Alternative block polyurethanes based on poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and poly(ethylene glycol)

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ABSTRACT

A series of amphiphilic alternative block polyurethane copolymers based on poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3/4HB) and poly(ethylene glycol) (PEG) were synthesized by a coupling reaction between P3/4HB-diol and PEG-diisocyanate, with different 3HB, 4HB, PEG compositions and segment lengths. Stannous octanoate was used as catalyst. The chemical structure, alternative block arrangement, molecular weight and distribution were systematically characterized by FTIR, ^{1}H NMR, GPC and composition analysis. The thermal property was studied by DSC and TGA. Platelet adhesion study revealed that the alternative block polyurethanes possess excellent hemocompatibility. CCK-8 assay illuminated that the non-toxic block polyurethanes maintain rat aortic smooth muscle cells (RaSMCs) good viability. The *in-vitro* degradation of the copolymers in PBS buffer solution and in lipase buffer medium was investigated. Results showed that the copolymer films exhibit different degradation patterns in different media from surface erosion to diffusion bulk collapsing. The synthetic methodology for the alternative block polyurethanes provides a way to control the exact structure of the biomaterials and tailor the properties to subtle requirements.

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1. Introduction

Biomaterials were defined as materials intended to interface with biological systems to evaluate, treat, augment, or replace any tissue, organ or function of the body [1]. Biodegradable polymers are widely used as biomaterials [2]. Increasingly, biodegradable block polyurethanes have been used as blood compatible materials [3]. Polyhydroxyalkanoates (PHA) are however a group of biodegradable polyesters accumulated by many bacteria as energy-storage, when an essential nutrient is limited [4–6]. Among them, poly(3-hydroxybutyrate) (PHB) is the most common short chain-length PHA [7,8] synthesized by various bacteria grown on different types of substrates [9,10], which was most actively explored for possibly medical applications [11]. However, PHB has several inherent deficiencies that limit its medical applications, including its brittleness due to high crystallinity and narrow thermo-processing window induced from its thermal instability. On the other hand, its sister copolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3/4HB) made of 3HB and 4HB units exhibit much improved and adjustable mechanical and processing properties over PHB homopolymer, via changing the ratio of 3HB to 4HB

content. These unique characters would make P3/4HB a promising candidate for large scale application of PHA materials from soft elastomers to commodity plastics. P3/4HB is now in mass production in China with over 10K tonnes/year. This would promote the PHA from academic research to industrial applications at a real possibility.

Addition to the commodity polymer applications of P3/4HB, much of our interesting is focused on the biomedical applications of P3/4HB or PHA [12–14]. Even though the biomedical applications of PHA have been investigated for many years and much attention was paid [11], the overall results seemed not to be satisfied for the practical use, compared with its chemosynthetic competitors such as poly(lactic acid) (PLA). The possible reason is due to the drawbacks mentioned above, the difficulties for the molecular weight and structural control from the fermentation methodology. Another reason is the lack of good enough biocompatibility and balanced mechanical properties of neat PHA. Under these circumstances, our research is to modify the properties, especially biocompatibility of the PHA to meet the needs of biomedical applications. One of our strategies is the synthesis of block copolymers based on PHA, with the emphasis on the block polyurethane copolymers. A series of block polyurethanes, triblock and star block copolymers have been prepared in our laboratory to explore the suitable biocompatibility and other properties [12,13,15,16] in order to achieve practical biomaterials. Among

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them, block polyurethane copolymers based on hydrophobic PHA with or without hydrophilic segment such as polyethylene glycol (PEG) [17–20] were prepared for their aggregation, cytotoxicity, thermal properties and biocompatibility.

However, almost all of these polyurethane block copolymers were achieved via the coupling reaction of terminal hydroxyl group of PHA-diols with or without PEG-diols by using diisocyanates as coupling agents [12,13,15]. Even though this method would provide the materials with improved properties, this approach actually lacks the block selectivity and provides the copolymers with blocks connected at a random manner. Thus this can only control the structure of the block polyurethane copolymers in a rough way, not able to construct the architecture of block copolymers at an accurate or alternative arrangement. The properties were not able to be finely toned. The alternative block polyurethane copolymers were based on the selective arrangement of the blocks, such as PHA and PEG blocks, which possess determined chemical structure as well as regular microstructure. The regular structure would endow the materials more predetermined properties. The alternative arrangement of the blocks would thus give us the capability for more sophisticated applications such as tailor-made biomaterials, molecular assembly, structural property relationship elucidation and so on. Even there were quite a few reports on the random block polyurethane copolymers based on PHA, however, a brief survey of past and present articles would enable us to find out that there was no scientific papers dealing with PHA-based alternative block polyurethanes up to now. In the motivation of achieving PHA block polyurethane copolymers with specifically designed structure, we initiated the preparation of PHA-based alternative block polyurethane copolymers. Our first attempt is the preparation via the terminal coupling reaction of hydroxyl group of P3/4HB-diols with isocyanate group of PEG-diisocyanates. This paper is to report the preparation, characterization and hemocompatibility of the alternative block polyurethane copolymers (abbreviation: P3/4HB-*alt*-PEG) based on P3/4HB and PEG. The non-platelet adhesion and good aortic smooth muscle cells viability on the materials would make it potential blood anticoagulation biomaterials.

2. Experimental procedure

2.1. Materials

Bacterial poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3/4HB) was synthesized by bacterial fermentation and provided by Tianjin Green Bio-Science Co. Ltd. (Tianjin, China). Two kinds of P3/4HB, i.e. P(3HB-*co*-20%-4HB) with 20% 4HB content and P(3HB-*co*-40%-4HB) with 40% 4HB content were utilized for this alternative block polyurethane preparation. Poly(ethylene glycol) (PEG, Sigma-Aldrich), 1,6-hexamethylene diisocyanate (HDI, Alfa-Aesar), stannous octanoate (Alfa-Aesar) were used as received. Toluene-*p*-sulfonic acid, 1,2-dichloromethane, diethyl ether, chloroform, ethylene glycol, methanol were all AR grade, purchased from YongDa Reagent Development Center (Tianjin, China) and used as received. Solvent 1,2-dichloroethane was distilled over calcium hydride and dried by 4 Å molecular sieves before use.

2.2. Synthesis of the P3/4HB-alt-PEG block copolymers

2.2.1. Preparation of prepolymer P3/4HB-diol

A method of PHA alcoholysis was employed for the P3/4HB-diol preparation as reported previously [21,22]. Briefly, P3/4HB 15 g was dissolved and refluxed in 150 mL chloroform at 75 °C in a 500 mL two-neck flash to which 5 g anhydrous toluene-*p*-sulfonic acid and 50 mL ethylene glycol were added dropwise. When P3/4HB was degraded to the desired molecular weight, the reaction solution was cooled to room temperature and precipitated into stirred distilled water (at ten times excess). The product was separated out by filtration under reduced pressure. After being washed by distilled water several times, the obtained product was dried under vacuum at 60 °C to constant weight. The average yield was about 80%.

2.2.2. Preparation of prepolymer PEG-diisocyanate

Diisocyanate terminated PEG was prepared according to a modified procedure. Amount 0.0010 mol PEG was dissolved in 10 mL 1,2-dichloroethane, then transferred to a 25 mL isobaric drop funnel, this solution was added dropwise to a 100 mL

4-neck flash in which a solvent of 5 mL 1,2-dichloroethane was placed in advance, and a slight excess diisocyanate HDI (0.44 g, 0.0026 mol), catalyst stannous octanoate (0.05 g) were injected sequentially. The reaction was carried out at 50 °C for 15 hours under a nitrogen atmosphere. The excess HDI was removed through azeotropic distillation using a short partial distillation apparatus operating at 50 °C and 1×10^{-2} mbar pressure [23]. At last only 2 mL of 1,2-dichloroethane solution was left in the flask. This PEG-diisocyanate 1,2-dichloroethane solution was kept in the flask and used for next-step reaction directly.

