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Synthesis and Characterization of pH-Sensitive Biodegradable Polyurethane for Potential Drug Delivery Applications

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ABSTRACT: To obtain a pH-sensitive multifunctional polyurethane micelle drug carrier, a novel pH-sensitive macrodiol containing acid-cleavable hydrazone linkers, poly(ε-caprolactone)−hydrazone−poly(ethylene glycol)−hydrazone−poly(ε-caprolactone) diol (PCL−Hyd−PEG−Hyd−PCL), was synthesized and characterized with proton nuclear magnetic resonance spectra (^{1}H NMR). A series of pH-sensitive biodegradable polyurethanes (pHPUs) were designed and synthesized using pH-sensitive macrodiol, L-lysine ethyl ester diisocyanate (LDI) and L-lysine derivative tripeptide as chain extender, which can provide an active reaction site for the development of positive target polyurethane micelles for drug delivery. The bulk structures of the prepared polyurethanes were carefully characterized with ^{1}H NMR, gel permeation chromatograph (GPC), differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FT-IR). The polyurethanes could be cleaved in acidic media (pH ∼ 4−6) as well as degraded in PBS and enzymatic solution, as demonstrated by ^{1}H NMR and weight loss, respectively. The cytotoxicity of their degradation products was evaluated using methylthiazoletetrazolium (MTT) assay in vitro, resulting in no apparent inhibition effect on the fibroblasts. These polyurethanes could self-assemble into micelles in aqueous solutions, as verified using dynamic light-scattering (DLS). Our present work provides a new method for the preparation of amphiphilic multiblock polyurethanes with pH-sensitivity and biodegradability. It could be a good candidate as biodegradable multifunctional carrier for active intracellular drug delivery.

1. Introduction

Polyurethanes (PUs) have been widely used for various biomedical applications, such as heart valves,[1] aortic grafts,[2] pacing leads insulation,[3] indwelling catheters,[4] intra-aortic balloons,[5] etc., due to their attractive physical properties and good biocompatibility. Besides their traditional applications, the development of biodegradable polyurethanes for novel biomedical applications, including ligament reconstruction prostheses,[6] temporary scaffolds,[7,8] controlled release systems of active ingredient,[9] etc., has been investigated extensively. Thus, biodegradable polyurethanes have been considered as one of the most attractive biodegradable polymers. The biodegradability of polyurethanes is a key issue for novel biomedical applications. It is generally achieved by incorporating labile and hydrolyzable moieties into the polymer backbones.[10] To fulfill this goal, polyols containing hydrolyzable bonds were usually employed as soft segments for these polyurethanes, such as hydroxyl-terminated oligomers of polycarolactone and polylactides, etc.[11] Similarly, biodegradable hard segments were also used to obtain biodegradable polyurethanes, nontoxic chain extenders to constitute the biodegradable hard segment were far wider than diisocyanate. For example, several combinations with aliphatic diisocyanate have been reported,[12] some of them are based on amino acids[13] or acid labile acetal/ketal-containing diamine.[14] Recently, biodegradable polyurethane micelle or pH-dependent polyurethane micelle based on carboxylic groups,[15] pH- and temperature-sensitive polyurethane hydrogels,[16] stimuli-sensitive[17] and shape memory polyurethanes[18] have been developed for biomedical applications. Our group has reported a series of novel cationic biodegradable multiblock poly(ε-caprolactone urethane) s, which were prepared using poly(ε-caprolactone) diols as soft segments, L-lysine ethyl ester diisocyanate, gemini quaternary ammonium (GA8), 1,4-butandiol as hard segments and methoxylpoly(ethylene glycol) as blocking agent.[15a] The bulk properties, self-assembly behavior, the size and its distribution, ζ potential, and critical micelle concentration (cmc) of these gemini polyurethane micelles were investigated. The results suggested that these micelles held great promise as biodegradable carriers for drug and gene delivery.[15b] Therefore, the studies mentioned above have significantly extended the application area of polyurethane.

Furthermore, pH-sensitive polymers are particularly promising candidates for anticancer drug delivery applications due to the presence of various pH values in different tissue in vivo. More and more attention has been paid to study pH-sensitive amphiphilic block copolymers for delivering anticancer drugs,[19−25] because the drug release rate in these polymers drug delivery system is much faster at an acidic pH of 5.0−6.0 than the physiological pH of 7.4. Polymers including: poly(L-histidiene) (polyHis), polyorthoesters and polyacetals or polyketals, have been used to prepare pH-sensitive nanocarriers. The swelling of ionized DOX-loaded polyHis micelles in the tumor site (pH = 5.0−6.0) could enhance DOX release.[20] As well-known, the hydrazone linkage, which is prone to acid cleavage, has been used for the conjugation of anticancer agents onto drug carriers or monoclonal antibodies to meet rapid cleavage of drug under acidic conditions in the vicinity of tumor tissues or within endosomes.[23,25−27] Thus, another approach to obtain pH-sensitive polymers is to use acidic cleavage hydrazone linkage, which can be introduced into polymer

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backbone, yielding new pH-responsive polymers for constructing nanocarriers.[23,28,29] The group lead by Kataoka prepared multifunctional polymeric micelles with hydrazone bonds, which can be cleaved by the intracellular acidic environment (pH = 5−6).[23,29] However, very little attention has been paid on the synthesis of novel pH-sensitive polyurethanes for drug delivery systems.[14,15c] Paramonov et al. reported a new generation of pH-sensitive polyurethanes and polyureas based on the same acid-degradable dimethyl ketal moiety embedded in the polymer main chain. The resultant polymers degraded significantly faster at acidic pH (pH = 5.0) than at physiological pH (pH = 7.4). Moreover, they could degrade into small molecule products under acidic conditions.[14] To the best of our knowledge, multiblock pH-sensitive polyurethanes based on hydrazone bond for the application as micelle drug carriers has never been reported in literature. Herein, pH-sensitive polyurethanes (pHPUs) were synthesized using PCL−Hyd−PEG−Hyd−PCL as soft segment, LDI and tripeptide as hard segment. The obtained polyurethanes were carefully characterized with ^{1}H NMR, GPC, FT-IR, DSC, and their degradation behavior in vitro was also investigated in PBS (pH = 7.4) and enzymatic solution. Regarding the potential application of the synthesized pHPUs as drug delivery carriers, their pH-sensitivity, biocompatibility, and the formation of micelles through self-assembly were preliminarily characterized with ^{1}H NMR, MTT assay, and DLS, respectively.

