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Novel pH-tunable Thermoresponsive Polymers Displaying Lower and Upper Critical Solution Temperatures

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Abstract: Novel pH-tunable thermoresponsive 3-azido-2-hydroxypropyl methacrylate-based polymers displaying lower critical solution temperature (LCST) and upper critical solution temperature (UCST) were successfully synthesized by a combination of atom transfer radical polymerization (ATRP) and a chemical modification reaction. Firstly, a novel monomer dimethyl 3,3'-(((1-(2-hydroxy-3-(methacryloyloxy)propyl)-1*H*-1,2,3-triazol-4-yl) methyl) azanediyl) dipropanoate (**HPMAB)** with a hydroxyl group was prepared from 3-azido-2-hydroxypropyl methacrylate (AHPMA) and N,N-bis(3-methoxycarbonyl-ethyl)-propargylamine (BMP) by click chemistry and polymerized via ATRP into a polymer bearing hydroxyl groups, P(HPMAB). Then, P(HPMAB) was subsequently reacted with excess succinic anhydride in the presence of pyridine to yield a polymer bearing carboxyl groups, P(PMAB-COOH). P(HPMAB) and P(PMAB-COOH) exhibited soluble-insoluble-soluble transition (S-I-S) with LCST from 53.4 to 79.1 ^{o}C and UCST from 79.4 to 88.3 ^{o}C in phosphate buffer solutions (PBS) with pH values from 4.7 to 7.8. The pH values in PBS were found to dramatically affect their characteristic themoresponsive behaviors. As a comparison, a similar functional polymer containing no hydroxyl or carboxyl groups, P(PMAB), was also synthesized by ATRP of the monomer dimethyl 3,3'-(((1-(3-(methacryloyloxy)propyl)-1*H*-1,2,3-triazol-4-yl)methyl) azanediyl) dipropanoate (**PMAB)** prepared from 3-azidopropyl methacrylate (APMA) and BMP by click chemistry, and only exhibited soluble-insoluble transition (S-I) with LCST from 41 to 60 ^{o}C in PBS with pH values from 4.7 to 7.8. P(HPMAB), P(PMAB-COOH) and P(PMAB) possessed excellent biocompatibility by

methyl tetrazolium (MTT) assays against NIH3T3 cells and could be regarded as biomedical materials.

Keywords: thermoresponsive polymer; atom transfer radical polymerization; click chemistry; lower critical solution temperature; biomedical materials

Introduction

Stimuli-responsive polymers, also called "intelligent" or "smart" polymers, which can show obvious and abrupt changes in response to small variations of environmental conditions such as temperature, pH, light and ionic, have attracted considerably research interesting due to their promising biomedical applications.[1-9] Among all of the aforementioned environmental stimuli, thermoresponsive mechanism has been extensively studied from both academic and industrial point of views because it is relatively convenient and effective stimuli in practical applications. In recent years, much effort has been devoted to the thermoresponsive polymers. Many thermoresponsive polymers with lower critical solution temperature (LCST) have been synthesized and studied, such as poly(N-isopropylacrylamide) (PNIPAAm) and the corresponding derivative polymer [10-12], poly(N-vinylcaprolactam),[13-15] poly(2-oxazoline)s,[16, 17] poly(2-oxazine)s,[18] pyrrolidone-based polymers,[19-22] oligo(ethylene glycol)-containing polymers[23-28], poly(ionic liquid)[29] and hyperbranched polymers[30-34] *etc*. All these polymers are soluble in aqueous solution below their LCST through their hydrogen bonding with water but become dehydrated and insoluble when heated above the LCST, resulting in fast phase transitions.

Another class of thermoresponsive polymers in aqueous solution shows an insoluble to soluble transition when heated, and this critical temperature is named as an upper critical solution temperature (UCST). Compared with polymers having LCST in aqueous media, polymers having UCST are relatively uncommon. Poly(acrylic acid) (PAA),[35] Poly(N-acryloyl glutamineamide),[36] poly(sulfobetaine),[37, 38] poly(6-(acryloxyloxy-methyl)uracil),[39] and ureido-derivatized polymers[40] are all reported to possess UCST. These polymers usually have a pair of interactive sites that cause the polymers to be insoluble at lower temperatures due to intramolecular and intermolecular interactions (such as hydrogen bonding and electrostatic attraction), which can be disrupted at higher temperatures due to intensified molecular motion within the polymer chains, resulting in a hydrated polymer.

There are also some thermoresponsive polymer systems that can show two or more phase

transitions upon heating and exhibit both LCST and UCST transitions in aqueous media.[41] These reported examples include a NIPAM-AA copolymer,[42] an AA-vinylacetamide copolymer,[43] a proline-based block copolymer,[44] are dual thermosensitive copolymers consisting of two responsive part. However, homopolymers that can exhibit both LCST and UCST transitions in aqueous media are quite rare.[45, 46] Nevertheless, this unique solution property will extend the application fields of temperature-responsive polymers because it will enable these polymers to respond to temperatures within a limited range. Therefore, it is not only scientifically but also technologically important to find a universal molecular design for polymers with a soluble-insoluble-soluble (S-I-S) transition (i.e. both LCST- and UCST- transitions). Poly(ethylene oxide) (PEO) was reported to exhibits S-I-S transitions as a feature of a closed-loop phase diagram.[47] However, it is difficult to utilize the unique properties of PEO in real applications because an UCST-type insoluble-soluble (I-S) transition of PEO appears at high temperatures (greater than 200 °C) and high pressures.