2.2.3. Synthesis of the P3/4HB-alt-PEG block copolymers

P3/4HB-*alt*-PEG block copolymers were synthesized via the coupling reaction of terminal hydroxyl group of PHA-diol and terminal isocyanate group of PEG-diisocyanate at equal molar ratio. Amount 0.0010 mol PHA-diol was dried by dissolution in 20 mL 1,2-dichloroethane and removed the water by azeotropic distillation, 10 mL solvent was removed. The remainder was transferred to an isobaric drop funnel. The reaction was started when the PHA-diol solution was added dropwise to the above mentioned ready-prepared PEG-diisocyanate solution in the 4-neck flask under a nitrogen atmosphere at 75 °C. After 72 h reaction, the flask was cooled to room temperature. The production was precipitated in diethyl ether, filtered, then redissolved in 1,2-dichloroethane, filtered to move the trace amount of insoluble by-product. In order to eliminate the stannous octanoate residue and the possible low molecular weight oligomers, the filtrate was again precipitated in a mixture of methanol and diethyl ether (1/20, v/v). Product was collected through filtration, washed by distilled water three times followed by drying under vacuum to constant weight at 60 °C. The yield was about 70%.

2.2.4. Preparation of alternative block polyurethane copolymer film

The film was prepared by a solvent-casting method. Typically, 5 wt% polymer solution in chloroform was filtered, placed in an Petri dish to draw off solvent naturally for two days. Then the film was dried under vacuum to constant weight at room temperature.

2.3. Materials characterizations

2.3.1. Infrared spectroscopy

Fourier transform infrared spectra (FTIR) were measured by Nicolet IR 200 (Thermo Electron, USA) spectrophotometer. Polymer sample 3 mg was dissolved in 1 mL chloroform, coated on a KBr pellet and dried before measurement. All samples were carried out with 64 scans at a resolution of 1 cm^{-1} at room temperature.

2.3.2. NMR spectroscopy

^{1}H NMR spectra were recorded at room temperature in D-chloroform (CDCl$_3$) at a concentration of 20 mg/mL on a Bruker AV 400 NMR spectrometer to determine the chemical structure and to estimate molecular weight of the polymer. Tetramethylsilane was used as the internal standard.

2.3.3. Molecular weight and distribution

Molecular weights and the distribution were estimated by a Waters 1525 gel permeation chromatography (GPC), calibrated by polystyrene standards of 1.22×10^3, 2.85×10^3, 1.35×10^4, 2.96×10^4, 1.97×10^5 and 5.58×10^5 g/mol number-average molecular weights, using chloroform as eluent at a flow rate of 1.0 mL/min. A polymer sample concentration of 2 mg/mL and an injection volume of 50 μL were used.

2.3.4. Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetry analysis (TGA) were performed on TA Instruments Q100 and Q50 respectively under nitrogen atmospheres. The DSC analysis was as following: a sample of 2 mg in an aluminum pan was cooled from room temperature to −60 °C by an auto cool accessory, the pan was heated from −60 °C to 180 °C at a 10 °C/min rate, isothermally maintained at 180 °C for 3 min, quenched to −60 °C, and reheated from −60 °C to 180 °C at 10 °C/min under a nitrogen flow rate of 50 mL/min. Data were collected during the second heating run. The glass-transition temperature (T_g) was taken as the midpoint of the heat capacity change. Melting point (T_m) was taken as the summit of melting peak and melting enthalpy (ΔH_m) was calculated from the area of the endothermic peak. Samples for TGA were heated at a heating rate of 10 °C/min from room temperature to 500 °C.

2.4. Platelet adhesion and cell viability

2.4.1. Platelet adhesion

Platelet adhesion experiments were carried out to study the blood compatibility of the synthesized alternative block polyurethanes [24]. Whole rabbit blood mixed with 3.8% sodium citrate solution (ratio: 9/1, v/v) was centrifuged at 1500 rpm for 10 min at 4 °C to obtain platelet-rich plasma (PRP), which was used in platelet adhesion test. The polymer films in the glass dish were sterilized with 75% ethanol, washed three times with PBS and equilibrated in PBS overnight. After being warmed to 37 °C, 1 mL PRP was added to the films, and films were then incubated at 37 °C for 1 h. The platelet-attached films were

washed three times by PBS and immersed in PBS containing 2.5% glutaraldehyde (pH = 7.4) overnight for fixation. They were subsequently dehydrated in an ethanol-gradient series (from 30%, 50%, 70%, 90%, 95% to 100%) for 15 min, respectively, and were dried under vacuum. The morphologies of the platelet adhered on the polymer film surfaces were observed by SEM. Two parallel films were performed for each polymer. Four different regions were randomly counted on each film, and result was taken as the average number of adhered platelets per square meter of surface.

2.4.2. Cell cultivation

The RaSMCs (Rat aortic smooth muscle cells) used in the experiments were kindly provided by Microorganism Lab of Tsinghua University (Beijing, China). Cells were cultured in DMEM medium (Gibco, US) supplemented with 10% FBS (Min Hai Bio-engineering, China), 100 U/mL penicillin (Sigma, US) and 100 U/mL streptomycin (Sigma, US) in a CO_2 incubator (Forma 3111, US) supplied with 5% CO_2 at 37 °C. Confluent cells were digested using 0.25% trypsin–0.02% EDTA, followed by centrifugation (1000 g for 3 min) to harvest the cells. Subsequently, the single cell suspension was used for cell number calculation using haemacytometer 1.0×10^4 cells in each film coated dish, cultivation was conducted for 24 and 48 h. Then, CCK-8 assay was used for cell viability study while SEM for the morphology of cells grown on the resulting films.

2.4.3. Cell viability

A cell count kit-8 (CCK-8 Beyotime, China) was employed in this experiment to quantitatively evaluate the cell viability. After RaSMCs were inoculated on the film coated dishes for 24 and 48 h, the original medium was replaced by 500 mL 10% FBS DMEM medium contains 50 mL CCK-8. It was incubated at 37 °C for 2 h to form water dissoluble formazan. Then 100 mL of the above formazan solution were taken from each sample and added to one well of a 96-well plate, six parallel replicates were prepared. The absorbance at 450 and 630 nm (calibrated wave) was determined using a microplate reader (Multiskan MK33, Thermo electron corporation, China). DMEM containing 10% CCK-8 was used as a control.

2.4.4. Cell morphology

The surface morphology of the polymer films was observed by scanning electron microscopy (SEM). Cell-seeded films were washed three times using phosphate buffered saline (PBS; Shanhai Sangon Biological Engineering Technology & Services Co., Ltd, China), then immersed in PBS with 2.5% glutaraldehyde (Alfa-Aesar, USA) overnight at 4 °C, followed by dehydration in ethanol gradient (from 30, 50, 70, 90, 95 to 100%) for 15 min each. The dehydrated cell-seeded films were dried via lyophilization and then used for the SEM observation.

2.5. Degradation test

Selected polymer films were submerged in aqueous buffer solutions (pH = 7.4) at 37 °C. The polymer films (∼10 mg, 5 mm diameter and 0.14–0.28 mm thick) were placed in small vials containing 2 mL buffer solution (pH = 7.4) under a constant temperature of 37 °C. The PBS buffer solution contained 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g of KH_2PO_4 in 1 L solution. Alternatively, 0.1 g/L lipase 100 T (Novozyme, Beijing, China, enzyme activity: 100 KLU/g) was added into another group of PBS buffer solution for studying the lipase degradation behavior. Buffer solution in small vials was renewed every week, and samples were removed periodically, washed with distilled water, dried in vacuum to a constant weight before analysis. The residual weight was calculated as: Residual weight $(\%) = 100 \times w_2/w_1$, where w_1 and w_2 the weights of films before and after degradation, respectively.

