed by water-initiated ring-opening polymerization and then it reacts with PHB-diol *via* transesterification to give the co-polymer? To answer these questions, control experiments were carried out in 1,4-dioxane with CL and novozym 435 at 70°C and at the same ratio of CL/enzyme/solvent as that used for the synthesis of sample B in Table 1. Reaction for 16 h without pre-drying gave PCL with M_n of 12000g/mol (GPC), while reaction under anhydrous conditions for 16 h afforded a PCL with M_n of 5000g/mol (GPC). From these experiments, the possible formation of PCL in our polymerization reactions can not be excluded. Based on the known mechanism, even only a very small amount of water could initiate the polymerization to give PCL. Further control reaction was to examine the possible transesterification between PHB-diol and PLC. Reaction of PHB-diol(M) (M_n of 3000 g/mol) and PCL (M_n of 5000 g/mol) in 1:1 molar ratio with Novozym 435 as catalyst was performed in dioxane under the anhydrous conditions. The M_n of the reaction mixture was 4400g/mol (GPC) at the beginning and dropped to 2200g/mol (GPC) at 48 h. The product was isolated by the same procedure used for ROP and analyzed by ^1H- and ^{13}C-NMR. The *m/u* signal ratio of the product in the ^1H-NMR spectrum was drop to 16.9 from 23.6 of PHB-diol(M), suggesting the degradation of PHB-diol. This must be caused by the Novozyme 435-catalyzed transesterification between PCL and PHB-diol, since no such degradation was observed in the same system without enzyme. In the



Scheme 2. Mechanism of enzyme-catalyzed ring-opening polymerization of *ε*-caprolactone with PHB-diol.

^{13}C-NMR, in addition to the signals at 168 and 172 ppm of the carbonyl group of the PHB and PCL block, two signals were observed at 169 and 171 ppm. These were the absorptions of the different type of carbonyl groups of random co-polymer. Thus, a mechanism of formation of PCL followed by transesterification with PHB-diol would generate co-polymers with decreased M_n, decreased *m*/*u* signal ratio in ^{1}H- NMR, and new signals in ^{13}C- NMR spectrum for random polymer units. Since all these phenomena were not observed in our polymer preparation, we can exclude such mechanism for our reaction.

The mechanism of enzyme-catalyzed ROP of CL was well known.[22] Accordingly, the steps of our ROP was proposed in Scheme 2. The first step is the opening of CL with lipase to give an acyl-enzyme intermediate (EM). Afterwards, the intermediate is reacted with an OH group of PHB-diol to form new molecule M and release the lipase. As the primary OH group is more reactive and has less stereo-hindrance than the secondary OH group, it is preferentially reacted with lipase to give compound M with a secondary OH end group from PHB block and a primary OH end group from CL. While the first two steps can be repeated, the compound MH could also react with EM to prolong the chain and release lipase. Also in this step, the primary OH group of CL/PCL in compound MH should be more reactive than the secondary OH group of PHB block, thus generating a *di*-block polymer with a secondary OH group at end of PHB block and a primary OH group at the end of PCL block. Although we can not exclude the possibility of initiating ROP of CL by the trace amount of water in the system such as in the enzyme hydration shell, the primary OH group of PHB-diol should be more reactive than water, thus effectively suppressing the water-initiated ROP. Even if PCL monomer and oligomer could be formed by water-initiated ROP, they could not react with the existing PHB-diol, its derivatives M and MH, and Poly(HB-co-CL) *via* enzymatic transesterification, since the enzyme active center is occupied through the formation of EM with CL in large excess. The only possibility remained for PCL monomer and oligomer is to react with EM for the elongation of the PCL chain, which is in competition with the elongation of Poly(HB-*co*-CL). In fact, only very small amount of PCL monomer and oligomer was observed in our experiments, and these side products were easily removed by precipitating Poly(HB-*co*-CL) in chloroform/*n*-hexane (1:9).