2. Materials and Methods

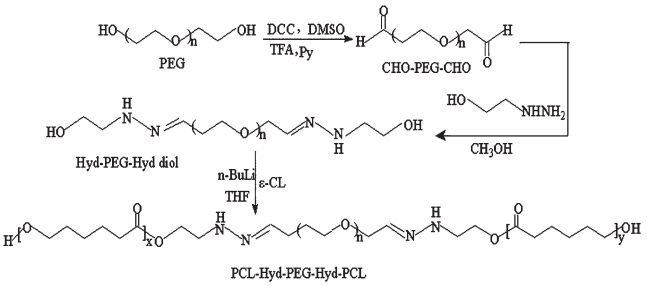
2.1. Materials. Unless otherwise noted, all chemical reagents were obtained from commercial suppliers and used without further purification. Poly(ethylene glycol) with M_n = 400 was purchased from Sigma−Aldrich. 2-Hydroxyethylhydrazine was obtained from Alfa Aesar, ε-caprolactone was purchased from Acros Organics (NJ), which was purified by drying over CaH_2 and distilled under reduced pressure. *N*-Butyllithium was purchased from Chemetall (Tai Wan). Lipase AK was purchased from Amano Pharmaceutical Co. Ltd., Japan. 3T3 mouse fibroblasts were obtained from type Culture Collection of Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Life (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). 3-(4,5-Dimethylthiazol-2-yl)diphenyltetrazolium bromide was purchased from Sigma−Aldrich. *N*,*N*-Dimethylacetamide (DMAc) was dried over CaH_2 for 2 days at room temperature, distilled under vacuum, and stored in the presence of 4 Å molecular sieves. Tetrahydrofuran (THF) was refluxed with sodium wires until benzophenone became blue and distilled out for use. LDI was synthesized with triphosgene according to ref 30, and $(Boc)_2$lysine and γ-aminobutyric acid methyl ester hydrochloride were synthesized using a conventional manner of preparing peptides in our laboratory.

2.2. Materials Characterization. ^{1}H NMR spectra were obtained on a Varian unity Inova-400 spectrometer (400 MHz) spectrometer, using tetramethylsilane (TMS) as an internal standard, with $CDCl_3$ or DMSO-d_6 as solvent.

Molecular Weight Determination. The number-average molecular weights (M_n), weight-average molecular weights (M_w) and molecular weight distributions were determined using gel permeation chromatography (GPC), which was performed with an HP1100 using two PLgel columns (10 μm, 104 Å, 10 μm, 500 Å). Molecular weights are relative to monodisperse polystyrene standards, and tetrahydrofuran (THF) was used as eluent.

FT-IR Spectroscopy. Each sample for infrared analysis was prepared by casting the polymer onto a clean potassium bromide disk from a single drop of 1% (w/v) DMAc. These samples were put into an oven at 40 °C for 24 h and 60 °C for 24 h under vacuum to remove the solvent completely. Infrared absorbance spectra were recorded using a Nicolet-560 spectrophotometer between 4000 and 600 cm^{-1} with the resolution of 4 cm^{-1}. Mass spectra (MS) were acquired using an HP1100-LC/MSD with atmosphere pressure chemical ionization (positive mode).

Scheme 1. Synthesis Route of PCL−Hyd−PEG−Hyd−PCL Oligopolymer



Differential Scanning Calorimetry (*DSC*). To characterize the thermal behavior of pHPUs, experiments were conducted on a Netzsch STA 449C Jupiter (Germany) at a heating rate of 10 °C/min in the range of −100 to +100 °C under a steady flow of nitrogen. Transitions were recorded from heating and cooling scans using a linear extrapolation method (T_m) and the midpoint inflection method (T_g). Since the pure soft segments used were less than 100% crystalline, percent crystallinity values reported were not absolute.

Size and Size Distribution Measurements. The size and its distribution of the polyurethane micelles were measured with a Zetasizer Nano ZS dynamic light-scattering (DLS) instrument (Malvern, U.K.). All the measurements were performed at 25 °C with an angle of 90°.

2.3. Synthesis of the pH-Sensitive Poly(ε-caprolactone)−Hydrazone−Poly(ethylene glycol)−Hydrazone−Poly(ε-caprolactone) Diol (PCL−Hyd−PEG−Hyd−PCL). *2.3.1. Aldehyde Poly(ethylene glycol) (CHO−PEG−CHO)*. PEG (2 g, 5 mmol) was dissolved in DMSO (20 mL) containing DCC (6.183 g, 30 mmol) and pyridine (0.81 mL, 10 mmol). TFA (0.38 mL, 5 mmol) was added, and the mixture was stirred for 48 h at 60 °C. Ethyl acetate (10 mL) was added, and dicyclohexylurea (DCU) was removed by filtration. The ethyl acetate solution was washed twice with 20 mL of distilled water, dried over anhydrous Mg_2SO_4 for 24 h, and concentrated under reduced pressure to give rude CHO−PEG−CHO. The obtained product (CHO−PEG−CHO) was purified with silica gel column chromatography using gradient petroleum ether/ethyl acetate (yield: 68%). FT-IR (cm^{-1}): 948.7 (m, δ, C−H), 1348.0 (m, ν, C−C), 1719.9 (s, ν, C═O), 2869.1, 2917.8 (m−s, ν, CH_2). ^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 3.66 ($-CH_2CH_2O-$); 3.82 ($-CH_2CHO$); 9.73 ($-CHO$).

2.3.2. Synthesis of Hyd−PEG−Hyd Diol. CHO−PEG−CHO (1.98 g, 5 mmol) was dissolved in methanol (15 mL), 2-Hydroxyethylhydrazine (0.837 g, 11 mmol) was added into the methanol solution, and the reaction mixture was allowed to stir for 1 h at room temperature. After the completion of reaction, methanol was evaporated in vacuum. The obtained Hyd−PEG−Hyd diol was purified with basic alumina column chromatography using gradient chloroform/methanol (yield: 74%). FT-IR (cm^{-1}): 1253.0 (m, β, O−H), 1692.5 (m, ν, C═N), 2869.1, 2911.8 (m−s, ν, CH_2), 3411.7 (m, ν, N−H). ^{1}H NMR (400 MHz, $CDCl_3$, δ in ppm): 2.62 ($-NHCH_2CH_2-$); 3.66 ($-CH_2CH_2O-$); 3.79 ($-CH_2OH$); 4.08 ($-OCH_2CH{=}N-$); 6.54 ($-CH{=}NNH-$); 7.05 ($-CH{=}NNH-$).