3. Results and discussion

3.1. Synthesis and characterization of the alternative block polyurethane copolymers

The preparation of the prepolymer PEG-diisocyanate was monitored through the FTIR analysis. Fig. 1 showed the O–H absorption change of PEG during different reaction time with 1,6-hexamethylene diisocyanate (HDI). It exhibited that the –OH group of PEG was consumed completely after 15 h reaction. The hydroxyl O–H absorption at 3500 cm^{-1} disappeared and switched to 3346 cm^{-1} which belongs to the urethane linkage (Fig. 1c). Such change indicated that the telechelic prepolymer PEG-diisocyanate was prepared.

The synthetic profile of the P3/4HB-based alternative block polyurethane copolymers is described in Scheme 1. The alternative block structure was achieved through the mutual coupling reaction of terminal hydroxyl group from P3/4HB-diol with
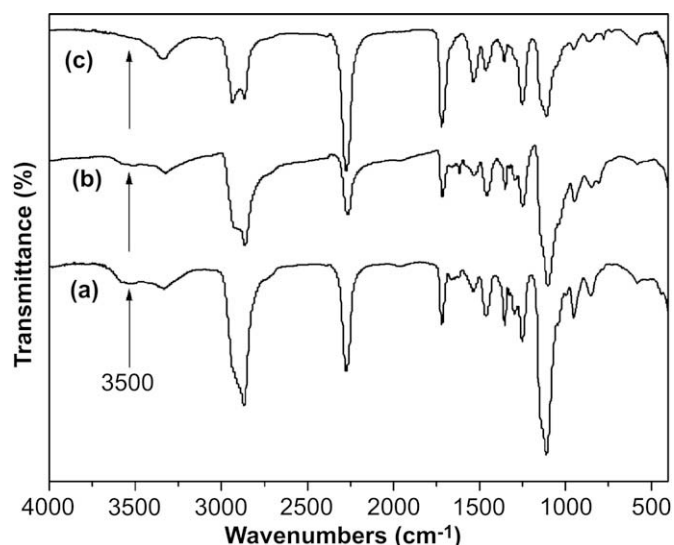

Fig. 1. FTIR spectra of PEG-2000 reacted with HDI at different time periods (a, 1 h; b, 8 h; c, 15 h).

terminal isocyanate group from PEG-diisocyanate. As either P3/4HB-diol or PEG-diisocyanate cannot react by itself, coupling reaction between P3/4HB-diol and PEG-diisocyanate is the only possible reaction in the system and the alternative block structure is the only product which can be postulated from the reaction. The alternative arrangement of P3/4HB and PEG segments should be attained. Previous investigator reported that excessive HDI (HDI:diol = 1.3:1) would lead to a cross-linked product [25], possibly due to the too much HDI used. In this study, we produced a linear soluble block copolymers via keeping the stoichiometric amount of molar ratio at 1:1 of P3/4HB-diol and PEG-diisocyanate.

The chemical structure, the molecular weight and distribution of the obtained alternative block copolymers were however further characterized by FTIR, ^{1}H NMR, GPC and composition analysis. The FTIR spectra of P3/4HB-alt-PEG, P3/4HB-diol and PEG are compared in Fig. 2. All the characteristic absorptions of P3/4HB and PEG segments can be clearly discerned in the IR spectrum of the P3/4HB-alt-PEG alternative block polyurethane copolymer (Fig. 2c). The C=O stretching band belonging to the P3/4HB segment appears at 1736 cm^{-1}, the C–H stretching vibration band and ether stretch assigned to the –OCH$_2$CH$_2$– repeating unit of PEG appear at 2883 and 1116 cm^{-1}. Compared with the P3/4HB-diol prepolymer, it was noticed that the disappearance of hydroxyl O–H absorption of 3500 cm^{-1} in the product, and two new characteristic absorptions at 3346 cm^{-1} and 1530 cm^{-1} belonging to N–H unit in urethane linkage were observed [25]. These revealed the formation of the polyurethane and the coupling reaction between the prepolymers happened.

The chemical structure of the P3/4HB-alt-PEG block polyurethane copolymers was also analyzed by ^{1}H NMR. Typically, the ^{1}H NMR spectrum and the peak assignments of sample B2-36-G-20 (Table 1) are present in Fig. 3. A strong proton signal at δ 3.65 ppm was attributed to the –OCH$_2$CH$_2$– repeating unit in PEG segment. Signals at δ 5.2 and 4.05 ppm were assigned to –OCH– in 3HB and –OCH$_2$– in 4HB units respectively. The urethane linkage in the product was also traced through ^{1}H NMR. Imino group –OOCNH– from reacted cyanate group assigned to δ 4.9 ppm, methylene next to the imino group –NH–CH$_2$– was presented at δ 3.2 ppm while another two methylene groups appeared at δ 1.45 ppm. All these
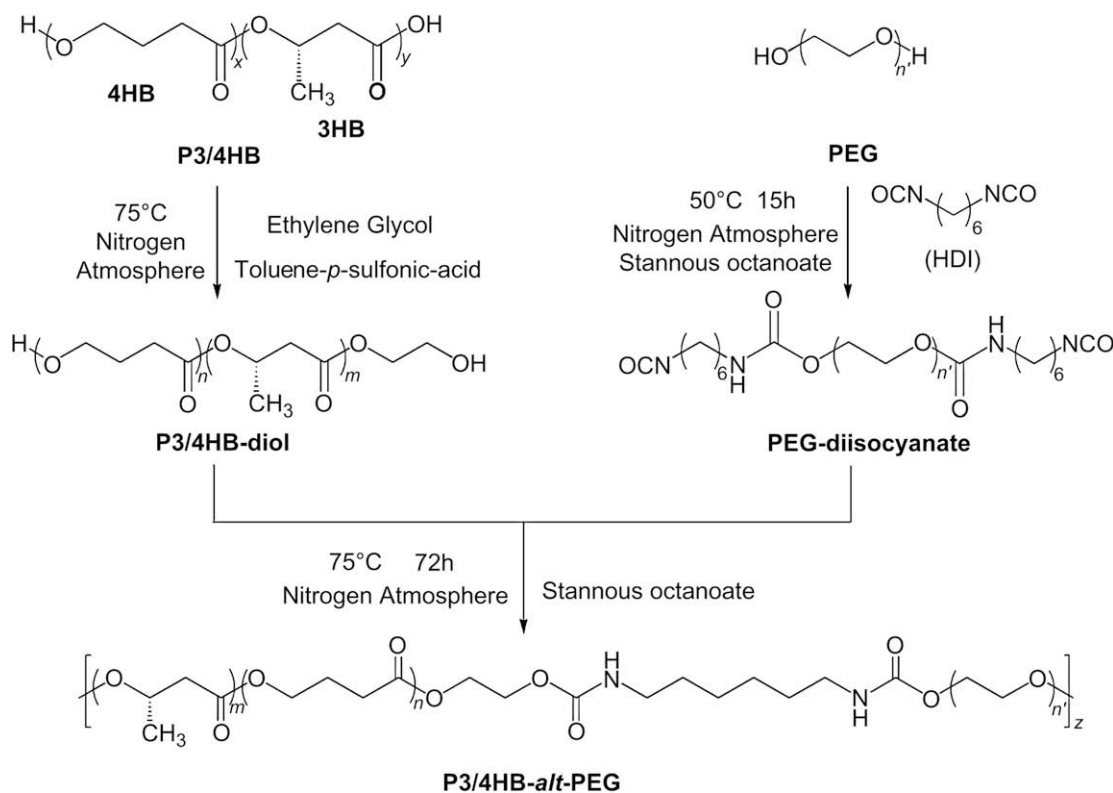
Scheme 1. Synthesis of alternative block polyurethane copolymers based on P3/4HB and PEG.

results, together with IR investigation, revealed that PEG-diisocyanate reacted with PHA-diol through the coupling reaction and the polyurethane was formed.

