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Article

Enzymatic Synthesis and Characterization of Thermosensitive Polyester with Pendent Ketoprofen

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Abstract: Three linear polyesters with pendant ketoprofen were synthesized by copolymerization of polyethylene glycol (PEG) with malic acid (thiomalic acid or aspartic acid) using lipase B acrylic resin from *Candida antarctica* (CAL-B) at 90 °C respectively. These thermosensitive polyesters exhibit a lower critical solution temperature (LCST) at 10–12 °C. The *in vitro* study demonstrated that these polyesters could release ketoprofen in neutral and alkaline medium but showed hydrolytic stability in acid medium. These results suggest that, with pendant drugs, these thermosensitive polyesters have potential applications in biomedical materials.

Keywords: enzymatic synthesis; CAL-B; thermosensitive polyester; ketoprofen; PEG

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1. Introduction

In the past decade, the stimulus-sensitive polymers have attracted rapidly growing interest based on their specific properties that can change dramatically at a critical point in response to external signals. Thermosensitive polymers are one type of stimulus-responsive polymers that have potential applications in the biomedical and pharmaceutical fields [1–4]. Among the thermosensitive polymer systems, polyethylene glycol (PEG) is a common choice as the hydrophilic block of copolymers due to its biocompatibility and resistance to both protein adsorption and cellular adhesion. Thus PEG-based amphiphilic block copolymers can potentially be used in the biomedical field, specifically in the area

of drug delivery [5,6]. Various strategies have been reported in the literature utilizing PEG to generate amphiphilic block copolymers. However, most of these strategies often involve organometallic catalysts, requiring additional processing to remove the trace amounts of catalyst.

Enzymatic catalysis has emerged as an attractive "green chemistry" alternative to conventional chemical catalysis. The enzyme-catalyzed method has now been applied to polymer synthesis and provides a new strategy for producing various polymers. Enzymatic polymerizations proceed with high efficiency and selectivity, leading to perfectly structure-regulated polymers in most cases [7–9]. Moreover, the environmental friendly enzymatic reaction takes place under relatively mild conditions without the use of toxic reagents [10–12]. There are several excellent reviews on this topic [13–17]. More recently, Puskas *et al.* reviewed precision synthesis of novel multifunctional poly(ethylene glycol) using enzymatic catalysis [12].

As a part of our continuous efforts toward the development of biocatalytic synthesis of polyesters with pendant ketoprofen, we have reported thermosensitive polyester based on 2-ketoprofen dimethyl malate and PEG [18] and linear polyesters based on ketoprofen glycerol ester, PEG with divinyl sebacate [19]. In the present study, we aimed to develop thermosensitive polyester as the carrier of ketoprofen bearing different linkage. The model drug ketoprofen was conjugated to malic acid (thiomalic acid or aspartic acid) respectively. Then three thermosensitive polyesters were synthesized based on PEG and malic acid (thiomalic acid or aspartic acid), catalyzed by lipase from *Candida antarctica* (CAL-B) under solvent-free conditions. The polyesters consist of hydrophilic PEG segments and hydrophobic drug segments, which exhibit a lower critical solution temperature (LCST) at 10–12 °C. The *in vitro* ketoprofen release behaviors of polyesters were investigated under three different pH conditions (pH 1.2, 7.4 and 10). These polyesters showed hydrolytic stability in acid environment while the release rate reached a maximum in alkaline environment. These results suggest that the thermosensitive polyesters with pendent ketoprofen could be used as promising prodrugs.

2. Experimental Section

2.1. Materials

Lipase B from *Candida antarctica* immobilized on acrylic resin (CAL-B) and PEG 800 were purchased from Sigma-Aldrich. Ketoprofen was obtained from Wuhan Gang Zheng Biology Technology Co. Ltd. (Wuhan, China). Chloroacylated ketoprofen was synthesized according to the literature [20]. L-malic acid, thiomalic acid and L-aspartic acid were obtained from Shanghai Jingchun Chemical Reagent Co. Ltd. (Shanghai, China). Malic acid dimethyl ester and thiomalic acid dimethyl ester were prepared by usual condensation method and purified by distillation under reduced pressures. Aspartic acid dimethyl ester was synthesized according to the literature [21]. All other chemicals used in this work were of analytical grade and were first dried over 3 Å molecular sieves for 24 h prior to use.