2.3.3. Synthesis of Poly(ε-caprolactone)−Hydrazone−Poly(ethylene glycol)−Hydrazone−Poly(ε-caprolactone) Diol (PCL−Hyd−PEG−Hyd−PCL). Hyd−PEG−Hyd (1.536 g, 3 mmol) was dissolved in anhydrous tetrahydrofuran (30 mL), *n*-butyllithium (0.67 mL, 6 mmol) was added to form macroinitiator, then ε-caprolactone (ε-CL) was added, and the reaction mixture was allowed to stir for 1.5 h at room temperature. Deionized water (150 mL) was added into the resulting solution and the precipitate was obtained by filtration. The precipitate (PCL−Hyd−PEG−Hyd−PCL) was dried in vacuum, and used without further purification. The synthesis route is shown in Scheme 1. ^{1}H NMR is the

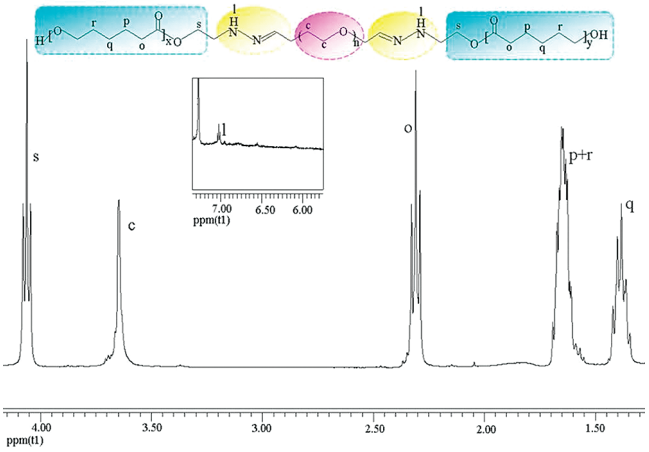


Figure 1. Structure and ^{1}H NMR spectrum of the PCL−Hyd−PEG−Hyd−PCL oligopolymer.

most convenient technique to determine the M_n of PCL diols[31] and acquire structural information. The number-average molecular weight (M_n) of PCL−Hyd−PEG−Hyd−PCL was calculated from ^{1}H NMR spectra according to eqs 1−3:

$$\frac{I_A}{x} = \frac{I_B}{y} \quad (1)$$

$$y = \frac{M_n(PEG) - 18}{44} \times 2 \quad (2)$$

$$\begin{aligned} &M_n(\text{PCL}-\text{Hyd}-\text{PEG}-\text{Hyd}-\text{PCL}) \\ &= M_n(\text{Hyd}-\text{PEG}-\text{Hyd diol}) + M_n(\text{PCL}) \\ &= M_n(\text{Hyd}-\text{PEG}-\text{Hyd diol}) + 114x \end{aligned} \quad (3)$$

where I_A and I_B are integral intensities of peaks at about 4.06 and 3.65 ppm, respectively. And x and y are degree of polymerization in the PCL and PEG blocks of PCL−Hyd−PEG−Hyd−PCL macrodiol. Structure and ^{1}H NMR spectrum of the PCL−Hyd−PEG−Hyd−PCL oligopolymer are shown in Figure 1.

2.4. Synthesis of Tripeptide Chain Extender. To a solution of $(Boc)_2$lysine (2.076 g, 6 mmol) in dichloromethane (20 mL) was added *N*-hydroxysuccinimide (HOSu, 0.736 g, 6.4 mmol), and then dicyclohexylcarbodiimide (DCC, 1.384 g, 6.7 mmol) was added stepwise after the mixture was cooled to 0 °C using an ice water bath to create an active-ester intermediate with carboxylic groups of $(Boc)_2$lysine. After stirring for 6 h at room temperature, a solution of γ-aminobutyric acid methyl ester hydrochloride (1.075 g, 7.0 mmol) in dimethylformamide (DMF, 2 mL) containing *N*-methyl morpholine (0.85 mL) was added into the mixture, and then the reaction was allowed to proceed overnight at room temperature. After completion of the reaction, the solvents were removed by rotary evaporator at 45 °C, then the residual oil was dissolved in cooled ethyl acetate (50 mL), instantaneously, dicyclohexylurea precipitate was formed. After the precipitate was filtered off, the ethyl acetate solutions were orderly washed with 3 M hydrochloric acid, saturated sodium bicarbonate solution, saturated salt water and deionized water. Finally, the solvents were evaporated in a rotary evaporator. The obtained product (2.924 g) was hydrolyzed in methanol (20 mL) containing 4 mol/L sodium hydroxide (3 mL) for 2 h and then the solution pH value was adjusted between 5−6 to achieve Boc-protected lysine dipeptide containing carboxylic group (2.677 g) at room temperature. The following synthesis processes were performed according to the same procedures described above to obtain Boc-protected lysine tripeptide containing carboxylic group. The tripeptide (2 g, 3.71 mmol) was dissolved in 60 mL of ethyl acetate saturated with hydrogen chloride and left to stand at room temperature for 12 h. The solution was concentrated under reduced pressure and hydrogen chloride was neutralized with saturated sodium bicarbonate solution to give tripeptide extender (yield: 87%). The synthesis route and structure of tripeptide chain extender are shown in Scheme 2. FT-IR (cm^{-1}): 1409.0, 1631.5 (s, ν, C=O), 1555.3 (s, δ, N−H), 793.2, 1631.5, 3286.6, 3433.0 (m−s, ν, β, ν_s, νas, NH_2). ^{1}H NMR (400 MHz, DMSO-d_6, δ in ppm): 1.33 (2H, m, c); 1.57(2H, m, d); 2.05 (2H, m, b); 2.30 (2H, t, e); 2.50 (2H, t, l); 2.54 (2H, t, i); 3.20 (4H, m, j); 3.57 (2H, s, f); 4.03 (1H, m, a); 5.75 (4H, m, k); 7.92 (1H, s, g).