Comparison of the feed ratio of P3/4HB-diol and PEG-diisocyanate in the reactants and the detected out composition of the obtained product can provide evidence for the alternative block structure. The molar ratio of P3/4HB and PEG in the obtained copolymers could be calculated by the related ^{1}H NMR signal integration. The PEG molar ratios in the reactants and in the

obtained copolymers are given in Table 1. It was noted that the molar compositions before and after the reaction are almost consistent, taking account of the possible identical terminal segments at two ends. The ratio of P3/4HB and PEG segments in the resulted copolymer is almost 1/1. This indicates that the reactants P3/4HB-diol and PEG-diisocyanate reacted completely, and the one-by-one alternative block structure was formed.

The alternative structure was also proved by the weight ratio analysis of P3/4HB and PEG components. The PEG weight contents in the reactant feed and in the obtained copolymers are displayed in Table 1. Compared with the weight percentage of PEG (w') in the copolymers detected out by TGA, the weight percentage values of PEG in feed (w) and in resulted copolymers were also the same (Table 1 and Fig. 6). The ratios of PEG and P3/4HB segment in the feed and in the resulted copolymer are almost 1:1. This revealed that the P3/4HB-diol and PEG-diisocyanate were reacted one to one and the alternative block arrangement was achieved.

The molecular weight and distribution of the alternative block copolymers were analyzed by GPC. The resulted data are given in Table 1. It can be seen that the alternative block copolymers with molecular weight ranging 4.58×10^4–1.19×10^5 and polydispersity distribution of 1.31–2.01 were obtained. These molecular weight and distribution would be enough to endow the P3/4HB-*alt*-PEG copolymers appropriate mechanical and processing properties to meet the requirements of biomedical devices. Typical GPC traces for the P3/4HB-*alt*-PEG copolymer sample together with its corresponding prepolymers are shown in Fig. 4. The unimodal bell shape GPC curves demonstrate the purity of the polymers. No residue left in the copolymer sample. The shift of the retention time to lower value proved the formation of the copolymers via polymerization reaction of the prepolymers. All these results firmly confirmed that the alternative block polyurethane copolymers were synthesized successfully.
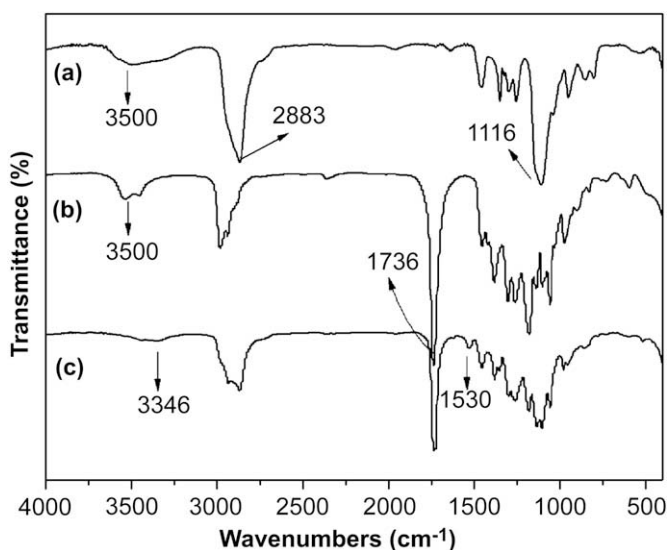
Fig. 2. FTIR spectra of (a) PEG-3400, (b) P3/4HB-diol ($M_w = 3600$) and (c) P3/4HB-*alt*-PEG block copolymers (B2-36-G-34, Table 1).

Table 1
Summary of alternative block polyurethane copolymers based on P3/4HB and PEG.

Series	Sample	R^a	R'^b	w^c	w'^d	PEG		Copolymer			M_w^i	PDI
						ΔH_f (J g^{-1})^e	X_c (%)^f	T_{d10} (°C)^g	T_m (°C)^h	T_g (°C)^h		M_w/M_n^i
B2 P(3HB-co-20%-4HB)	P(3HB-co-20%-4HB)-diol	–	–	–	–	–	–	264.60	–	−15.59	3600^k	–
	B2-36-G-20^j	1.00	1.06	34.74	33.85	21.82	10.64	272.00	32.66	−30.97	51,100	1.34
	B2-36-G-34	1.00	1.07	47.49	45.14	55.10	26.88	277.90	41.71	−39.90	48,900	1.38
	B2-36-G-46	1.00	1.13	54.97	47.27	63.75	31.10	275.30	47.88	−42.22	45,800	1.51
	B2-76-G-20	1.00	0.97	20.48	21.52	25.41	12.40	262.12	30.84	−24.16	86,000	2.01
	B2-30-G-34	1.00	0.88	51.77	56.67	46.96	22.91	270.84	42.10	−32.08	69,800	1.31
B4 P(3HB-co-40%-4HB)	P(3HB-co-40%-4HB)-diol	–	–	–	–	–	–	241.70	–	−26.92	2500^k	–
	B4-25-G-20	1.00	0.99	42.88	43.78	8.716	4.25	265.70	44.82	−35.03	91,900	1.32
	B4-25-G-34	1.00	0.86	56.06	56.31	48.48	23.65	273.00	46.81	−38.74	98,600	1.67
	B4-25-G-46	1.00	0.96	63.32	63.24	69.62	33.96	276.40	53.21	−44.26	65,500	1.69
	B4-80-G-20	1.00	1.09	19.67	19.14	22.07	10.77	263.12	32.01	−29.62	82,400	1.81
	B4-80-G-34	1.00	1.02	29.39	30.69	37.02	18.06	268.63	42.03	−33.08	119,300	1.70

[a] R: P3/4HB/PEG molar ratio in feed.
[b] R': P3/4HB/PEG molar ratio in product determined by NMR.
[c] w: the mass percentage of PEG content in feed.
[d] w': the mass percentage of PEG in product detected by TGA.
[e] Melting enthalpy determined from the DSC second heating run.
[f] Crystallinity percentage according to the following equation: $X_c\% = 100 \times \Delta H_f/w_i \cdot \Delta H_f^\circ$, ΔH_f is the melting enthalpy of PEG, and ΔH_f° is the melting point enthalpy of completely crystallized PEG with reference values of 205.0 J/g, w_i is the weight fraction of PEG in the copolymer.
[g] Decomposition temperature at 10% weight loss determined by TGA.
[h] Determined from DSC second heating run.
[i] Determined by GPC in CHCl$_3$.
[j] Sample abbreviation B2-36-G-20 means that the feeding P(3HB-co-20%-4HB)-diol segment $M_n = 3600$, 20%-4HB content; PEG segment $M_n = 2000$, and so on.
[k] Calculated from NMR integration.