2.2. Characterization

Proton nuclear magnetic resonance (^{1}H-NMR) and carbon NMR (^{13}C-NMR) spectra were obtained on a Bruker DMX 400 (Bruker, Rheinstetten, Germany). Spectra were run in deuterated chloroform ($CDCl_3$) and referenced to an internal tetramethylsilane (TMS) standard. High resolution mass

spectrometer (HRMS) data were determined on a Bruker Daltonics Bio TOF (Bruker Daltonics, Bremen, Germany). The quantitative analysis of samples was carried out by high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) on a reverse phase column (Welchrom-C18, 5 μm, 4.6 mm × 150 mm, Welch, MD, USA) using Shimadzu LC-2010AHT equipped with a UV detector at 254 nm. For the analysis of ketoprofen, methanol/water 70:30 (*v*/*v*) was used as eluent (flow rate, 0.8 mL/min). Gel permeation chromatography (GPC, Waters, Massachusetts, USA) was performed with a Waters 515 HPLC pump and a Waters 2410 refractive index detector (RID). Tetrahydrofuran (THF) was used as an eluent with a flow rate of 0.35 mL/min^{-1}.

2.3. Synthesis of 2-Ketoprofen Malic Acid (Thiomalic Acid or Aspartic Acid) Dimethyl Ester

Malic acid (thiomalic acid or aspartic acid) dimethyl ester (17 mmol) was dissolved in dichloromethane (50 mL) and placed in a round bottom flask. Triethylamine (1.7 g, 17 mmol) was added, followed by the dropwise addition of chloroacylated ketoprofen (4.6 g, 17 mmol) at room temperature. The reaction mixture was stirred for 10 h. The solvent was removed under vacuum and the product was isolated by silica gel column chromatography with an eluent consisting of petroleum ether/ethyl acetate (3/1, *v*/*v*). All reactions were detected by thin layer chromatography plates using petroleum ether/ethyl acetate (3/1, *v*/*v*) as eluent.

2-Ketoprofen malic acid dimethyl ester (**2a**): Yellow liquid (yield: 73%); ^{1}H-NMR ($CDCl_3$, ppm): 7.82–7.43 (m, 9H, Ar–H), 5.51 (m, 1H, –CH), 3.88 (m, 1H, –CH), 3.75–3.56 (m, 6H, –OCH_3), 2.86 (m, 2H, –CH_2), 1.55 (d, J = 7.2 Hz, 3H, –CH_3). ^{13}C-NMR ($CDCl_3$, ppm): 196.5 (CO–Ar), 172.9 (CHCO), 169.4 (–COO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 68.9 (–OCH), 52.6 (–OCH_3), 45.0 (–CH), 35.8 (–CH_2), 18.3 (–CH_3). HRMS (*m*/*z*) calculated for $[M + H]^+$ 399.1444, found 399.1422.

2-Ketoprofen thiomalic acid dimethyl ester (**2b**): Yellow liquid (yield: 91.2%) ^{1}H-NMR ($CDCl_3$, ppm): 7.82–7.43 (m, 9H, Ar–H), 4.52 (m, 1H, –CH), 3.98 (m, 1H, –CH), 3.73–3.65 (m, 6H, –OCH_3), 2.98–2.81 (m, 2H, –CH_2), 1.58 (d, J = 7.2 Hz, 3H, –CH_3). ^{13}C-NMR ($CDCl_3$, ppm): 198.0 (–COS), 196.0 (CO–Ar), 170.1 (CHCO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 53.6 (–CH), 52.9, 51.9 (–OCH_3), 40.9 (–SCH), 36.4 (–CH_2), 18.3 (–CH_3). HRMS (*m*/*z*) calculated for $[M + Na]^+$ 437.1035, found 437.1028. For $[M + H]^+$ 415.1215, found 415.1208.

2-Ketoprofen aspartic acid dimethyl ester (**2c**): Colorless liquid (yield: 72.4%) ^{1}H-NMR ($CDCl_3$, ppm): 7.82–7.43 (m, 9H, Ar–H), 6.59 (m, 1H, –NH), 4.83 (m, 1H, –NCH), 3.73–3.56 (m, 7H, –OCH_3, –CH), 3.05–2.78 (m, 2H, –CH_2), 1.55 (d, J = 7.2 Hz, 3H, –CH_3). ^{13}C-NMR ($CDCl_3$, ppm): 196.3 (CO–Ar), 173.3 (–CONH), 171.2 (CHCO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 52.6, 51.8 (–OCH_3), 48.5 (–NCH), 46.4 (–CH), 35.7 (–CH_2), 18.3 (–CH_3). HRMS (*m*/*z*) calculated for $[M + H]^+$ 398.1604, found 398.1587.