In this work, we report the synthesis of novel pH-tunable thermoresponsive homopolymers based on the novel monomer dimethyl 3,3'-(((1-(2-hydroxy-3-(methacryloyloxy)propyl)-1*H*-1,2,3-triazol-4-yl) methyl) azanediyl) dipropanoate (HPMAB**)** by atom transfer radical polymerization (ATRP), as shown in Scheme 1. P(HPMAB) bearing hydroxyl groups and the corresponding derivative P(PMAB-COOH) bearing carboxyl groups, exhibited a soluble-insoluble-soluble transition (i.e. both LCST- and UCST-transitions) in phosphate buffer solutions (PBS). Since P(PMAB-COOH) has both carboxyl and tertiary amine groups, the polymer can be recognized as a typical model polymer to investigate the pH values on the thermoresponsive behaviors. The pH values on their thermoresponsive behaviors were carefully investigated by cloud point determination using a UV-Vis spectrometer. These results may provide guidelines for the design of the pH-tunable thermoresponsive polymers. In addition, the methyl tetrazolium (MTT) assays against NIH3T3 cells confirmed that the cytotoxicity of these polymers was very low, and which have potential applications as biomedical materials.

Experimental Section

Materials

Glycidyl methacrylate (GMA, 99%, Acros), 1,1,4,7,7-pentamethyl diethylenetriamine (PMDETA, 99%, Aldrich), ethyl 2-bromoisobutyrate (EBiB, 99%, Lancaster) and sodium azide (NaN_3, 99%,

Acros) were used as received without further purification. Copper(I) bromide (CuBr, A.R., Shanghai Chemical Reagent Co.) was purified by stirring in glacial acetic acid overnight, filtered, washed with ethanol and then dried in a vacuum oven at 60 ^{o}C overnight. N,N'-Dicyclohexyl carbodiimide (DCC, A.R.), copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, A.R.), sodium bicarbonate ($NaHCO_3$, A.R.), Methyl acrylate (MA, A.R.), methacrylic acid (A.R.) and succinic anhydride (A.R.) were purchased from Shanghai Chemical Reagent Co., Ltd. and used as received. 4-(Dimethylamino) pyridine (DMAP, 99%), sodium ascorbate (NaAsc, A.R.), and propargylamine (98%) were purchased from Aldrich and used as received. The methylation agent, (trimethylsilyl)diazomethane (approx. 2M in diethyl ether) was purchased from Acros and used as received. The phosphate buffer solutions (PBS) with different pH values were prepared from sodium dihydrogen phosphate and disodium hydrogen phosphate by different molar ratio. Other chemical reagents were purchased from Shanghai Chemical Reagent Co., Ltd. and purified by conventional procedures if needed.

Synthesis of Monomers

Synthesis of 3-azido-2-hydroxypropyl methacrylate (AHPMA)

Typical procedures employed for the preparation of AHPMA were as follows: NaN_3 (3.7 g, 57.0 mmol), $NaHCO_3$ (3.8 g, 45.2 mmol) and 60 mL of THF/H_2O mixed solvent (5:1 v/v) were charged in a 150 mL round-bottom flask, and then GMA (5.4 g, 37.8 mmol) was added slowly into the mixture by a syringe with stirring. The mixture was stirred at room temperature for 48 h. After removing insoluble salts by filtration, the reaction solution were evaporated by rotary evaporation. The residue was extracted twice by CH_2Cl_2 and the combined organic phase was dried over Na_2SO_4. The solvent was removed and the product was further purified by a silica column chromatography using CH_2Cl_2/ethyl acetate (9:1 v/v) as the eluent to afford 5.6 g of the targeted substance AHPMA (yield: 80 %). ^{1}H NMR ($CDCl_3$, δ/ppm): 1.96 (s, 3H, -C**H**$_3$), 2.61 (d, 1H, -O**H**), 3.45 (d, 2H, -C**H**$_2$N$_3$), 4.08 (m, 1H, -C**H**OH), 4.25 (d, 2H, -COOC**H**$_2$-), 5.63 (s, 1H, C**H**$_2$=C-), 6.15 (s, 1H, C**H**$_2$=C-).

Synthesis of N,N-bis(3-methoxycarbonyl-ethyl)-propargylamine (BMP)

This compound was synthesized following the procedure reported by Lee et al.[48, 49] MA(40.0 g, 46.5 mmol) and methanol (50 mL) were added into a 150-mL round bottom flask equipped with magnetic stirrer bar. The flask was then immersed into an ice-water bath. A solution of progargylamine (1.1 g, 20.0 mmol) in methanol (25 mL) was introduced dropwise to the flask through a dropping funnel over 30 min. The mixed solution was stirred for another 1 h at 0 ^{o}C, and then for an additional 48 h at

room temperature under a nitrogen atmosphere. The reaction solution was evaporated on a rotary evaporator. The residues were further purified by a silica column chromatograph using ethyl acetate/petroleum ether (1:2 v/v) as the eluent to afford 4.3 g of the targeted substance BMP as a colorless oil (yield: 96%). ^{1}H NMR ($CDCl_3$, δ/ppm): 3.66 (s, 6H, -OC**H**$_3$), 3.47 (s, 2H, CHCC**H**$_2$N-), 2.81 (t, 4H, -N(C**H**$_2$CH$_2$COO)$_2$-), 2.49 (t, 4H, -N(CH$_2$C**H**$_2$COO)$_2$-), 2.20 (s, 1H, C**H**CCH$_2$N-).