3.2. Thermal property

The thermal transitions and thermal stability of the P3/4HB-alt-PEG block copolymers were evaluated by DSC and TGA respectively. The typical DSC thermograms of the P3/4HB-alt-PEG block copolymers are shown in Fig. 5 with the resulted data given in Table 1. As the P3/4HB used in this study are amorphous, only PEG is the crystal moiety. Thus, the only melting point (T_m) detected out should be corresponding to the crystallization of PEG segment with the T_m of 32.01–53.21 °C, which is in the range of body temperature and would be suitable for some biomedical applications. The percentage crystallinity (X_c) of the PEG moiety was calculated through melting enthalpy. Compared with the B4 series products from P3/4HB with 40% 4HB (Table 1), P3/4HB-alt-PEG copolymers
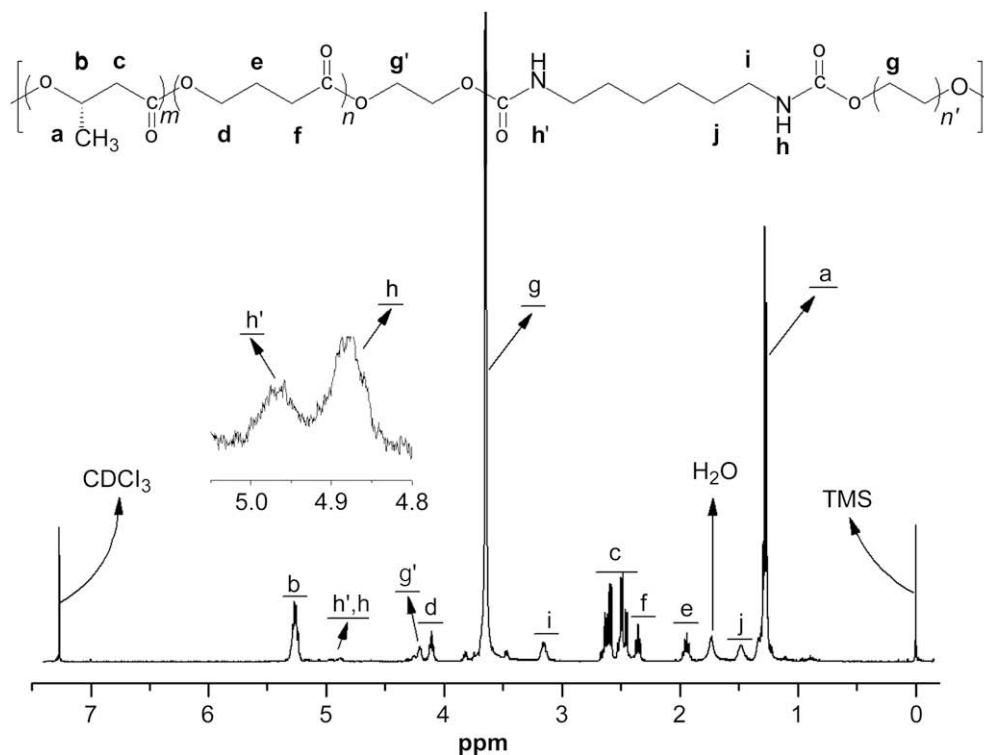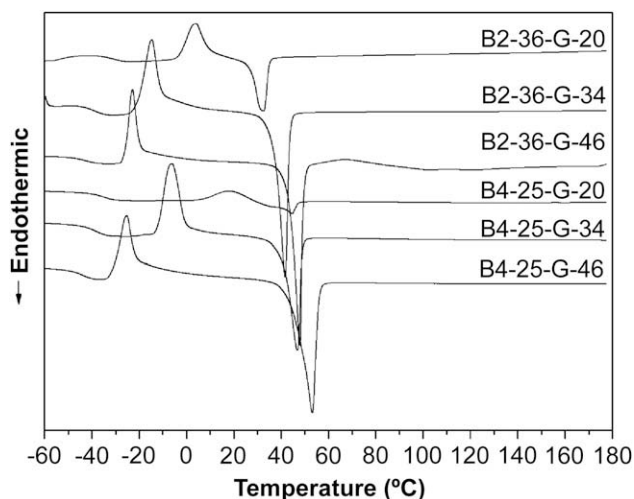

Fig. 3. ^{1}H NMR spectrum of P3/4HB-alt-PEG block copolymers (B2-36G-34, Table 1) in CDCl$_3$.
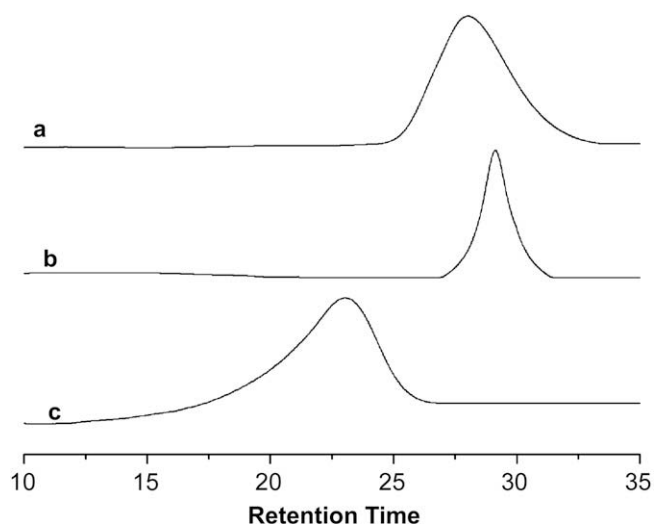
Fig. 4. GPC diagrams of P3/4HB-*alt*-PEG block copolymer and its P3/4HB-diol and PEG prepolymers: (a) P(3HB-*co*-20%-4HB)-diol ($M_w = 3600$); (b) PEG ($M_w = 3400$); (c) B2-36-G-34 ($M_w = 48,900$, Table 1).

from P3/4HB with less 4HB content (B2 series 20% 4HB) gave the higher X_c and T_g, but lower T_m. The higher crystallinity X_c in the system would hinder the mobility of the chains and thus elevate the glass-transition temperature T_g. For samples B2-36-G-46 and B4-25-G-46, although they have the same PEG segment length of molecular weight 4600, the former gave the lower X_c of 31.10% and higher T_g of −42.22 °C, compared to latter of 33.96% and −44.26 °C. This phenomenon would be due to that higher 4HB content P3/4HB in B4 series has the lower T_g value. And the PEG4600 is long enough for better crystal formation. The lower T_g means the higher chain mobility. This would increase the mobility of PEG segment and thus increase the crystallinity of PEG domain. The better crystals formed would give the higher T_m at 53.21 °C.

Results also gave that both X_c and T_m of the PEG phase increased with an increase of PEG content or shorter P3/4HB segment. This is due to the P3/4HB components and urethane linkage which would hinder the crystallization of PEG as a result of the dilution effect and depress of the PEG chain arrangement and mobility [26]. The shorter PEG segment could not form enough size crystal lamellar and hence exhibits lower T_m and lower crystallinity. The

appearance of the cold crystallization in the DSC thermograms (Fig. 5) would hint that the crystallization rate of PEG moiety was depressed. This result also revealed that the crystallization and further the permeability of the materials can be tailored through adjusting the chemical composition.

All the investigated P3/4HB-*alt*-PEG samples had only a single T_g between the T_gs of the neat components P3/4HB and PEG, and their T_g shifted to lower temperature with increasing PEG content. These results indicated a relative miscibility of P3/4HB and PEG block segments in the copolymers. The T_g inner-shift could be attributed to the enhanced mobility of P3/4HB segment with the dispersion of lower T_g (−65 °C) PEG segment into the P3/4HB domain [27]. This influence was more obvious in the samples originated from the longer-chain prepolymers than corresponding samples derived from shorter chain prepolymers. This shift in the T_g was taken as an indication for the miscibility encountered in phase mixing. The shorter the chain of two block segments, the more restrictive the chain mobility. Thus, the higher the expected T_g in the copolymers [28]. The exception of sample B2-36-G-46 possesses long PEG segment but higher T_g. The reason would be the higher crystallinity of the sample as stated above. The disappearance of the cold crystallization would also hint the higher crystallization rate in this sample.