Physical properties of *di*-block poly[(*R*)-3-hydroxybutyrate-*co*-*ε*-caprolactone]s

The melting temperature (T_m) and glass transition temperature (T_g) of several poly(HB-*co*-CL)s, PCL, and PHB-diols were measured by DSC. The data were summarized in Table 2 and 3. As shown in Figure 8, T_m and T_g were determined from the second heating curve. T_m of PHB-diol(P) was found to be 134°C and 149°C, and T_m of PCL with an M_n(GPC) of 12000 g/mol was 57°C. Poly[HB(55wt%)-*co*-CL(45wt%)] (sample E) and poly[HB(28wt%)-*co*-CL(72wt%)] (sample D) showed T_m from both PHB and PCL blocks, with the values of 147-149 °C and 50-54°C. T_m of the PHB block is a broad peak in DSC, and in some case it was split into two peaks. T_g of PHB-diol(P) and the PCL was determined to be -5°C and -63°C, respectively. The polymers D and E showed a T_g of -60°C and

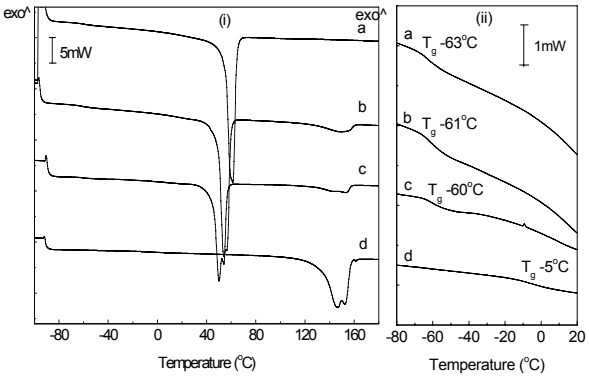


Figure 8. DSC spectra of (a) PCL with M_n 12000 (GPC), (b) Poly[HB(28wt%)-*co*-CL(72wt%)] (sample D in Table 2), (c) Poly[HB(55wt%)-*co*-CL(45wt%)] (sample E in Table 2), and (d) PHB-diol(P): (i) The second heating curve; (ii) The enlarged second heating curve.

-61°C, respectively, which was obviously the T_g from the PCL block. The T_g of PHB block was not detectable in all polymers prepared in this study. As shown in Table 3, all polymers containing 44-74wt% of PCL block have a T_g between -57°C and - 61°C for the elastomer domain. Thus, introduction of the PCL block significantly improved the thermal properties of block co-polymers for potential thermoplastic application.

Table 3. Physical properties of selected poly(HB-*co*-CL)s, PHB-diol and PCL

Polymer	Code	Initiator	M_n (GPC) g/mol	M_n (NMR) g/mol	HB:CL[a] wt%	T_m °C	T_g °C
PHB-diol(M)			3000	2380		137 / 147	-4
PHB-diol(P)			3700	2010		134 / 149	-5
Poly(HB-*co*-CL)	A	PHB-diol(M)	6700	6100	32:68	54 / 120 / 139	-57
Poly(HB-*co*-CL)	B	PHB-diol(M)	5400	3900	56:44	53 / 123 / 145	-57
Poly(HB-*co*-CL)	C	PHB-diol(M)	8400	7500	26:74	53 / 121 / 140	-56
Poly(HB-*co*-CL)	D	PHB-diol(P)	7900	7100	28:72	50 / 147	-60
Poly(HB-*co*-CL)	E	PHB-diol(P)	5600	3400	55:45	54 / 149	-61
PCL		water	12000			57	-63

a: The ratio was calculated based on NMR analysis.

In Figure 9, the DSC curves of different samples were compared, including PCL, PHB-diol(M), a mixture of PHB-diol(M) and PCL with weight ratio of 1:1, and poly[HB(56wt%)-*co*-CL(44wt%)] (sample B). While the 1:1 mixture demonstrated exactly the same T_m values as those of PHB-diol and PCL, the block co-poly(HB-*co*-CL) showed lower T_m than PHB-diol and PCL. This confirmed once again the formation of block-copolymer.