Synthesis of monomer HPMAB

AHPMA (1.0 g, 5.4 mmol), BMP (1.3 g, 5.7 mmol) and 45 mL of THF/H_2O mixed solvent (4:1 v/v) were charged in a 100 mL round-bottom flask. After the mixture was bubbled with nitrogen for 30 min, $CuSO_4 \cdot 5H_2O$ (143 mg, 0.57 mmol) and NaAsc (226 mg, 1.14 mmol) were then introduced into the flask and the mixture was further bubbled with nitrogen for 10 min. The flask was sealed under nitrogen and the reaction mixture was stirred at room temperature for 24 h. THF was removed by rotary evaporation and the reaction mixture was extracted by CH_2Cl_2 (3×15 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. The crude product was further purified by a silica column chromatography using methanol/ethyl acetate (9:1 v/v) as the eluent to afford1.8 g of a transparent product (yield: 83%). ^{1}H NMR ($CDCl_3$, δ/ppm): 1.97(s, 3H, -C**H**$_3$), 2.46 (t, 4H, -NCH$_2$C**H**$_2$COO-), 2.79 (t, 4H, -NC**H**$_2$CH$_2$COO-), 3.65 (s, 6H, -OC**H**$_3$), 3.75 (s, 2H, triazole-C**H**$_2$N-), 4.25 (d, 2H, -COOC**H**$_2$CH-), 4.36 (d, 2H, -CHC**H**$_2$-triazole), 4.63 (m, 1H, -C**H**OH), 5.64 (s, 1H, C**H**$_2$=C-), 6.17 (s, 1H, C**H**$_2$=C-), 7.60 (s, 1H, -triazole). ^{13}C NMR ($CDCl_3$, δ/ppm): 18.2 (-**C**H$_3$), 32.7 (-NCH$_2$**C**H$_2$COO-), 48.9 (-N**C**H$_2$CH$_2$COO-), 49.1 (-O**C**H$_3$), 51.5 (triazole-**C**H$_2$N-), 53.3 (-CH**C**H$_2$-triazole), 65.4 (-**C**HOH), 68.7 (-COO**C**H$_2$CH-), 124.2 (**C**H$_2$=C-), 135.7 (CH$_2$=**C**-), 126.2 and 145.1 (-triazole), 167.0 (-**C**OOCH$_2$CH$_2$CH$_2$-triazole), 172.9 (-NCH$_2$CH$_2$**C**OOCH$_3$).. HRMS (ESI-TOF) calcd for $C_{18}H_{28}N_4O_7$ $[M + H]^+$ m/z =413.2031, found 413.2057; $[M + Na]^+$ m/z =435.1850, found 435.1889.

Synthesis of PMAB

Synthesis of 3-azido-1-propanol

NaN_3 (3.4 g, 52.2 mmol), 3-bromo-1-propanol (6.6 g, 47.5 mmol) and 30 mL of water were charged in a 100 mL round-bottom flask equipped with magnetic stirrer bar. The flask was immersed in a thermostatic oil bath at 80 ^{o}C under a nitrogen atmosphere for 12 h. The solution was extracted by diethyl ether and dried over $MgSO_4$. The crude product was further purified by a silica column chromatography using CH_2Cl_2 as the eluent to afford 4.3 g of a transparent product (yield: 90%).

^{1}H NMR ($CDCl_3$, δ/ppm): 2.05 (m, 2H, $HOCH_2C\mathbf{H}_2CH_2N_3$), 3.48 (t, 2H, $HOCH_2CH_2C\mathbf{H}_2N_3$), 3.78 (t, 2H, $HOC\mathbf{H}_2CH_2CH_2N_3$).

Synthesis of 3-Azidopropyl methacrylate (APMA)

3-Azido-1-propanol (3.3 g, 32.6 mmol), methacrylic acid (2.8 g, 32.5 mmol), DMAP (0.4 g, 3.3 mmol) and 25 mL of CH_2Cl_2 were charged in a 100 mL round-bottom flask. A solution of DCC (7.4 g, 36.0 mmol) in CH_2Cl_2 (25 mL) was added to the flask under stirring at room temperature and the mixture was stirred for 48 h at room temperature. The mixture was filtered and extracted using subsequently distilled water. The organic layer was dried with $MgSO_4$. The crude product was further purified by a silica column chromatography using CH_2Cl_2/petroleum (1:1 v/v) as the eluent to afford 4.8 g of a transparent product (yield: 87%). ^{1}H NMR ($CDCl_3$, δ/ppm): 1.95-2.05 (m, 5H, -C$\mathbf{H}_3$ and -$COOCH_2C\mathbf{H}_2CH_2N_3$), 3.45 (d, 2H, -$COOCH_2CH_2C\mathbf{H}_2N_3$), 4.30 (d, 2H, -$COOC\mathbf{H}_2CH_2CH_2N_3$), 5.57 (s, 1H, C$\mathbf{H}_2$=C-), 6.13 (s, 1H, C$\mathbf{H}_2$=C-).

Synthesis of monomer PMAB

APMA (2.6 g, 15.4 mmol), BMP (3.5 g, 15.4 mmol) and 75 mL of THF/H_2O mixed solvent (4:1 v/v) were charged in a 150 mL round-bottom flask. After the mixture was bubbled with nitrogen for 30 min, $CuSO_4 \cdot 5H_2O$ (769 mg, 3.1 mmol) and NaAsc (1.22 g, 6.2 mmol) were then introduced into the flask and the mixture was further bubbled with nitrogen for 10 min. The flask was sealed under nitrogen and the reaction mixture was stirred at room temperature for 24 h. THF was removed by rotary evaporation and the reaction mixture was extracted by CH_2Cl_2 (3×25 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. The crude product was further purified by a silica column chromatography using ethyl acetate as the eluent to afford 5.1 g of a transparent product (yield: 82%). ^{1}H NMR ($CDCl_3$, δ/ppm): 1.97(s, 3H, -C$\mathbf{H}_3$), 2.33 (m, 2H, -$COOCH_2C\mathbf{H}_2CH_2$-triazole), 2.50 (t, 4H, -$NCH_2C\mathbf{H}_2COO$-), 2.81 (t, 4H, -$NC\mathbf{H}_2CH_2COO$-), 3.67 (s, 6H, -OC$\mathbf{H}_3$), 3.81 (s, 2H, triazole-C$\mathbf{H}_2$N-), 4.21 (d, 2H, -$COOCH_2CH_2C\mathbf{H}_2$-triazole), 4.47 (d, 2H, -$COOC\mathbf{H}_2CH_2CH_2$-triazole), 5.61 (s, 1H, C$\mathbf{H}_2$=C-), 6.13 (s, 1H, C$\mathbf{H}_2$=C-), 7.58 (s, 1H, -triazole). ^{13}C NMR ($CDCl_3$, δ/ppm): 18.3 (-$\mathbf{C}H_3$), 29.5 (-$COOCH_2\mathbf{C}H_2CH_2$-triazole), 32.6 (-$NCH_2\mathbf{C}H_2COO$-), 47.1 (-$N\mathbf{C}H_2CH_2COO$-), 48.7 (-$COOCH_2CH_2\mathbf{C}H_2$-triazole), 49.0 (-O$\mathbf{C}H_3$), 51.5 (triazole-$\mathbf{C}H_2$N-), 61.1 (-COO$\mathbf{C}H_2CH_2CH_2$-triazole), 122.7 ($\mathbf{C}H_2$=C-), 136.0 (CH_2=$\mathbf{C}$-), 125.9 and 145.0 (-triazole), 167.1 (-$\mathbf{C}OOCH_2CH_2CH_2$-triazole), 172.8 (-$NCH_2CH_2\mathbf{C}OOCH_3$). HRMS (ESI-TOF) calcd for $C_{18}H_{28}N_4O_6$ $[M + H]^+$ m/z =397.2082, found 397.2109; $[M + Na]^+$ m/z