The TGA curves of the P3/4HB-*alt*-PEG block copolymers are shown in Fig. 6 and Table 1. All the copolymer samples gave a two-

Fig. 5. DSC thermograms (2nd heat run) of P3/4HB-*alt*-PEG block polyurethanes (10 °C/min).
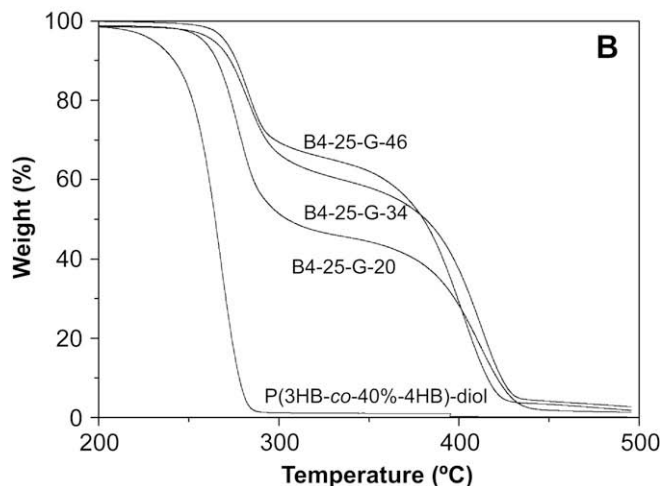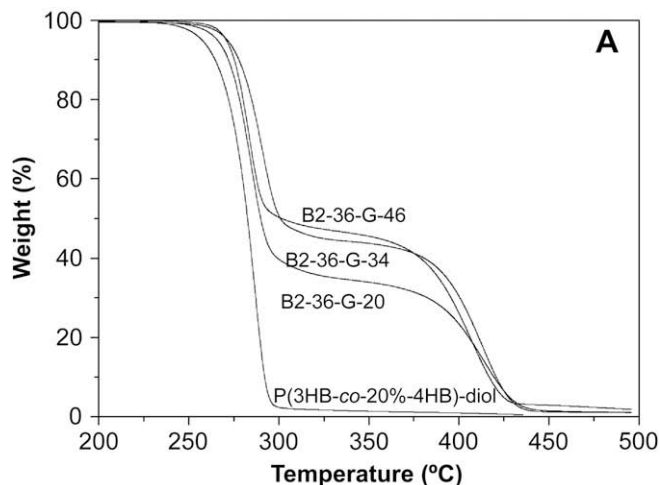
Fig. 6. TGA thermograms of P3/4HB-*alt*-PEG block polyurethanes and the P3/4HB-diol prepolymers.
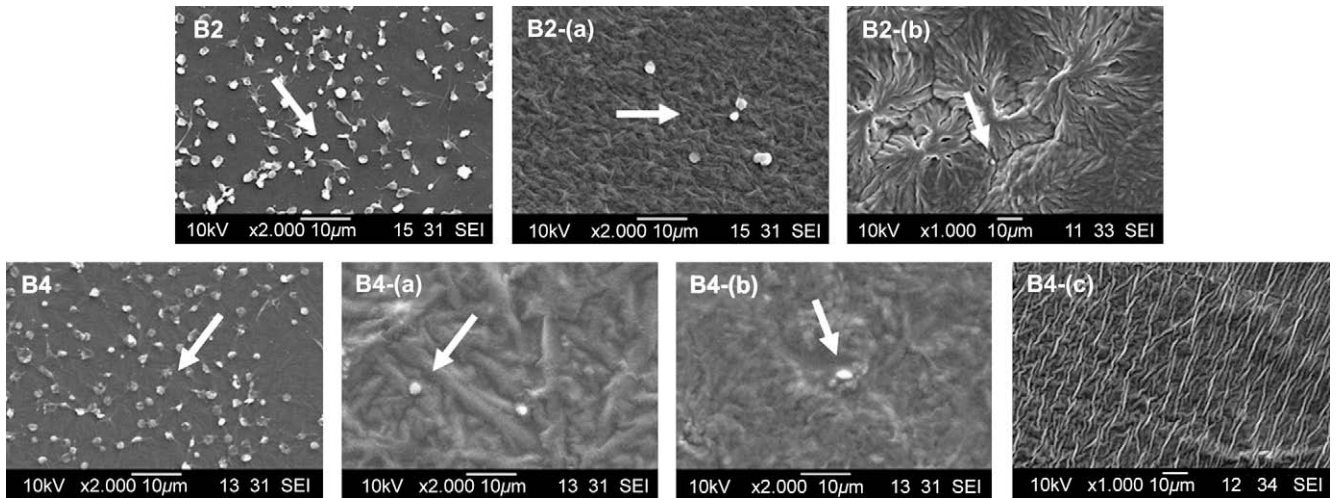
Fig. 7. The morphology of rabbit platelets (indicate by arrows) cultured on the surfaces of the control films and P3/4HB-*alt*-PEG block copolymer films: B2, P(3HB-*co*-20%-4HB); B4, P(3HB-*co*-40%-4HB); B2-(a), B2-76-G-20; B2-(b), B2-36-G-20; B4-(a), B4-80-G-20; B4-(b), B4-80-G-34; B4-(c), B4-25-G-20.

stage thermal decomposition profile. The first stage of weight loss accounts for PHA decomposition and the second stage for PEG, as PEG possesses obvious higher thermal stability. This phenomenon was also used to check the weight composition of the copolymers as stated above. Compared with the thermal property of the pre-polymers, it was noted that the P3/4HB-*alt*-PEG copolymers possess 10–20 °C higher decomposition temperature than that of the P3/4HB-diols. This may be due to the PEG segment and urethane linkage in the backbone, which would enhance the thermal stability of the P3/4HB segmental block. Also, this would widen the processing window for P3/4HB materials.

3.3. Platelet adhesion and cell viability

Platelet adhesion on the P3/4HB-*alt*-PEG block copolymer films was examined to characterize the hemocompatibility of the materials. SEM was used to observe the platelet number and morphology attached on the tested copolymer film surfaces (Fig. 7). As a control, the number of rabbit platelets adhered on neat P(3HB-*co*-20%-4HB) and P(3HB-*co*-40%-4HB) films was determined and the values were $(49.3 \pm 1.6) \times 10^5/m^2$ and $(44.2 \pm 5.6) \times 10^5/m^2$, respectively. The platelets adhering on the control films showed out their

pseudopods (Fig. 7, B2, B4), indicating that the platelets were activated so as to form thrombus and cause coagulation of blood. However, for the P3/4HB-*alt*-PEG polyurethane copolymer films, platelets were barely adhered on the surfaces. Very few even no
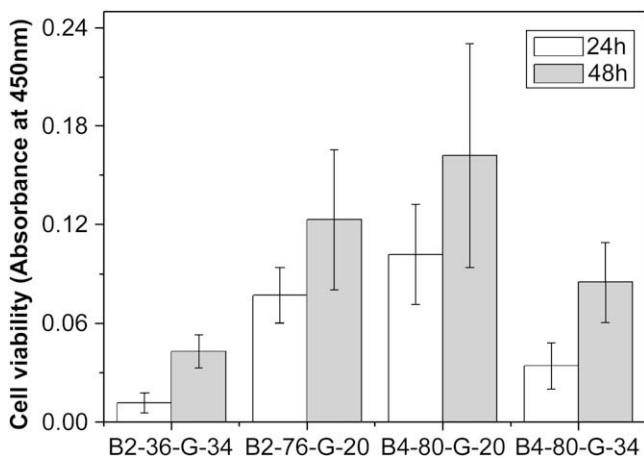