Scheme 2. Synthesis Route of Tripeptide Chain Extender

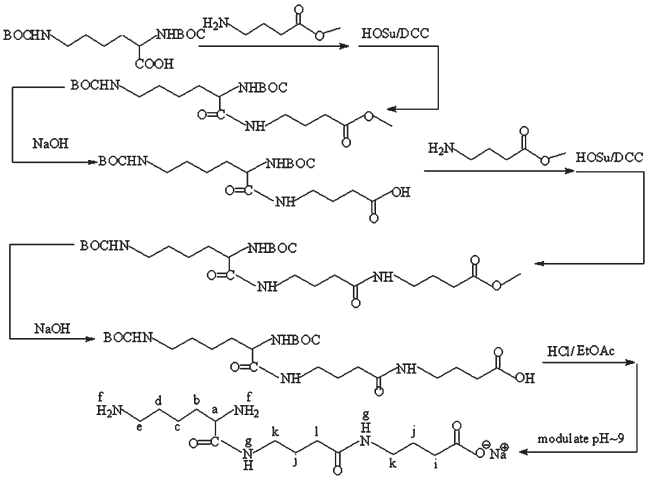


Table 1. Feed Composition and Molecular Weights of pH-Sensitive Polyurethanes with Various Amounts of LDI in Hard Segments

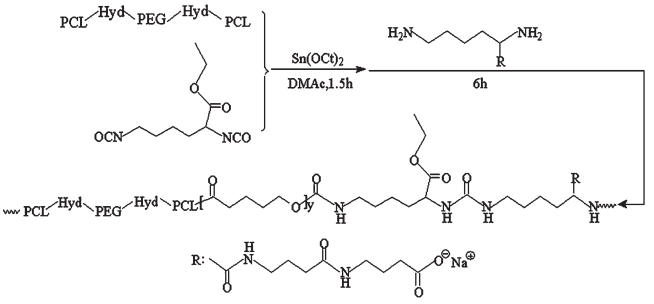
	molar ratio			molecular weights		
samples	LDI	macrodiol	tripeptide	M_n (g/mol)	M_w (g/mol)	M_w/M_n
pHPU1	2	1	1	5016.5	13 202	2.6
pHPU2	2.1	1	1	4631.0	10 846	2.3
pHPU3	2.2	1	1	6140.4	14 753	2.4
pHPU4	3.15	2	1	5716.3	12 984	2.3

MS (APCI, positive) *m*/*z*: theoretical 316 g/mol, observed 317 g/mol

2.5. Synthesis of pH-Sensitive Polyurethanes. pH-sensitive polyurethanes (pHPUs) based on LDI, PCL−Hyd−PEG−Hyd−PCL, and tripeptide chain extender were synthesized using a two step solution polymerization method in DMAc. The feed ratios shown in Table 1. LDI was added into a DMAc solution of PCL−Hyd−PEG−Hyd−PCL under dry nitrogen atmosphere at 60 °C, the reaction was allowed to proceed in the presence of 0.1% stannous octoate and stirred for 1.5 h. Chain extension was carried out by adding chain extenders tripeptide, and the reaction was kept at 60 − 65 °C for 6 h. After the solution was cooled to room temperature and stored for 3 days, the polymer was precipitated in deionized water and dried under vacuum at 60 °C for 3 days. The synthesis route is shown in Scheme 3.

2.6. Degradation Test. Degradation of polymer in vitro was evaluated as weight loss of the hydrated polymer film. The polyurethane films (~20 mg, ~15 mm diameter, and ~0.2 mm thick) were prepared via a solution casting method.[15a] The polymer films were placed in small vials containing 10 mL of phosphate buffer solution (PBS, 100 mM, and pH 7.4). Alternatively, Lipase AK (10 mg/mL, 2 mL) was added into another group of PBS buffer solution with 0.1 wt % $MgCl_2$ (2 mL) for studying the lipase degradation behavior.[32,33] The vials were then incubated with cyclic shaking at 52.5 °C, where the optimum enzyme activity of Lipase AK was found for the degradation of PCL.[33] The films were extracted at selected time intervals, washed with distilled water, dried at 25 °C in vacuum to a constant weight before analysis. The residual weight was calculated as: weight loss (%) = $((m_0 - m_t)/m_0) \times 100\%$, where

Scheme 3. Synthesis Route of pH-Sensitive Polyurethane (pHPU)



m_0 and m_t represent the weights of films before and after degradation, respectively. Two samples of pHPU2 and pHPU4 were measured to obtain the mean weight loss.

2.7. Evaluation of pH-Sensitive Polyurethanes. To confirm that the hydrazone bonds of these polyurethanes are acid-cleavable under mildly acidic condition, the pHPU2 was taken as an example. 3 mL DMAc solution of pHPU2 (20 mg/mL) was incubated with 1 mL various pH value HCl (pH ~ 4, 5, 6) and PBS (0.2 mol/L, pH ~ 7.4) overnight at room temperature, respectively. Then, the solutions were concentrated under reduced pressure and further dried in a vacuum oven for 24 h at 60 °C. The spectras of cleaved pHPU2 were run on the 400 MHz NMR in DMSO-d_6.

2.8. Cell Culture and Cytotoxicity of Degradation Products. 3T3 mouse fibroblasts were grown as adherent cultures and maintained in DMEM supplemented with 15 v/v % heat-inactivated FBS, 2.5% HEPES buffer and antibiotics (1% PC/SM) at 37 °C cell culture incubator in a humidified atmosphere containing 5% CO_2 (Sanyo Incubator, MCO-18AIC, Japan).

Methylthiazoletetrazolium (MTT) cell proliferation and viability assay was used to evaluate the cytotoxicity of pHPU2 degradation product, and 2-Hydroxyethylhydrazine was used as positive control. The pHPU2 was completely degraded under accelerated conditions,[15a] described as follows: 100 mg of pHPU2 was placed in 1.5 mL NaOH solution (1 mol/L) and incubated at 70 °C for approximately 48 h to obtain the degradation products (named pHPU2-base). To further cleave the hydrazone bond in the degradation products, the degradation solution was equally divided into two parts. First, 5 mL of HCl (1 mol/L) was added into one of them for 1 h and the corresponding acid degradation product was prepared (named pHPU2-acid). Both pHPU2-base and pHPU2-acid solutions were adjusted to pH 7.4 with HCl (1 mol/L) or NaOH (1 mol/L) and filtered through a 0.2 μm membrane filter for sterilization and then diluted by 10, 100, 1000, and 10000 times with culture media. Briefly, 1000 cells/well (100 μL/well) were plated in 96-well plates and incubated for 24 h to allow the cells to attach. Then, the cells were exposed to a serial concentrations of the solutions (100 μL/well) at 37 °C for 1 d, 3 d, and 5 d, respectively, followed by the addition of 20 μL of MTT solution (5 mg/mL, PBS) and incubation for another 4 h. Cells incubated with media were used as control. The cell culture media was removed by aspiration and replaced with 200 μL of DMSO to dissolve the precipitates. The resulting solution was measured for absorbance at 490 nm using a microplate reader, and the cell viability was calculated from the following equation:

$$\text{cell viability}(\%) = \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

where $\text{OD}_{\text{sample}}$ is the OD value from a well treated with sample and $\text{OD}_{\text{control}}$ that from a well with culture medium. The data reported represent the means of triplicate measurement.