2.4. Copolymerization of 2-Ketoprofen Malic Acid (Thiomalic Acid or Aspartic Acid) Dimethyl Ester with Peg

The reactants were dried via vacuum prior to use. **2a** (**2b** or **2c**) (5 mmol) and PEG 800 (4 g, 5 mmol) were placed in a round bottom flask and the temperature was raised to 90 °C to homogenize the reaction mixture. Lipase CAL-B (10% *w*/*w* of monomers) was added to the mixture and the reaction

was allowed to proceed with continuous stirring under 350 mbar vacuum. The reaction was quenched by adding dichloromethane and removing the enzyme by filtration. The filtrate was dissolved in methanol and reprecipitated in the hexane for purification. The purified product was dried *in vacuo* for 24 h to obtain a highly viscous liquid of polyester. Characterization of the products was performed using NMR ($CDCl_3$) (see supplementary information, Figures S1–S6) and GPC measurements.

2-Ketoprofen malic acid PEG polyester (**3a**): ^{1}H-NMR ($CDCl_3$, ppm): 7.82–7.43 (m, 9H, Ar–H), 5.51 (m, 1H, –CH), 4.20–4.32 (m, 4H, $COOCH_2$), 3.88 (m, 1H, –CH), 3.64 (brs, 72H, OCH_2CH_2O PEG main chain), 2.86 (m, 2H, $–CH_2$), 1.55 (m, 3H, $–CH_3$). ^{13}C-NMR ($CDCl_3$, ppm), 196.3 (CO–Ar), 172.9 (CHCO), 168.9 (–COO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 72.6 ($–CH_2$ PEG end group), 70.5 (OCH_2CH_2O PEG main chain), 68.5 (–OCH, $–CH_2$), 64.7, 64.6 ($–OCH_2$), 61.6 ($–CH_2OH$ PEG end group), 45.2 (–CH), 35.8 ($–CH_2$), 18.4 ($–CH_3$).

2-Ketoprofen thiomalic acid PEG polyester (**3b**): ^{1}H-NMR ($CDCl_3$, ppm): 7.77–7.36 (m, 9H, Ar–H), 4.46 (m, 1H, –CH), 4.31–4.07 (m, 4H, $COOCH_2$), 3.92 (m, 1H, –CH), 3.70–3.44 (brs, 77H, OCH_2CH_2O PEG main chain), 3.08–2.67 (m, 2H, $–CH_2$), 1.52 (m, 3H, $–CH_3$). ^{13}C-NMR ($CDCl_3$, ppm): 197.8 (–COS), 195.9 (CO–Ar), 170.2 (CHCO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 72.1 ($–CH_2$ PEG end group), 70.3 (OCH_2CH_2O PEG main chain), 68.5 (–OCH, $–CH_2$), 64.9, 63.9 ($–OCH_2$), 61.4 ($–CH_2OH$ PEG end group), 53.5 (–CH), 41.1 (–SCH), 36.2 ($–CH_2$), 18.2 ($–CH_3$).

2-Ketoprofen aspartic acid PEG polyester (**3c**): ^{1}H-NMR ($CDCl_3$, ppm): 7.86–7.40 (m, 9H, Ar–H), 4.82 (m, 1H, –NCH), 4.35–4.07 (m, 4H, $COOCH_2$), 3.82 (m, 1H, –CH), 3.76–3.49 (brs, 77H, OCH_2CH_2O PEG main chain), 3.09–2.53 (m, 2H, $–CH_2$), 1.53 (m, 3H, $–CH_3$). ^{13}C-NMR ($CDCl_3$, ppm): 196.2 (CO–Ar), 173.3 (–CONH), 170.3 (CHCO), 140.9, 137.7, 137.1, 132.7, 131.7, 129.9, 128.5 (–CH), 72.4 ($–CH_2$ PEG end group), 70.4 (OCH_2CH_2O PEG main chain), 68.4 (–OCH, $–CH_2$), 64.6, 63.5 ($–OCH_2$), 61.2 ($–CH_2OH$ PEG end group), 48.5 (–NCH), 45.9 (–CH), 35.9 ($–CH_2$), 18.4 ($–CH_3$).