Fig. 8. CCK-8 assays of RaSMCs' proliferation on tested films after 24 h and 48 h of cultivation.
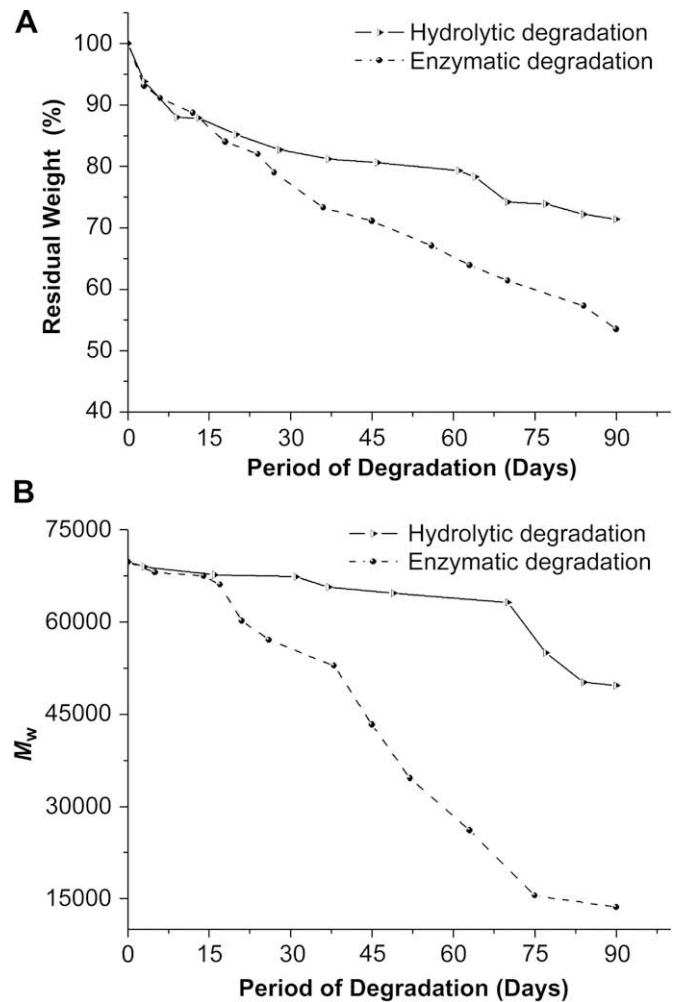
Fig. 9. (A) Residual weight and (B) molecular weight change of the P3/4HB-*alt*-PEG polyurethane copolymer films (B2-30-G-34) during *in-vitro* degradation.
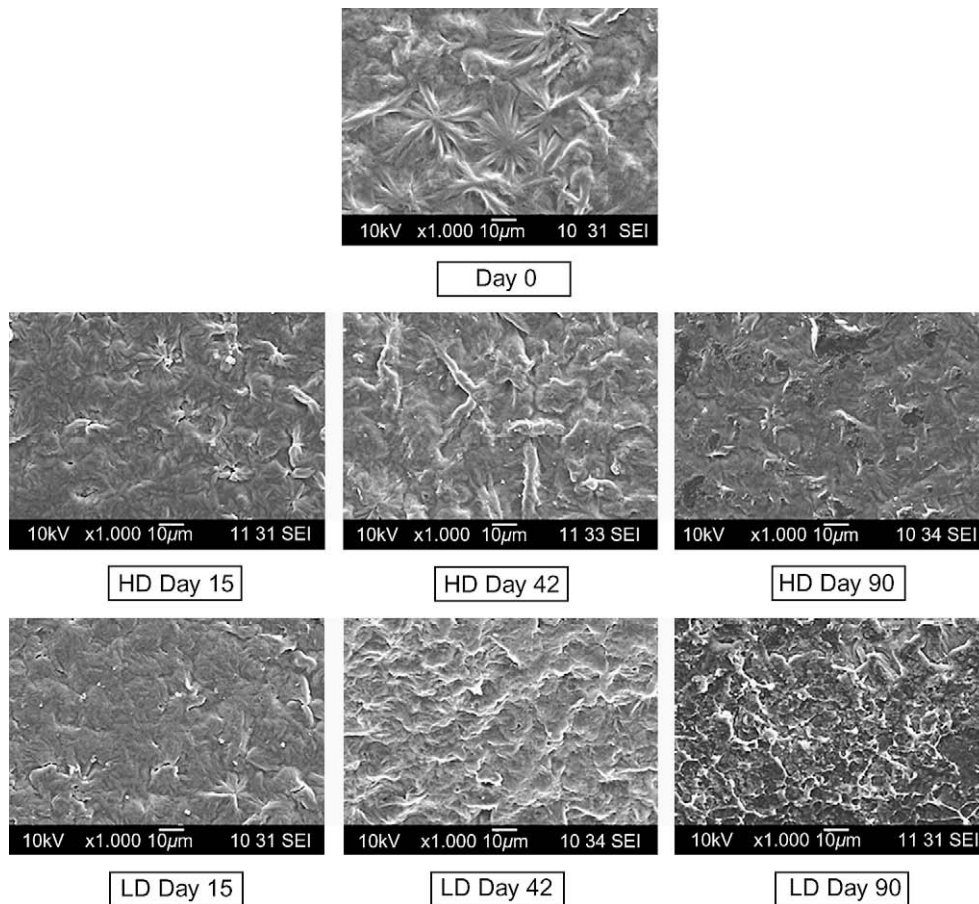
Fig. 10. The surface morphology of sample B2-30-G-34 copolymer film at different periods of degradation (HD, hydrolytic degradation; LD, lipase degradation).

platelets were found on the P3/4HB-*alt*-PEG copolymer film surfaces and no pseudopod was observed, indicating that the platelets were not activated and could not cause the blood coagulation. The platelet adhesion density was from 0 to $(2.2 \pm 0.02) \times 10^5/m^2$. This result proved that the obtained P3/4HB and PEG-based alternative block polyurethanes P3/4HB-*alt*-PEG possess excellent hemocompatibility. This hemocompatibility is even much better than that of our previously synthesized P3/4HB and PEG random block polyurethane counterparts [12]. Compared with the PEG content in Table 1 and the platelet adhesion in Fig. 7, it was noted that the higher the PEG content in the P3/4HB-*alt*-PEG copolymer gave the lower platelet adhesion. There are many factors to affect the hemocompatibility. For these P3/4HB-*alt*-PEG copolymers, the good hemocompatibility may be due to their improved hydrophilicity, flexibility of PEG chains and the surface patterned microstructures [29]. Our attempt to determine the contact angle of the copolymer films was failed due to the encountered rapid water absorption of the films. According to Nagaoka [30], PEG segments can form PEG-hydration with water, the quick movement of PEG-hydration influences the hydrodynamic properties of the blood-materials micro-domains so as to prevent protein conglutination and denaturation on materials surface. This demonstrates that these P3/4HB-*alt*-PEG block copolymers possess excellent blood compatibility due to the PEG block introducing and the alternative structure. Our investigation found that even the higher PEG content could give the copolymers higher hemocompatibility, however this would reduce the film forming ability and deleterious mechanical property of the copolymers (data not given) at the same time.