2.9. Statistical Analysis. Results were expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for evaluating statistical significance. Statistical analysis was performed with SPSS 17.0. Statistical significance was set while $P < 0.05$.

2.10. Size and Size Distribution Measurements. The pH-sensitive polyurethane (pHPU) micelles were prepared with a modified dialysis method. The pHPUs (25 mg) were first dissolved in 5 mL of DMAc by stirring overnight and 15 mL of deionized water was dropwisely added to the polymer solutions under magnetic stirring followed by dialysis against deionized water with a molecular weight cutoff (MWCO) of 3500 for about 3 d to remove the organic solvent at room temperature.

3. Results and Discussion

3.1. Synthesis and Characterization of pH-Sensitive Polyurethanes. To obtain pH-sensitive polyurethanes, new triblock macrodiol, PCL−Hyd−PEG−Hyd−PCL was designed and synthesized. The hydrazone linkages between PCL and PEG blocks could be cleaved in acidic solutions to fulfill the pH-sensitivity of the macrodiol. PEG blocks would help the proton of acid attack hydrazone bonds due to their hydrophilicity. The structure and ^{1}H NMR results of PCL−Hyd−PEG−Hyd−PCL are shown in Figure 1. Peaks at 1.40, 1.65, 2.30 and 4.06 ppm are assigned to the methylene protons of (−CH$_2$*CH$_2$*CH$_2$−), (−*CH$_2$*CH$_2$*CH$_2$*−), (−*CH$_2$*COO−), and (−COO*CH$_2$*−) in PCL blocks, respectively. The sharp peak at 3.65 ppm is attributed to the methylene protons of (−*CH$_2$*-*CH$_2$*O−) in PEG blocks. The weak peaks at 4.23 and 7.05 ppm are attributed to the methylene protons of (−O*CH$_2$*CH$_2$-NH−) and (−CH$_2$*NHN*═CH−) covalently linked to the PCL blocks, respectively. As calculated from their ^{1}H NMR spectra,[31] the molecular weight of the PCL−Hyd−PEG−Hyd−PCL is 3380.7. Additionally, tripeptide chain extender with two primary amine groups was successfully synthesized. The structure of final product was identified with FT-IR, ^{1}H NMR, MS as shown in the section of Materials and Methods. It should be noted that a carboxyl group ended the longer side chain of tripeptide chain extender could provide an active reaction site for the development of multifunctional polyurethane micelles for drug delivery applications to bond targeting molecules, such as antibody, RGD peptide and folic acid etc.,[34] and be in favor of the formation of polyurethane micelles with the enhanced hydrophlicity of the polymer as well. Thus, a series of pHPUs with various molecular weights were synthesized by changing the stoichiometry of the PCL−Hyd−PEG−Hyd−PCL, tripeptide, and LDI. Table 1 demonstrates the theoretical composition of pH-sensitive polyurethanes (pHPUs) with various amounts of LDI in hard segments. Moreover, ^{1}H NMR, FT-IR, and GPC were used to characterize the pHPUs. In Figure 2, the peak at 3.98 ppm (−*CH$_2$*O−) is assigned to methylene protons of the PCL units. Peaks at 4.07 and 1.17 ppm are assigned to methylene (−*CH$_2$*-OCO−) and methyl (−*CH$_3$*) protons in the ethoxyl group of LDI units, respectively. Peaks at 5.76, 6.18, and 6.69 ppm are assigned to (−NHCO*NH*−), (−*NH*CONH−), (−OCO*NH*−) units, respectively. In addition, FT-IR spectra of pHPUs were recorded and shown in Figure 3. In the amine adsorption regions, the formation of urethane and urea groups by the presence of two new characteristic absorption bands at 3367 and 1558 cm^{-1} belonging to hydrogen bonded N−H vibration in urethane and urea linkages are observed.[35] It is also observed that the evidence of free (non-hydrogen bonded) N−H groups vibration at approximately 3435 cm^{-1}.[36] In the carbonyl region, the strong band at approximately 1727 cm^{-1} corresponding to ν(C═O) of ester groups, and a new adsorption peak at around 1645 cm^{-1}of hydrogen bonded carbonyl in urea groups are distinctly observed in Figure 3. The adsorption peaks range from 1850−1600 cm^{-1}, which are mainly overlapped by adsorption of carbonyl in urethane, urea and ester groups

of LDI and PCL,[36,37] could fit the subpeaks of relevant groups. Herein, using pHPU2 and pHPU4 as examples, assignments of adsorption peaks in these areas are shown in Figure 3C, and the percentages of various groups are listed in Table 2. 58.61% carbonyl of PCL ester group in pHPU2 is lower than that of pHPU4 (61.3%), 13.17% carbonyl of LDI in pHPU2 is higher than that of pHPU4 (12.3%), both of which are in agreement with their theoretical composition listed in Table 1. However, the microphase separation of pHPU4 is superior to that of pHPU2 as concluded from the calculation of subpeak area percentage, the ratios of hydrogen bonded carbonyl to free carbonyl in urea and urethane groups of pHPU4 (1.90) are higher than that of pHPU2 (1.36) (see Table 2).[37] These results demonstrate that pH-sensitive polyurethanes are successfully synthesized with various degree of microphase separation. The molecular weights and molecular weight distributions of pHPUs obtained are determined by GPC using THF as the mobile phase and polystyrene as reference, and the results are listed in Table 1. The number-average molecular weights (M_n) are in the range of 4500−6200 and the molecular weight distributions are between 2.2 and 2.7. Clearly, the molecular weight data reported here is lower than that of the poly(ε-caprolactone urethane) s in our previous work,[15a] and molecular weight distributions are wider despite of the fact that high molar ratio of isocynate group to hydroxyl group and amino group was employed in the synthesis prescription. As such a high molar ratio could enhance the molecular weight of general polyurethanes because the excessive isocynate group would induce cross-link reaction occurred in their synthesis precess (Table 1). The possible reason for the decrease in molecular weight and increase in molecular distribution is that the tripeptide chain extender is not well dissolved in DMAc. Thus, the propagation of polyurethane chains is hindered in the process of these polymers extension. Therefore, the synthetic technology should be taken into account for further optimization.