2.5. Temperature Responsive Behavior

The optical transmittance of the polyester in an aqueous solution (0.5 wt %) was measured by UV-Vis spectrometer (Hitachi U-1900, Tokyo, Japan) detection of the change in transmittance (λ = 500 nm) at a heating/cooling rate of 0.5 °C·min^{-1}. The temperature at which the change in absorbance of the solution was a maximum was defined as the lower critical solution temperature (LCST).

2.6. In Vitro *Drug Release*

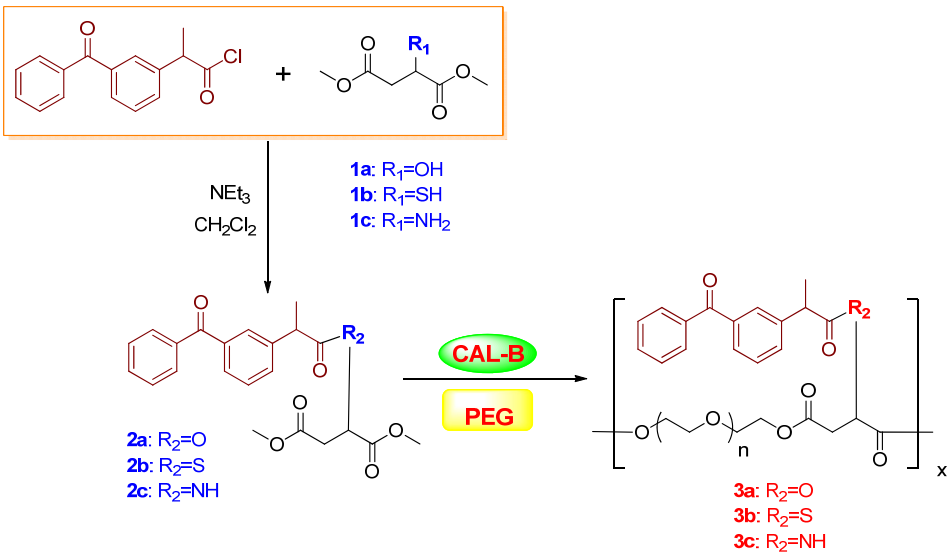
The hydrolysis of the polyester was studied using pH 7.4 phosphate buffered saline (PBS) and pH 10 carbonate buffer and pH 1.2 hydrochloric acid aqueous solutions. For the hydrolysis studies, the polyester was suspended in 70 mL of buffer solution which was shaken at 37 °C. At appropriate time intervals, samples were withdrawn and diluted with mobile phase for direct analysis by High Performance Liquid Chromatography (HPLC). Hydrolysis rates of the polyester were determined by monitoring the production of parent drug with HPLC.

3. Results and Discussion

3.1. Enzyme-Catalyzed Polycondensation for the Preparation of Polyester

The two step strategy applied for the synthesis of thermosensitive polyesters with pendant ketoprofen is shown in Scheme 1. First, **2a** (**2b**, **2c**) was synthesized by the esterification of chloroacylated ketoprofen with **1a** (**1b**, **1c**). The products were purified by silica gel chromatography and characterized by HRMS and NMR spectrometries (see Figures S7–S15). Then enzyme-catalyzed polycondensation of PEG 800 with **2a** (**2b**, **2c**) were performed in solvent-free system with high yields. PEG can be safely administrated *in vivo*, and covalent conjugation of PEG to drug can increase the plasma residence time [22–25]. The CAL-B was chosen as catalyst, as it is well-known with high catalytic activity and stability.

Scheme 1. Enzymatic copolymerization of polyethylene glycol (PEG) and 2-ketoprofen malic acid (thiomalic acid or aspartic acid) dimethyl ester.



Toluene was widely used as solvent in enzymatic copolymerization, but no polycondensation was obtained in this reaction while using toluene as solvent. Then the solvent-free condition was chosen for the further investigation. Equilibrium exists between polymerization and degradation in enzyme-catalyzed reactions. Therefore, the removal of polycondensation products is indispensable for obtaining polymer with high molecular weight. The by-product of the reaction is methanol which is easily removed under vacuum during the reaction.