=419.1901, found 419.1941.

Polymerization reactions

Synthesis of P(HPMAB)

P(HPMAB) was synthesized by ATRP in ethyl acetate. Typically, an oven-dried Schlenk tube was charged with HPMAB (1.0 g, 2.4 mmol), EBiB (11.7 mg, 0.06 mmol), PMDETA (20.8 mg, 0.12 mmol), CuBr (17.2 mg, 0.12 mmol), ethyl acetate (2 mL) and a magnetic stirrer. After degassing with three freeze-pump-thaw cycles, the tube was sealed under vacuum and then placed in a thermostatic oil bath at 60 ^{o}C for 20 h. The reaction was stopped and quickly cooled down to room temperature with cold water. The mixture was further diluted with THF, removed copper salts through a plugged column of neutral aluminum oxide and precipitated into an excess amount of diethyl ether. The sample was purified by reprecipitating three times from THF to diethyl ether and dried in a vacuum oven overnight. The homopolymer was denoted as P(HPMAB). Yield: 87%, M_n (GPC)=1.59×10^4, M_w/M_n=1.21.

Synthesis of P(PMAB-COOH)

P(PMAB-COOH)was prepared from the corresponding P(HPMAB) reacted with excess succinic anhydride in anhydrous pyridine, as shown in Scheme 1. In a typical run, P(HPMAB) (0.4 g, 0.025 mmol) and succinic anhydride(0.5 g, 5.0 mmol) were dissolved in anhydrous pyridine (5 mL). The solution was stirred over night at room temperature. The product was recovered by precipitation in diethyl ether and further purified by reprecipitating from DMF to diethyl ether for three times and dried at room temperature under vacuum to a constant mass (Yield: 84 %). The corresponding derivative polymer was denoted as P(PMAB-COOH). For GPC measurement, a part of P(PMAB-COOH) was modified by methylation using (trimethylsilyl)diazomethane in a mixture of THF/methanol at room temperature according to the reported method.[50] The sample was purified by reprecipitating three times from THF to diethyl ether and dried in a vacuum oven overnight. M_n (GPC)=2.13×10^4, M_w/M_n=1.20.

Synthesis of P(PMAB)

P(PMAB) was synthesized by ATRP in ethyl acetate. Typically, an oven-dried Schlenk tube was charged with PMAB (1.0 g, 2.5 mmol), EBiB (11.7 mg, 0.06 mmol), PMDETA (20.8 mg, 0.12 mmol), CuBr (17.2 mg, 0.12 mmol), ethyl acetate (2 mL) and a magnetic stirrer. After degassing with three freeze-pump-thaw cycles, the tube was sealed under vacuum and then placed in a thermostatic

oil bath at 60 ^{o}C for 20 h. The reaction was stopped and quickly cooled down to room temperature with cold water. The mixture was further diluted with THF, removed copper salts through a plugged column of neutral aluminum oxide and precipitated into an excess amount of diethyl ether. The sample was purified by reprecipitating three times from THF to diethyl ether and dried in a vacuum oven overnight. The homopolymer was denoted as P(PMAB). Yield: 85%, M_n (GPC)=1.35×10^4, M_w/M_n=1.25.

Cell culture

NIH 3T3 cells (a mouse embryonic fibroblast cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% FBS (fetal bovine serum) and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2 atmosphere.

Cell viability assay

NIH 3T3 cells were used to measure the cytotoxicity of the synthetic materials. The obtained polymers were dissolved in DMEM with different concentration from 1.0 to 220 μg/mL, respectively. NIH 3T3 cells were seeded in 96-well plates with approximately 6000 cells/well in 200 μL medium, and incubated for 48 h at 37 °C. Then, the culture medium was removed and replaced with 200 μL of fresh medium containing serial concentrations of the polymers to incubate the cells. After 48 h of incubation at 37 °C, 20 μL of 5 mg/mL MTT assays stock solution in PBS was added to each well and the cells were incubated for another 4 h at 37 °C. Then, the medium containing unreacted dye was removed carefully and 200 μL per well DMSO was added to dissolve the obtained blue formazan crystals. The absorbance was measured in a BioTek Elx800 at a wavelength of 490 nm.