The growth and proliferation of the rat aortic smooth muscle cells (RaSMCs), a cell grown on the inner surface of blood vessels, on the P3/4HB-*alt*-PEG copolymer films were investigated to characterize the cell compatibility of the materials. The results are given in Fig. 8. No significant absorbance or cell viability difference was observed after 24 h incubation compared with the previous experiments [31] based on PHB, PLA, P(3HB-*co*-20%-4HB) and
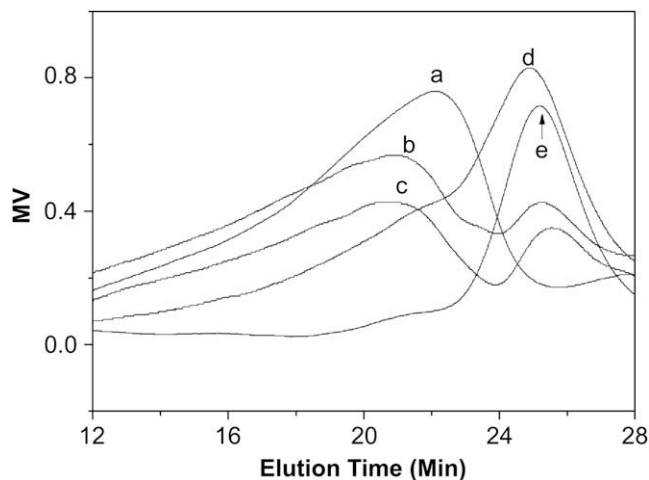

Fig. 11. GPC profiles of sample B2-30-G-34 copolymer films after different periods of enzymatic degradation: a) 0 days; b) 7days; c) 42 days; d) 70 days; e) 90 days.

P(3HB-*co*-40%-4HB). However, the RaSMCs began to show remarkable growth and proliferation after 48 h incubation on different P3/4HB-*alt*-PEG block copolymers films, based on the CCK-8 assay. This demonstrated that the P3/4HB-*alt*-PEG polyurethanes possess the ability to maintain the cell viability and growth, which also indicated the non-toxicity of the P3/4HB-*alt*-PEG polyurethanes to RaSMCs. Meanwhile, B2-36-G-34 showed the less cell viability among the tested materials and cells grew the strongest on B4-80-G-20 in all time period. Interestingly, cell viability was observed to decrease with increasing PEG content in the P3/4HB-*alt*-PEG block copolymers. Although PEG content of B4-80-G-20 is similar to B2-76-G-20, CCK-8 assay showed that cells grown on B4-80-G-20 were higher compared with B2-76-G-20, which might hint that the 4HB component had an ability to stimulate elastin in RaSMCs steadily [31]. From above discussion, it can be seen that the P3/4HB-*alt*-PEG polyurethane copolymers possess excellent hemocompatibility and very good RaSMCs viability, suggesting the materials are good candidate for blood anticoagulation applications such as vascular grafts. Our on-going work is to adjust the materials via tailoring the compositions to balance the properties of satisfied biocompatibility, mechanical property, processing ability and device fabrication.

3.4. In-vitro degradation

Degradation of the P3/4HB-*alt*-PEG copolymers was carried out using the solution-cast films submerged in 0.01 M phosphate buffer solution (pH = 7.4) at 37 °C, the buffer contained 0.1 g/L lipase 100 T, respectively. The residual weight, molecular weight and morphology changes of the selected copolymer sample (B2-36-G-34) before and after degradation were studied (Figs. 9–12). As expected, the P3/4HB-*alt*-PEG copolymer displayed a much faster degradation rate than neat P3/4HB, because the introduction of PEG block endows the materials higher hydrophilicity. Compared with hydrolysis and lipase degradation, the latter showed a similar degradation rate with that of the hydrolysis at the initial 15 days degradation, however much faster degradation rate was observed after 15 days degradation (Fig. 9). This phenomenon was also observed in our previous investigation [16]. Fig. 10 shows the surface morphology of the polymer film at

the different stages of degradation. At the initial 15 days, the surface texture of lipase degradation sample does not make much difference from the hydrolytic sample. After 15 days, surface change of the enzymatic degradation process is becoming more and more manifest on tested film. This result, along with the decreasing residual weight and molecular weight of the films indicates that the degradation process of the P3/4HB-*alt*-PEG copolymer was accelerated by subjecting the degradation under lipase conditions, as the lipase could accelerate the degradation of the ester bond of P3/4HB. The abrupt molecular weight reduction with the rapid residual weight decrease hints that the P3/4HB-*alt*-PEG copolymers degradation was diffusion-controlled pattern under lipase degradation, after a 15 days induction period. The hydrolytic degradation however exhibits almost slow and linear decrease of both residual weight and molecular weight. This indicates that the hydrolysis would be the surface erosion pattern.

The GPC curve of the degraded residual film (Fig. 11) revealed a change of unimodal to bimodal molecular weight distributions of the copolymers during enzymatic degradation, with a new-born lower molecular weight component. With the degradation proceeding, almost all of the residual samples degraded into lower molecular weight component (Fig. 11d,e). This also supports that the degradation under lipase is bulk diffusion-controlled. The TGA analysis of the degraded residue showed that with degradation, the left residue contains more and more P3/4HB component, as the PEG-riched fragments are more liable to dissolve out in the buffer medium (Fig. 12). The surface morphology of the degraded copolymer films was given in Fig. 10. Compared with the hydrolysis, the enzymatic degradation surface became rougher, and highly porous surface was observed with 90 days degradation. As stated above, the hydrolysis gives the lower degradation and surface erosion pattern, thus tends to keep the shape intact of the degraded articles. Even the erosion of original surface patterned texture was still observed up to 42 days degradation. The enzymatic degradation however exhibits a much higher degradation rate and a bulk diffusion-controlled pattern. This would keep the shape of the degraded articles for a certain period and then the shape of the articles bankrupts rapidly, when the bulk degrades to a certain degree.
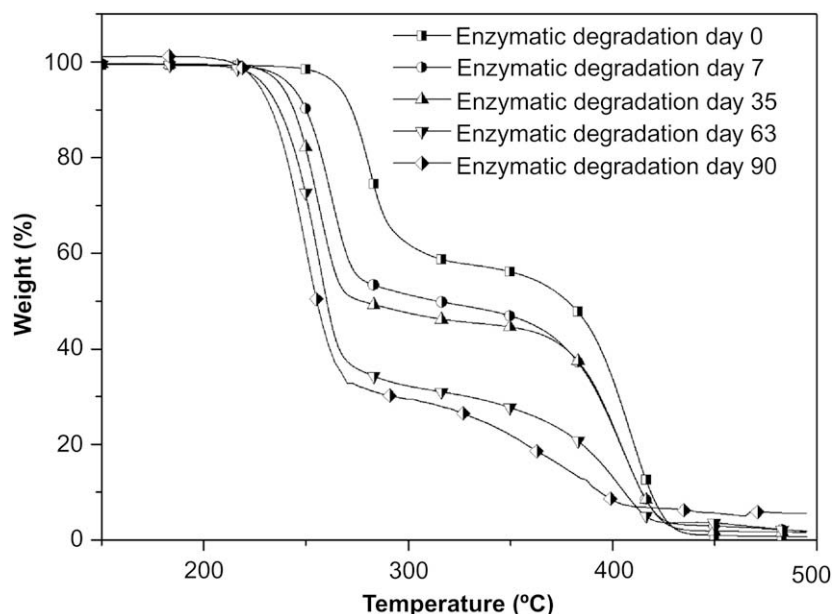

Fig. 12. TGA thermograms of sample B2-30-G-34 copolymer film after different periods of enzymatic degradation.

4. Conclusions

Amphiphilic and alternative block polyurethanes P3/4HB-*alt*-PEG based on polyhydroxyalkanoates P3/4HB and PEG were synthesized by coupling reaction of the terminal hydroxyl and isocyanate groups. These polyurethanes have a sufficiently high molecular weight, a narrow molecular weight distribution and appropriate crystallinity to allow films and coatings to be made. The P3/4HB-*alt*-PEG block polyurethanes possess excellent hemocompatibility and different *in-vitro* degradation patterns. The CCK-8 assay showed that the materials are non-toxic and maintain good RaSMCs viability. The alternative block architecture and the synthetic methodology would enable us to easily realize the exact control of chemical structure and properties of the biomaterials, and achieve the practical and reproducible structure–property relationship.

Acknowledgments

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