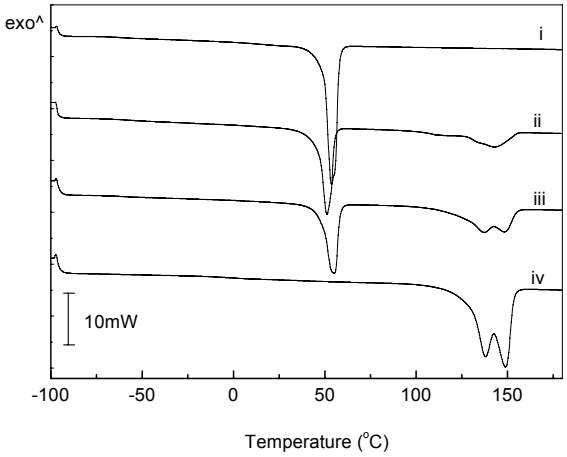


Figure 9. DSC spectra of different polymers: (i) PCL with M_n of 5000; (ii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 1); (iii) Mixture of PHB-diol(M) and PCL with M_n of 5000 (GPC); (iv) PHB-diol(M).

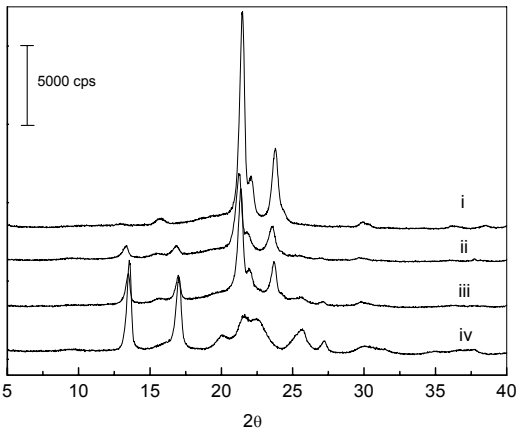


Figure 10. WAXD spectra of different polymers: (i) PCL with M_n of 12000 (GPC); (ii) Poly[HB(26%)-*co*-CL(74%)] (sample C in Table 1); (iii) Poly[HB(56wt%)-*co*-CL(44wt%)] (sample B in Table 1); (iv) PHB-diol(M).

The crystallinity of the polymers was investigated by the WAXD. As shown in Figure 10, poly[HB(56wt%)-*co*-CL(44wt%)] (sample B) and poly[HB(26wt%)-*co*-CL(74wt%)] (sample C) showed very similar patterns to that of PHB-diol and PCL, but with decreased intensity: two major peaks from PCL at 2θ of 21.4° and 2θ of 23.8° and two major peaks from PHB-diol at 2θ

of 13.6^{o} and 2θ of 17.0^{o} were founded in these copolymer. This indicated that the crystalline structures of the copolymers were similar to each of the two homopolymers. Thus, the polymer should show T_m from both PHB and PCL blocks. The crystallinities of poly(HB-*co*-CL)s, PHB-diol, PCL were estimated based on the ratios of crystalline peak area and amorphous peak area by using the affiliated software of SHIMADZU 6000 X-ray diffractometer. While PHB-diol and PCL showed crystallinities of 21% and 26% respectively, the two *di*-block poly(HB-*co*-CL)s sample B and C demonstrated a slightly decreased crystallinity of 19%.

Conclusion

Novel *di*-block co-polyesters containing PHB and PCL blocks were synthesized in high yield, for the first time, by enzymatic ring-opening polymerization of ε-caprolactone with PHB-diol. The structures of the *di*-block polymers with two different OH end groups were established by IR, ^{1}H-NMR and ^{13}C-NMR analyses. Poly(HB-*co*-CL)s with 44-74%(w/w) PCL demonstrated good thermal properties with T_g of about -60°C and T_m of 120-149°C and 50-54°C, being potentially useful thermoplastic biomaterials. Incorporation of PCL into the PHB-derived polyesters significantly improved the T_g of the materials.

Low molecular weight PHB-diol with a primary and a secondary OH end group was proven to be very useful for highly selective ring-opening polymerization, being the first example of using a telechelic macro-diol containing ester groups as an initiator. The primary OH end group of PHB-diol was found to initiate the ring-opening polymerization of ε-caprolactone, while the secondary OH end group was not reacted thus remaining as an end group of the final polymer. No enzymatic transesterification of PHB-diol and PCL happened and no random co-polymers formed during the polymerization. Optimal enzymatic polymerization conditions were established for the preparation of block co-polymers with different block ratio. This type of novel and selective ring-opening polymerization provides with new synthetic methods for preparing novel *di*-block co-polymers with functional end groups which could also be modified for other applications.

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