To study the effect of different reaction conditions on the polymerizations, a set of experiments were designed with different polymerization temperatures and reaction times. This polymerization was significantly influenced by the reaction temperature. When the polycondensation reactions were performed at 70 °C under vacuum, the molecular weight of products was 3810 Da for **2a** and 3500 Da for **2b**, but no polycondensation was obtained for **2c**. When the temperature rise up to 90 °C, all the substrates could copolymerize with PEG and the M_w of products was increased. Then we chose 90 °C for the further investigation of the polycondensation because the higher temperature could increase the enzyme activities.

Table 1 shows the behavior of three substrates (**2a**, **2b**, **2c**) on the synthesis of polyester (**3a**, **3b**, **3c**) using CAL-B. All the reactions were gently stirred at 90 °C under vacuum, and the polyesters were obtained in good yield (80%–90%). Among these three monomers, **2b** produced the highest molecular weights and **2c** produced the highest yield (entries 6 and 9). In most cases, the molecular weight increased as a function of reaction time, but the molecular weight of **3a** observed in 96 h was decreased (entry 3). The reason for this may be that the degradation of **3a** takes place more frequently with longer reaction time. Since the highest yield was obtained in 24 h (entry 1), we chose this reaction condition (90 °C, 24 h) to synthesis **3a**. The same reaction condition was chose to synthesis **3b** to obtain the narrow molecular weight distribution product (entry 4). However, the molecular weight of **3c** is only 2560 Da in 24 h (entry 7). The reaction time was prolonged to 96 h to make sure the molecular weight of **3c** much closer to **3a** and **3b** (entry 9). This may be a result of the difference in enzyme activities for the malic acid, thiomalic acid and aspartic acid.

Table 1. Synthesis of polyester by enzyme-catalyzed polycondensation [a].

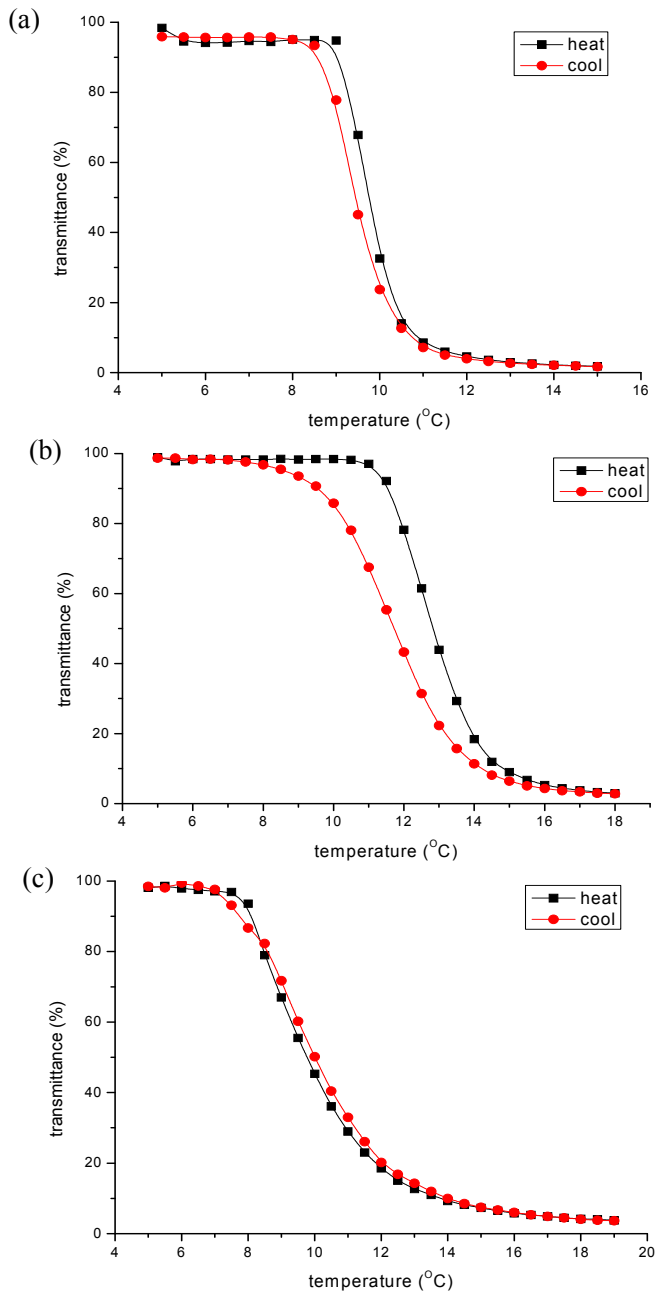
Entry	Substrate	Time (h)	M_w (g/mol) [b]	M_w/M_n	Yield (%) [c]
1	**2a**	24	6,250	1.9	85.1
2	**2a**	48	8,970	4.8	80.8
3	**2a**	96	6,560	2.4	79.6
4	**2b**	24	8,570	2.6	86.7
5	**2b**	48	11,230	3.1	87.0
6	**2b**	96	13,760	3.1	89.9
7	**2c**	24	2,560	2.9	91.7
8	**2c**	48	2,430	3.0	91.0
9	**2c**	96	4,270	1.6	92.8