Characterizations

The molecular weights and polydispersity (M_w/M_n; M_w = weight-average molecular weight; M_n = number-average molecular weight) of the polymers were recorded on a gel permeation chromatograph (GPC) equipped with two Mixed-B columns (Polymer Laboratory Corp., pore size = 10 μm; column size = 300 × 7.5 mm) and a refractive index detector (Perkin-Elmer Series 200) using DMF (0.01 mol L^{-1} LiBr) as the eluent at 40 ºC with a flow rate of 1 mL min^{-1}. The column system was calibrated by a set of mono-dispersed standard PMMA. ^{1}H NMR spectra were measured on a 500 Bruker NMR instrument using $CDCl_3$ as the solvent and TMS as a reference standard for chemical shifts, and ^{13}C NMR spectra were recorded on a 125 MHz with solvent resonance as the

internal standard ($CDCl_3$ at 77.1 ppm). High-resolution mass spectra (HRMS) were recorded using ESI-Q-TOF-MS measurements which were performed with a micrOTOF-Q II (Bruker Daltonics) mass spectrometer. The ESI-Q-TOF mass spectrometer was running at 3.7 kV at a desolvation temperature of 180 ºC. The mass spectrometer was operating in the positive ion mode with a time-of-flight mass analyzer (TOF-MS) and the standard electrospray ion (ESI) source was used to generate the ions. The samples were prepared with the concentration of 1.0 mg/mL and the solvent was methanol. There was no salt addition prior to analysis, but ionization occurred readily from the sodium content that is naturally present in the glass. The thermoresponsive behaviors of the polymer aqueous solutions were analyzed at the concentration of 1.0 mg/mL by cloud point determination. The absorbance at 550 nm was continuously recorded at a heating rate of 0.5 $^{o}C\ min^{-1}$ using a Perkin Elmer Lambda 20 UV-Vis spectrometer.

Results and Discussion

Synthesis and Characterization

The synthesis of all monomers and corresponding polymers is schematically illustrated in Scheme 1. Click reaction was used to prepare the novel monomers HPMAB and PMAB. P(HPMAB) and P(PMAB) were synthesized by the ATRP of corresponding monomers HPMAB and PMAB using ethyl 2-bromoisobutyrate (EBiB) as an initiator in the presence of CuBr/PMDETA catalyst system. The derivative polymer of P(HPMAB), P(PMAB-COOH), was obtained by the reaction of the corresponding P(HPMAB) with excess succinic anhydride in the presence of pyridine. The molecular structures of the novel monomers HPMAB and PMAB were confirmed by NMR and HRMS technology. The molecular structures of P(HPMAB), P(PMAB-COOH) and P(PMAB) were confirmed by ^{1}H NMR and GPC.

Scheme 1

Figure 1

Figure 2

Figure 1 shows ^{1}H NMR spectra of PMAB and HPMAB. The characteristic resonance peaks of the 1,2,3-triazole ring and the vinyl substituent of HPMAB and PMAB were clearly observed at 7.60 and 5.64-6.17 *ppm* (Fig. 1: f and a), respectively. The integral of each proton agrees well with the expected structures. The purity and structure of HPMAB and PMAB were further confirmed by ^{13}C

NMR and HRMS spectra (seeing ESI, Fig. S1 and S2). The obtained results indicated the successful synthesis of the monomers HPMAB and PMAB. Figure 2 shows the typical ^{1}H NMR spectra of P(PMAB), P(HPMAB), and P(PMAB-COOH). The peaks at 7.72 *ppm* (Fig. 2: e) representing the proton on the 1,2,3-triazole rings were clearly observed. The resonance signals of the protons originated from the structure of BMP appeared at 3.64, 2.77, 2.47 *ppm*, assigned to methoxyl group (Fig. 2: k), methylene group (Fig. 2: i) adjacent to tertiary amine group and methylene group (Fig. 2: j) adjacent to the ester group, respectively. Compared with the ^{1}H NMR spectrum of P(HPMAB) (Fig. 2B), a new peak at the ^{1}H NMR spectrum of P(PMAB-COOH) (Fig. 2C) appeared at 5.46 *ppm* (Fig. 2C: n), which was assigned to methine group (Figure1C: n) adjacent to the ester group formed from the reaction between the hydroxyl of P(HPMAB) and succinic anhydride. To ensure 100% conversion of the hydroxyl of P(HPMAB), excess succinic anhydride was used in the modified reaction. The integral ratio of the proton signal from methine group at 5.46 *ppm* (Fig. 2C: n) to that from triazole groups at 7.72 *ppm* (Fig. 2C: e) within the error of ^{1}H NMR measurement was close to 1:1. This primary result indicated that all hydroxyl groups were consumed and participated in the ring-opening reaction of succinic anhydride. P(PMAB-COOH) can be modified by methylation using (trimethylsilyl)diazomethane in a mixture of THF/methanol at room temperature and the methylated P(PMAB-COOH) was confirmed by ^{1}H NMR (seeing ESI, Fig. S3). The degree of esterification was more than 96% according to ^{1}H NMR spectroscopy by comparing the integration of the methyl group resonance (-COOC**H**$_3$) at 3.52-3.76 *ppm* (seeing ESI, peak "b" in Fig. S3) with the intensity of the methine group resonance (-NC**H**C-) at 7.65-8.00 *ppm* (seeing ESI, peak "a" in Fig. S3). The molecular weight and molecular weight distribution of the resulting P(PMAB), P(HPMAB) and methylated P(PMAB-COOH) were obtained from the GPC measurement using DMF as an eluent solvent at the presence of LiBr and the obtained results were listed in Table 1. By the GPC analysis of the resulting polymers (Fig. 3), the traces were unimodal and the polydispersity index was lower (<1.30), indicated the polymerization was a controlled/"living" process. From the results of ^{1}H NMR and GPC analysis, P(PMAB), P(HPMAB) and P(PMAB-COOH) were successfully synthesized. P(PMAB), P(HPMAB) and the methylated P(PMAB-COOH) can be soluble in most organic solvents, such as THF, dioxane, $CHCl_3$, CH_2Cl_2, DMSO and DMF.