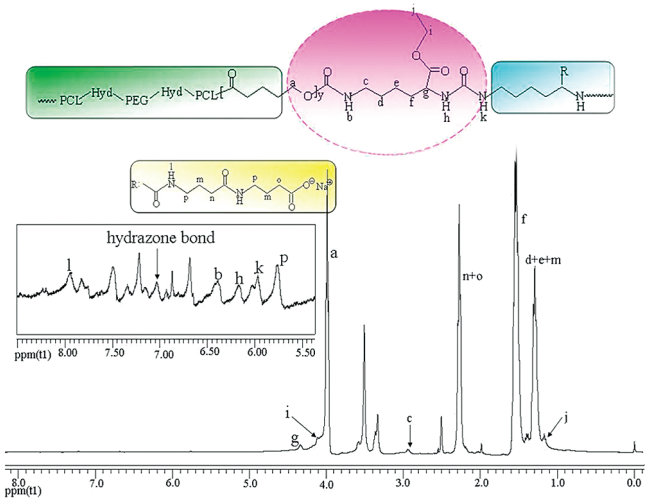


Figure 2. Structure and ^{1}H NMR spectrum of pH-sensitive polyurethane (pHPU).

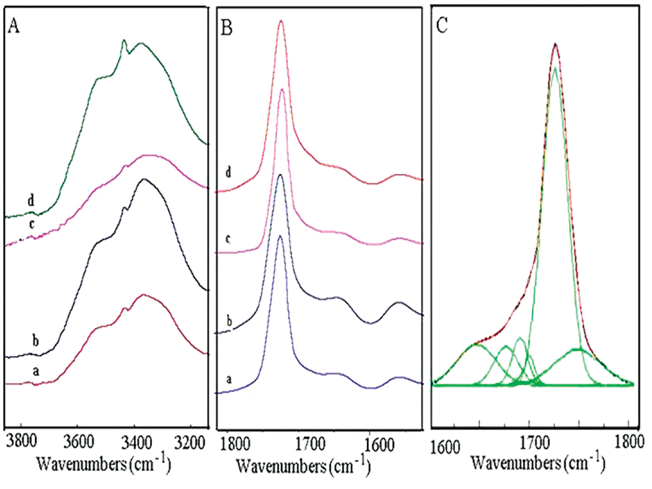


Figure 3. FT-IR spectra of pH-sensitive polyurethanes: amine (A) and carbonyl (B) regions of the pHPUs; a split of carbonyl region between 1600 and 1850 cm^{-1} of pHPU4 (C). Key: (a) pHPU1, (b) pHPU2, (c) pHPU3, and (d) pHPU4.

3.2. Thermal Analysis. In polyurethane materials, the change of the glass transition temperatures (T_gs) of soft segments has been used as an indicator for the degree of microphase separation.[38] DSC was used to measure the thermal properties of pHPUs. The results of DSC measurements are listed in Table 3 and shown in Figure 4. T_gs of the soft segments, ranging from −53.3 to −58.7 °C, are higher than pure PCL−Hyd−PEG−Hyd−PCL (T_g = −63.2 °C), indicating that these polyurethanes obtained have a certain degree of mixing between hard and soft segment in the PCL-based polyurethane matrix.[39,40] However, the T_gs of these pHPUs are much lower comparing with that of the PCL-based polyurethanes we reported previously.[15a,41] The lowest T_g is obtained for pHPU4. The DSC results suggest that these polyurethanes have a high degree of microphase separation.[39,40] No thermal transition related to hard segment is observed in all DSC curves of the studied samples. This is expected as LDI and tripeptide extender are structurally asymmetric and also contain ethyl ester side chains as well as longer peptide chains, which may significantly inhibit chain packing in the hard segment.[42] Multiple melting endotherms are observed in the pHPUs because of the difference in crystal sizes or less perfection of crystals resulting from the PCL−Hyd−PEG−Hyd−PCL soft segment, of which crystallization ability was restrained from alternate bond between hard segments and soft segments of polyurethanes. Similar phenomenon in PCL-based polyurethane ionomers, PCL homopolymer and PCL blends has been reported. It is mainly caused by the presence of polymorphism, melting−recrystallization process, and variation of morphology (lamellar thickness, perfection of crystals).[43]

Table 2. Absorption Band Assignments between 1600 and 1850 cm^{-1} and Their Respective Integration Areas of pHPU2 and pHPU4

frequency, cm^{-1}	absorption band assignment	integration area (%)	
		pHPU2	pHPU4
1645	ν(C=O) hydrogen bonded carbonyl in urea groups	15.2	11.7
1676	ν(C=O) free carbonyl in urea groups	7.4	6.9
1690	ν(C=O) hydrogen bonded carbonyl in urethane linkage	1.0	5.5
1700	ν(C=O) free carbonyl in urethane linkage	4.6	2.2
1727	ν(C=O) carbonyl in ester group of soft segments	58.6	61.3
1744	ν(C=O) of ethyl ester groups of LDI	13.2	12.3

Table 3. Thermal Properties of pH-Sensitive Polyurethanes

samples	T_g (°C)	T_{m_1} (°C)	T_{m_2} (°C)	$\triangle H_{max}$ (J/g)	X_c (%)
PCL−Hyd−PEG−Hyd−PCL	−63.2	37.3	47.3	66.9	57.9
pHPU1	−55.7	40.5	45.5	52.8	57.0
pHPU2	−53.4		45.7	48.4	65.6
pHPU3	−53.1		50.9	63.7	71.1
pHPU4	−58.7		51.3	56.3	58.3

T_g is defined as the midpoint of the glass transition. T_{m_1} and T_{m_2} represent melting peak at lower temperature region and that at higher temperature region, respectively. The crystallinity of the PCL is calculated from the equation: $X_c = \Delta H_m / (\Delta H_{m_0} fw) \times 100\%$, where ΔH_m is the melting enthalpy measured by DSC, ΔH_{m_0} (136 J/g) is the melting enthalpy for 100% crystalline PCL,[50] and fw is the weight fraction of the PCL component in the polyurethanes.

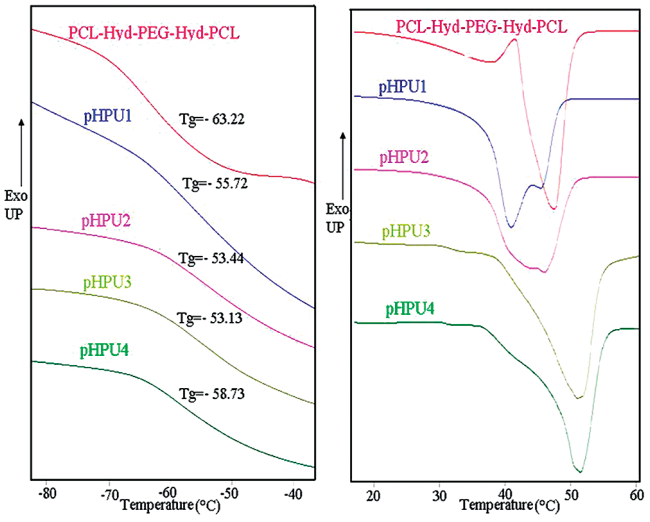


Figure 4. Thermal transitions detected by DSC analysis in pHPUs.