Notes: [a] Reaction conditions: polymerization of **2a** (**2b**, **2c**) and PEG using CAL-B (10% *w/w* of monomers) in bulk at 90 °C under vacuum; [b] From GPC (see Figures S16–S18, Tables S1–S3); [c] Methanol/hexane (1/6 vol %) insoluble part.

3.2. Temperature Responsivity of Polyester in Aqueous Solution

PEG with a small molecular weight is dehydrated as the temperature increases, some block copolymers that consist of PEG and hydrophobic segments exhibit temperature responsive phase transition behavior [26–30]. The temperature responsive behavior of the polyester was investigated by measuring the change of the solution transmittance using a UV-Vis spectrometer. The plot of transmittance (*T%*) as a function of temperature was shown in Figure 1. As expected, all of the polyester aqueous solutions displayed apparent phase transitions (change in turbidity). This suggests the polyesters have a good thermosensitive property.

Figure 1. Plot of transmittance as a function of temperature measured for polyester aqueous solution (0.5 wt %) at a heating/cooling rate of 0.5 °C·min^{-1}: Black dots, heating curve; red dots, cooling curve. (**a**) Polyester **3a**; (**b**) Polyester **3b**; and (**c**) Polyester **3c**.



The **3a** solution was transparent at 5 °C, but when the temperature was increased to 15 °C, the solution became turbid. The transmittance decreased sharply in the solution during the heating process and the LCST was about 9 °C. When the temperature was reduced back to 5 °C, the precipitates disappeared and the solution became transparent, which indicated reversibility of this transition process. It is known that the LCST of thermosensitive polymers depends on the molecular weight. Compared with our previous study [18], an increase in the molecular weight of 3810 to 6250 Da caused a 10 °C decrease in the LCST.

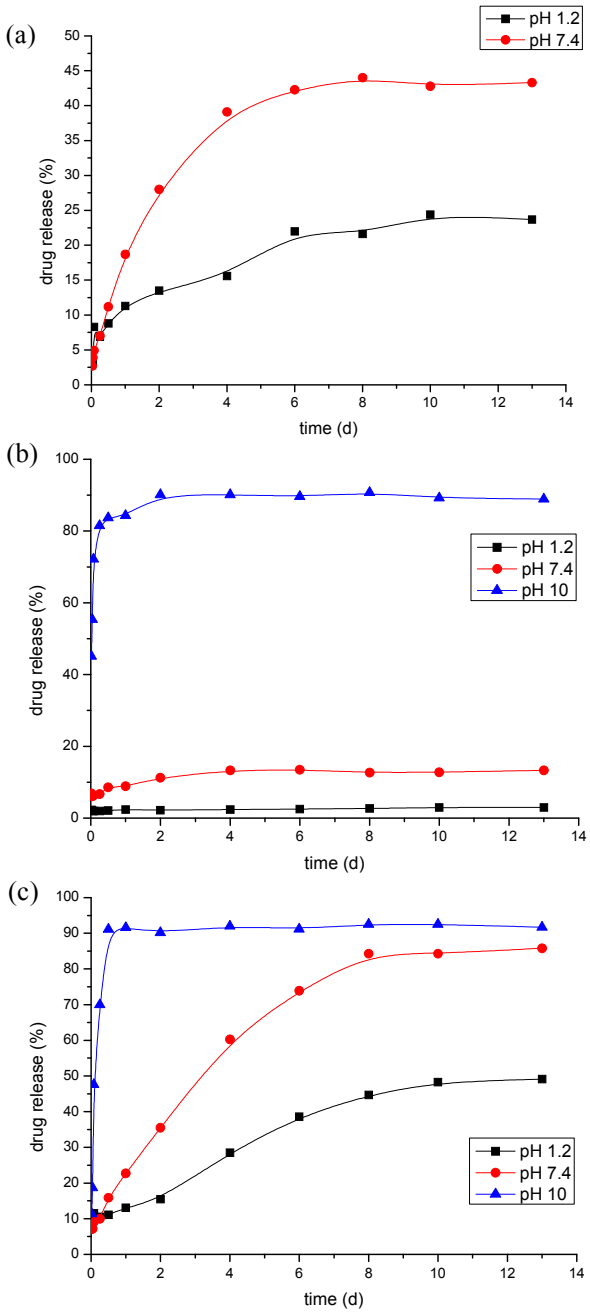
The **3b** solution proved transparent below 11 °C, and the transmittance dropped when the temperature was increased. In contrast, a higher LCST close to 12 °C is observed for **3b** and the phase