Table 1

Figure 3

Thermo-Responsive Behavior

In this study, we initially evaluated thermally induced phase separation behaviors of P(PMAB), P(HPMAB) and P(PMAB-COOH) in water (polymer concentration: 1 mg/mL) by cloud point determination, as monitored by UV (550 nm), in which the heating rate of 0.5 ^{o}C min^{-1} was continuously recorded. Figure 4 shows the transmittance changes of P(PMAB), P(HPMAB) and P(PMAB-COOH) at 1.0 mg/mL in water corresponding to the heating processes. As can be seen in Figure 4, P(PMAB) and P(HPMAB) failed to show a phase transition in water. However, P(PMAB-COOH) was soluble in water in low temperature (<46.2 ^{o}C) and the transmittance decreased drastically during 46.2-53.4 ^{o}C upon heating at the rate of 0.5 ^{o}C min^{-1}, indicating that a LCST-type soluble-insoluble (S-I) phase transition (ca. 49.7 ^{o}C) occurred. The white turbid solution (transmittance <10%) was kept constant during 53.4-78.1 ^{o}C. When the opaque solution was further heated, the transmittance increased sharply during 78.4-82.3 ^{o}C, suggesting the presence of an UCST-type insoluble-soluble (I-S) phase transition (ca. 80.3 ^{o}C). In addition, polymer concentration obviously affected the LCST-type phase transition temperature of P(PMAB-COOH), which decreased with the increasing of the concentration (seeing ESI, Fig. S4). It may be reasonable that the polymer can assemble more easily at the higher concentration upon heating, which resulted in a lower the LCST-type phase transition temperature. Therefore, P(PMAB-COOH) showed a characteristic dual thermoreponsive properties, in which LCST and UCST can be detected on heating. This demonstrated that the carboxyl groups in P(PMAB-COOH) are a key factor in determining the phase transition behavior.

Table 2

Figure 4

Since P(PMAB), P(HPMAB) and P(PMAB-COOH) have tertiary amine groups, they can be regarded as week polyelectrolytes and the corresponding hydrophilicities may be affected by the pH values of aqueous medium.[8, 51, 52] Hence, their thermoresponsive properties are expected to be affected by the pH values of aqueous medium. To order to clarify this point, the thermally induced phase transition behaviors of P(PMAB), P(HPMAB) and P(PMAB-COOH) were investigated in phosphate buffer solution (PBS) at different pH values. Figure 5 represents the transmittance against temperature curves for P(PMAB), P(HPMAB) and P(PMAB-COOH) at 1.0 mg/mL in PBS at

different pH values corresponding to the heating processes. As can be seen in Figure 5, P(HPMAB) and P(PMAB-COOH) showed a LCST-type soluble-insoluble phase transition and an UCST-type insoluble-soluble phase transition whereas only a LCST-type soluble-insoluble phase transition can be observed for P(PMAB) on heating in PBS with the pH values from 4.7 to 7.8. All results are summarized in Table 2. P(PMAB) and P(HPMAB) can show thermally induced phase transition

Figure 5

behavior in PBS and the phenomena cannot be detected in water, indicating that ionic strength in PBS is a crucial role in determining the phase transition behavior.[51] The LCST-type phase transition temperatures of P(PMAB) were very narrow and increased from 40.5 to 58.8 °C with the pH values from 4.7 to 7.8 (Fig. 5a). In addition, the hysteresis of the LCST-type phase transition temperature can be observed during the cooling process (seeing ESI, Fig. S4). The type of hysteresis is relatively common with thermally responsive polymers[40, 44, 46] and may be ascribed to slow rehydration and chain disentanglement in the particle and aggregates. The LCST-type and UCST-type phase transition temperatures of P(HPMAB) increased from 53.4 to 79.1 °C and from 83.0 to 86.4 °C, respectively, with the pH values from 4.7 to 7.8 (Fig. 5b). For P(PMAB), the interactions between polymer chains with water possibly weakened on heating and polymer chains aggregated under the electrostatic interaction of counterions. Then, the LCST-type phase transition behavior was induced. Compared with P(PMAB), the existence of the hydroxyl groups in P(HPMAB) should be contributed to the formation of the soluble-insoluble-soluble (S-I-S) phase transition. On heating, the hydrogen bonding interactions between P(HPMAB) with water weakened and dissociated, and the intra- and inter-chain hydrogen bonds were formed. The LCST-type phase transition behavior of P(HPMAB) were induced under the coordination of the electrostatic interaction of counterions with polymer chains. With further increase in solution temperature, the intra- and inter-chain hydrogen bonding cooperatively dissociated and the electrostatic interaction weakened because of the activated motion of the polymer chains, which leaded to UCST-type phase transition behavior of P(HPMAB). Similar pH-dependent changes in the phase transitions were also observed in P(PMAB-COOH), in which the LCST-type and UCST-type phase transition temperatures changed from 62.2 to 78.3°C and from 79.4 to 88.3 °C, respectively, with the pH values from 4.7 to 7.8 (Fig. 5c). Figure 5d shows the pH ranges for the LCST-type and UCST-type phase transition temperatures of three polymers as a function of temperature. Both P(HPMAB) and P(PMAB-COOH) had a higher LCST-type phase transition

temperature than P(HPMAB) under the same solution conditions, suggesting that the addition of hydroxyl groups in P(HPMAB) and carboxyl groups in P(PMAB-COOH) leads to an obvious increase in LCST-type phase transition temperature. It may be due to the fact that P(HPMAB) and P(PMAB-COOH) have hydroxyl groups and carboxyl groups, respectively, resulting in the increase of the water solubility.[44, 53, 54] On the other hand, P(PMAB-COOH) had a higher LCST-type phase transition temperature than P(HPMAB) in pH below 7.0, and the LCST-type phase transition temperature of P(PMAB-COOH) was lower than that of P(HPMAB) in pH more than 7.0. It may be attributed to the partial ionization of carboxylic acid moiety when pH value is more than 7.0. In the UCST-type phase transition temperatures, P(HPMAB) and P(PMAB-COOH) exhibited similar pH-dependent changes (Fig. 5d) and the effect of pH value on the UCST was relatively small. It might be due to the fact that the motion of the polymer chains were enormously activated in higher solution temperature, and the effect of the hydrogen bonding and the electrostatic interaction on the UCST might be ignored. On the other hand, the UCST-type phase transition temperature of P(PMAB-COOH) by and large was lower than that of P(HPMAB) under our research solution conditions. It may be due to the fact that carboxyl groups are more hydrophilic than hydroxyl groups.