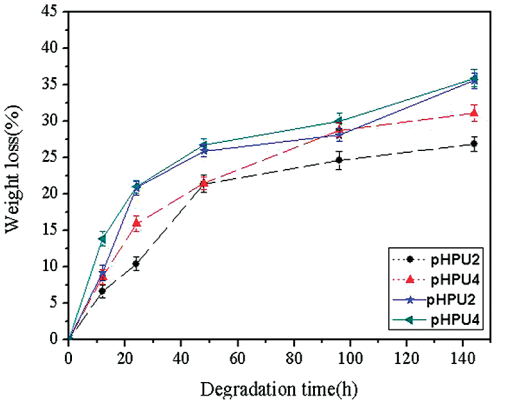


Figure 5. Weight loss of the pHPU as a function of degradation time for pHPU2 and pHPU4; dash lines and symbols represent data for hydrolysis in the absence of enzymes, while solid lines and symbols represent data for enzymatic hydrolysis. Error bars represent means ± standard deviation for $n = 3$.

And the degree of crystallinity of the PCL was calculated (Table 3).[15a,44] These results show that the PCL segments in pHPUs are only partially crystallized mainly owing to the chemical structure and length of the hard and soft segments, the thermal history and processing conditions.[40−42] Nevertheless, their crystallinity is over 50%, indicating that most of the PCL segments are in the crystalline state. All these results further verify that the pHPUs have a better microphase separation. The possible reason is that urea groups and tripeptide side chains in the hard segments largely enhances the incompatibility between hard segments and soft segments.[45]

3.3. Hydrolytic and Enzymatic Degradation. It has been confirmed that poly(ε-caprolactone urethane)s can be degraded by hydrolysis and enzyme, and the degradation rate is strongly affected by their components and aggregation structure.[15a,46] Also, the degradation characteristics of pHPUs obtained have been preliminarily investigated in vitro. Figure 5 shows the weight loss of pHPU2 and pHPU4 as a function of degradation time. The hydrolysis rates of the two samples observed in Lipase AK PBS are higher than that in PBS.[15a,32] 31.1% and 35.9% of weight loss are detected after hydrolytic and enzymatic degradation for 144 h of pHPU4, respectively. The results indicate that the pHPUs are also facile to degrade in enzymatic solution, which is in agreement with reported literatures that Lipase AK is able to accelerate the PCL-based polymers biodegradation.[15a,32,33] Furthermore, it is valuable to note that pHPU4 is degraded faster than pHPU2 by enzymatic hydrolysis and PBS due to the lower crystallinity of pHPU4 (see Table 3). Another reason is that the PCL−Hyd−PEG−Hyd−PCL content in pHPU4 is higher than that in pHPU2 (see Table 1, Table 2). As it has been demonstrated that the biodegradation of a polymer backbone primarily depends on the hydrolysis of polyester chain as well as its content within the polymer backbone, which ultimately determines the rate of hydrolysis.[47]

3.4. pH-Sensitivity and Micellization of Polyurethanes. pH-sensitive polymers are currently considered as one of the most promising materials for anticancer drug carriers, as they can utilize the decrease in pH in tumor surrounding to trigger the release of anticancer drugs to reduce the side effects of drug and increase drug bioavailability.[29] In this work, the pH-sensitivity of the synthesized pHPUs was investigated using ^{1}H NMR. After the pHPU2 sample is incubated under various pH conditions (pH ∼ 4, 5, 6, 7.4), new single peaks at 10.20−10.35 ppm assigned to the proton of aldehyde group (−C*H*O) are observed in the spectra of these samples incubated in pH ∼ 4, 5, 6 solutions, and the peak magnitude increases with decreasing pH of the solutions (Figure 6). In contrast, the peaks at 7.05 ppm of hydrazone proton (−CH=NN*H*CH_2−) decreases and even disappears as pH value decreases to 4 in the ^{1}H NMR spectra (Figure 6). It should be noted that the full ^{1}H NMR spectra of these samples (see Figure S in Supporting Information) show that the peaks between 0.0 and 5.0 ppm are almost not changed, which demonstrates that the rest of the polymer chain remains unchanged. These results indicate that the hydrazone bonds in the polyurethanes obtained can be cleaved under weak acidic conditions and the cleavage rate is accelerated at lower pH values. However, the hydrazone bonds in pHPU2 sample are stable at pH∼7.4 (under physiologic condition) for 24 h (Figure 6). As well-known, the weak acid cleavable characteristic is extremely valuable for drug carrier materials because the chemical stimuli can be used to trigger anticancer drug to be rapidly released from pH sensitive drug carriers in tumor tissues or within endosomes and lysosomes effectively,[23,29,48] where the proton concentration is 10 to 100 times higher (pH 5.0−6.0) compared to health tissue or cells

(pH ~ 7.4). The self-assembly of pHPUs into micelles was preliminarily characterized with DLS. The average size and size distribution of the pHPUs micelles are listed in Table 4 and shown in Figure 7. Their average sizes are less than 170 nm, and the polydispersity indexes (PdI) of these micelles are in the range of 0.1−0.3, which shows a narrow pattern from the DLS measurements. The results demonstrate that the pH-sensitive polyurethanes are good candidates as biodegradable carriers for active intracellular drug delivery.