transition is achieved in about a 4 °C range. However, the phase transition of **3c** occurs over a broader temperature range as compared to **3a** and **3b**, spanning at least 10 °C with solution changing from clear to totally turbid.

3.3. In Vitro *Hydrolysis of Polyester*

The *in vitro* ketoprofen hydrolysis release behaviors of polyesters were carried out at different pH conditions (pH 1.2, 7.4 and 10.0). The release of ketoprofen from the polyester in different buffer was investigated at 37 °C. Figure 2 shows the degree of hydrolysis of polyester as function of time at different pH values.

Figure 2. Release profiles of ketoprofen from polyester in different solution (pH 1.2, 7.4 and 10.0) at 37 °C. (**a**) Polyester **3a**; (**b**) Polyester **3b**; and (**c**) Polyester **3c**.



As shown in Figure 2a, at pH 7.4, 42.3% of ketoprofen was released from the polyester **3a** after 6 days. A slow release of 23.7% was observed over 13 days at pH 1.2. The slow and incomplete release suggesting that the ester linkage between polymer and ketoprofen was stable in acid and neutral environment. However, a high burst release and complete hydrolysis (88.5%) was reached during the first 30 min in pH 10 carbonate buffer (data not shown).

The hydrolysis behavior of polyester **3b** was shown in Figure 2b. A very slow release of 13.3% was observed in pH 7.4 PBS over 13 days, but there is no drug was found in the pH 1.2 hydrochloric acid aqueous solutions over 13 days. This implied that the thioester bond between polymer and ketoprofen was potentially resistant to acid and neutral environment. However, the rate of drug release from polyester **3b** in pH 10 carbonate buffer was much faster than in pH 1.2 and 7.4 buffer: 90.1% of ketoprofen was released after 2 days.

As shown in Figure 2c, 84.3% of ketoprofen was released from the polyester **3c** after 8 days in pH 7.4 PBS while a slow release of 49.1% was observed in pH 1.2 hydrochloric acid aqueous solution over 13 days. The rate of drug release in pH 10 carbonate buffer was much faster: complete hydrolysis (91.1%) was reached in 12 h.

These polyesters showed hydrolytic stability in acid environment while the release rate reached a maximum in alkaline environment. It was found that the release rate of ketoprofen was increased as the pH increased: pH 1.2 showed the slowest release rate and pH 10 showed the highest release rate. In addition, the influence of the different linkage between polymer and ketoprofen was observed: **3a** (ester bond) could release drug in neutral and alkaline medium, **3b** (thioester bond) only release in alkaline medium, and **3c** (amide bond) could release in acid, neutral and alkaline medium.

4. Conclusions

The thermosensitive polyester, consisting of hydrophilic PEG segments and hydrophobic drug segments, was synthesized by enzyme-catalyzed polycondensation. Under the selected conditions, polyesters with molecular weights of several thousands were obtained in good yields. The release behaviors at different pH conditions were investigated. The results showed that the polyesters were hydrolyzed and the release rate of drugs at alkaline medium was higher than other medium. In principle, other drugs containing carboxylic functional groups can be linked to PEG using the same polyester system in this study to control the drug release rate.

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Conflicts of Interest

The authors declare no conflict of interest.

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