Cytotoxicity assay

For biomedical applications, it is necessary to evaluate the potential toxicity of the three synthetic polymers, P(PMAB), P(HPMAB) and P(PMAB-COOH). Here, *in vitro* cytotoxicity of P(PMAB), P(HPMAB) and P(PMAB-COOH) with different administered concentration ranging from 1.0 to 220 μg/mL against NIH 3T3 cells was studied through MTT assay. As shown in Figure 6, the cell viability after 48 h of incubation with P(PMAB) and P(HPMAB) below 220 μg/mL remains almost 100% compared with the untreated cells, suggesting very lower cytotoxicity of P(PMAB) and P(HPMAB). Although P(PMAB-COOH) due to the carboxyl groups possess a higher cytotoxicity than P(PMAB) and P(HPMAB) when the concentration is more than 28.0 μg/mL, the viability of NIH3T3 cells is more than 75% with the concentration 220 μg/mL, indicating that P(PMAB-COOH) shows a low cytotoxicity. The in vitro evalutions confirmed that the three synthetic polymers are highly biocompatible and may be regarded as biomedical materials.

Figure 6

Conclusion

Well-defined pH-tunable thermosensitive homopolymers P(PMAB), P(HPMAB) and P(PMAB-COOH) were successfully synthesized using ATRP and click reactions. P(PMAB-COOH) bearing carboxyl groups exhibited a LCST-type and an UCST-type phase transition in water, whereas P(PMAB) and P(HPMAB) failed to show a phase transition in water. It is suggested that the carboxyl groups in P(PMAB-COOH) are a key factor in determining the phase transition behavior. To the best of our knowledge, this is the first report that the homopolymer system in water can exhibit both LCST and UCST transition. On the other hand, P(HPMAB) and P(PMAB-COOH) exhibited a LCST-type and an UCST-type phase transition in phosphate buffer solutions (PBS), and P(PMAB) containing no hydroxyl or carboxyl groups only exhibited a LCST-type in PBS. The phase transition temperatures including LCST and UCST of polymers in PBS can be easily tuned by changing the pH values. Therefore, P(PMAB-COOH) exhibited dual responses to temperature and pH. At the same time, the in vitro evalutions also confirmed that the three synthetic polymers were less cytotoxic and may be regarded as biomedical materials.

Acknowledgement

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Table 1 Characterization of the Synthesized Polymers

Polymers [a]	yield [b]	M_n [c]	M_w/M_n [c]
P(PMAB)	85%	13 500	1.25
P(HPMAB)	87 %	15 900	1.21
P(PMAB-COOH)	84 %	21 300 [d]	1.20 [d]

a).P(PMAB-COOH) were prepared from P(HPMAB)

b).Determined by gravimetric method.

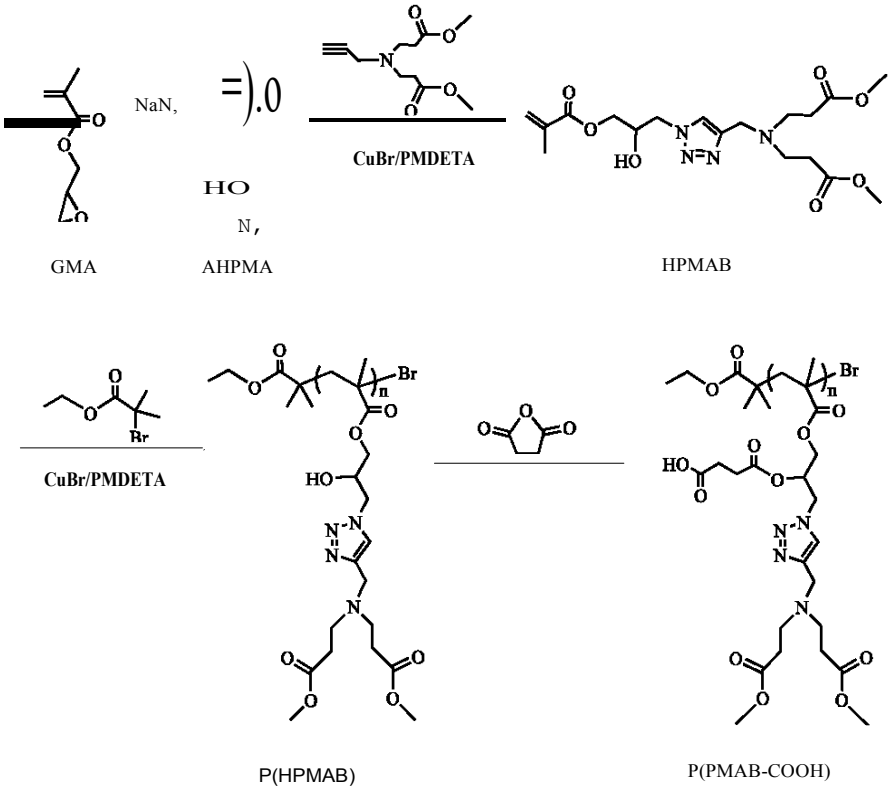
c). Determined by GPC in DMF.

d). The results of the methylated P(PMAB-COOH) was determined by GPC in DMF.