3.5. Cytotoxicity of Degradation Products. To evaluate the biocompatibility of the degradation products of pHPUs, experiment is carried out to evaluate its cytotoxicity. An accelerated degradation in a strong basic solution and then in an acid medium was carried out to further decompose hydrazone bonds in the degradation products and yield the maximum release of degradation products. The MTT assay was empolyed to evaluate the cytotoxicity of the degradation products: pHPU2-base and pHPU2-acid. The effect of the concentration of degradation product on the proliferation of 3T3 mouse fibroblasts is investigated as shown in Figure 8. The results indicate that there is no apparent inhibition effect on the 3T3 fibroblasts even at the highest concentrations of pHPU2-base and pHPU2-acid (1 mg/mL) in this work. Moreover, the cell viability of the pHPU2-base and pHPU2-acid is not significant difference as the dilution ratio of the degradation solution is lower than 1000 times, while the dilution ratio is higher than 10000 times, the cell proliferation of pHPU2-base is higher compared with that of pHPU2-acid after 5 days incubation (Figure 8). This is probably caused by the presence of 2-hydroxyethylhydrazine and more salt in pHPU2-acid sample. To better understand the biocompatibility of these degradation products, the cytotoxicity of 2-hydroxyethylhydrazine was also evaluated for comparison. The cytotoxic effect of 2-hydroxyethylhydrazine is stronger at higher concentrations. However, the cytotoxicity remarkably decreases and reaches acceptable level with further dilution (see Figure 8). In addition, an amino acid-derived diisocyanate (LDI) was used in an attempt to avoid the release of toxic or carcinogenic compounds.[49] Consequently, these pHPUs could meet safety requirements of biodegradable carriers for the target-specific drug delivery for clinical applications at low dosage. The ongoing animal testing results will be reported elsewhere shortly to further demonstrate their biocompatibility.

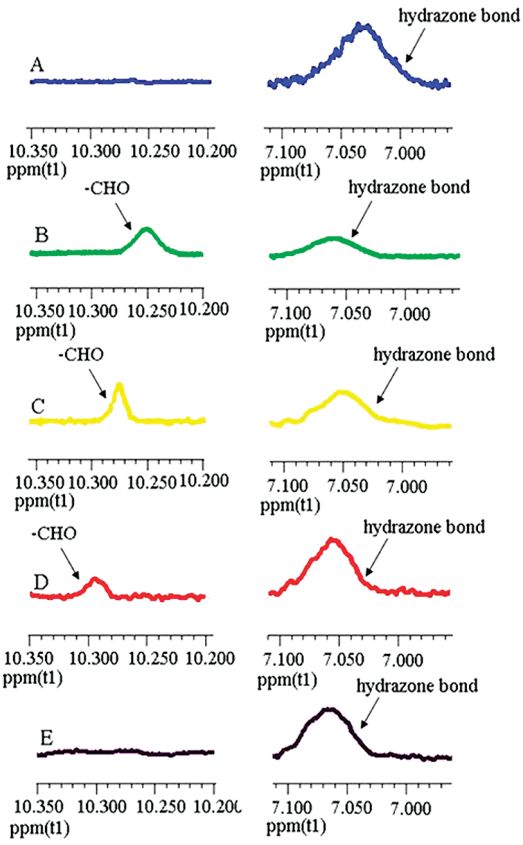


Figure 6. ^{1}H NMR spectra of hydrazone bond broken after and before (A) in pHPU2 under pH ~ 4 (B), pH ~ 5 (C), pH ~ 6 (D), and pH ~ 7.4 (E).

Table 4. Particle Sizes and Size Distributions of the Obtained Polyurethane Micelles

samples	Z-Average(d.nm)	PdI
pHPU1	140.6	0.159
pHPU2	114.3	0.162
pHPU3	121.9	0.229
pHPU4	161.6	0.114

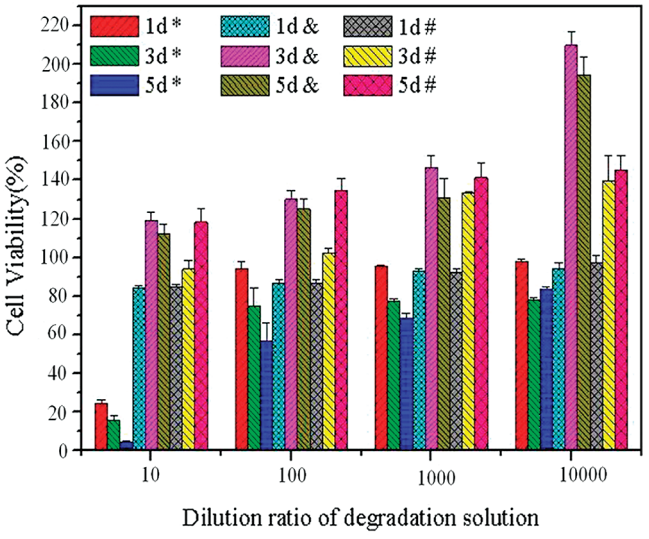


Figure 8. Cell viability measured by MTT assay after 1, 3 and 5d incubation with various concentrations of 2-Hydroxyethylhydrazine (∗), pHPU2-base (&), and pHPU2-acid (#) . Error bars represent means ± standard deviation for $n = 3$. Statistical significance: $p < 0.05$.

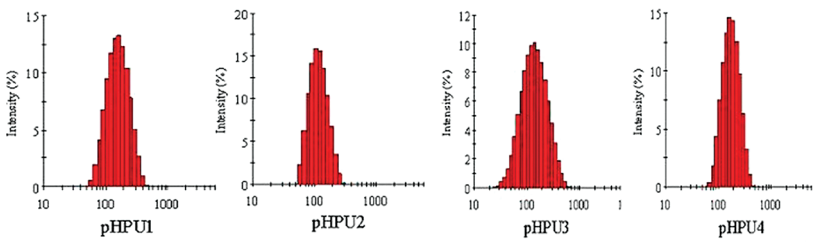


Figure 7. Particle sizes and size distributions of the obtained polyurethane micelles.

4. Conclusions

In summary, a series of pH-sensitive polyurethanes have been successfully synthesized using pH-sensitive macrodiol PCL−Hyd−PEG−Hyd−PCL, LDI, and a new type of tripeptide chain extender. The obtained pHPUs is demonstrated to have a number-average molecular weights of 4500−6200 and high microphase separation as shown by DSC and FT-IR studies. Additionally, the pHPUs could be cleaved in acid media (pH ∼ 4−6) and degraded in PBS and enzymatic solution. Besides, these polyurethanes have good biocompatibility and they can self-assemble into micelles as verified with MTT assay and DLS, respectively. Thus, these pH-sensitive polyurethanes with good micellization are favorable candidates for multifunctional active intracellular drug delivery. Our ongoing works have confirmed that the covalent attachment of antibody to the pH-sensitive polyurethane micelles and they can target tumor cells in vitro. Further studies are currently being carried out in our group to investigate the drug-delivery characteristics of these multifunctional polymeric micelles and their antitumor effects on animals in vivo.

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Supporting Information Available: ^{1}H NMR full spectra of the pHPU2 after incubation under acidic conditions (pH ∼ 4, 5, 6, 7.4). This material is available free of charge via the Internet at http://pubs.acs.org.

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