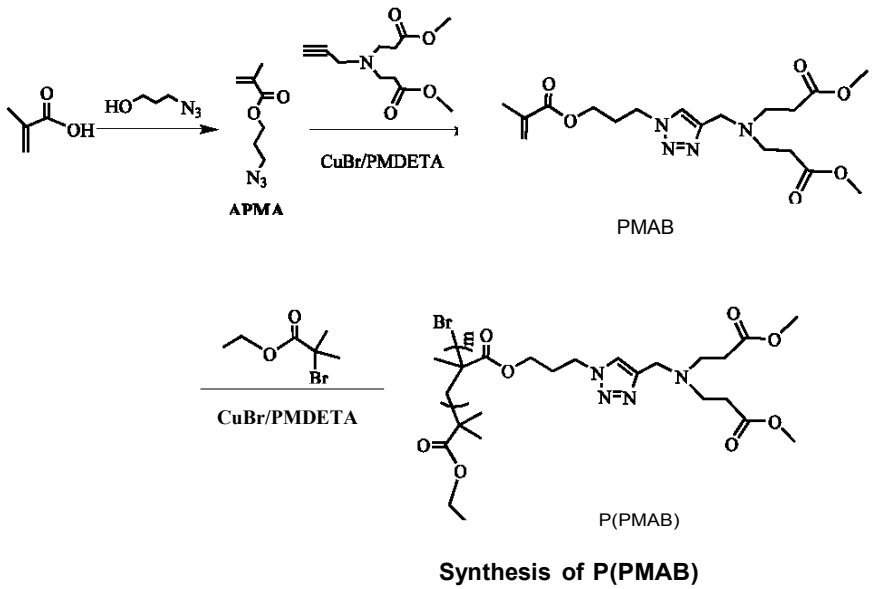
Table 2 LCST and UCST of polymers at the concentration of 1.0 mg/mL under different pH values

Polymers	LCST and UCST (°C) [a]				
	pH=4.7	pH=6.4	pH=6.8	pH=7.4	pH=7.8
P(PMAB)	40.5	48.9	51.7	57.3	58.8
P(HPMAB)	53.4 (83.0)	73.4 (85.7)	74.9 (86.4)	78.3 (87.7)	79.1 (86.4)
P(PMAB-COOH)	62.2 (79.4)	74.0 (83.5)	76.0 (85.3)	77.0 (86.0)	78.3 (88.3)

a). The values of LCST and UCST were determined at 50% transmittance. UCST values were shown in parentheses.



Synthesis of P(HPMAB) and P(PMAB-COOH)



Synthesis of P(PMAB)

Scheme 1 Synthetic route for P(HPMAB), P(PMAB-COOH) and P(PMAB).

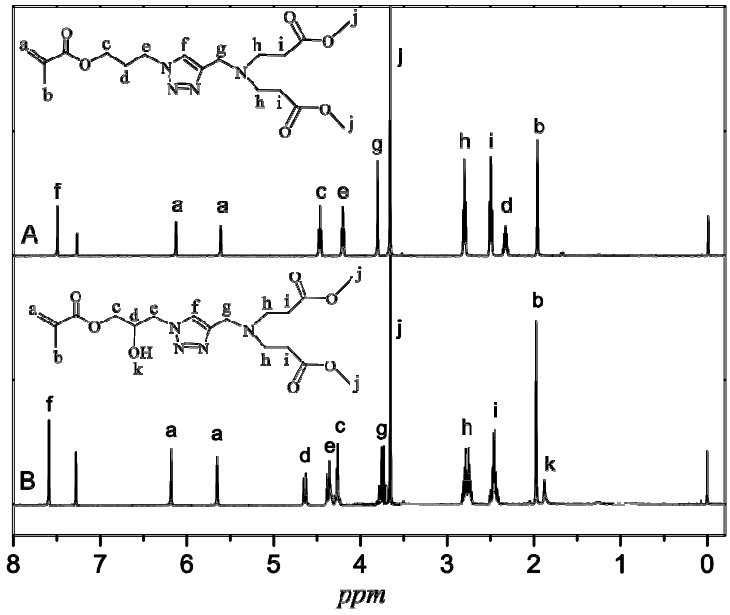


Figure 1 ^{1}H NMR spectra of the monomers PMAB (A) and HPMAB (B).

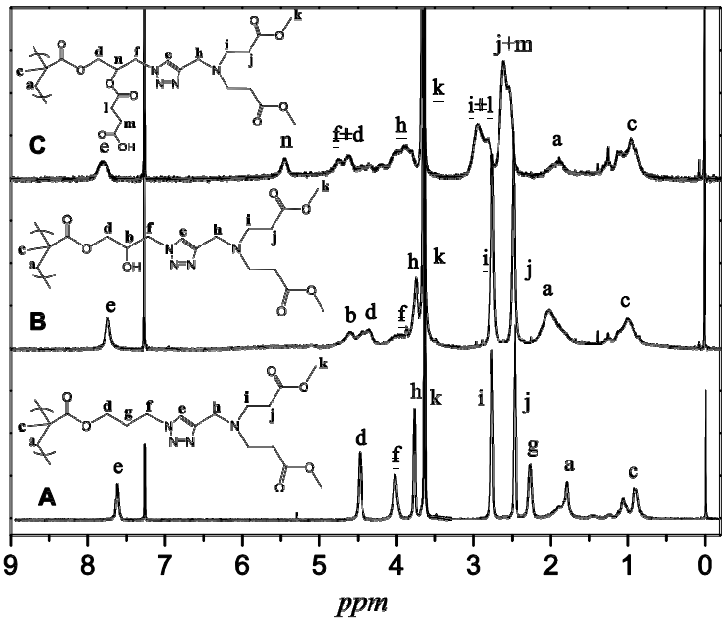


Figure 2 ^{1}H NMR spectra of the homopolymers P(PMAB) (A), P(HPMAB) (B) and P(PMAB-COOH) (C).

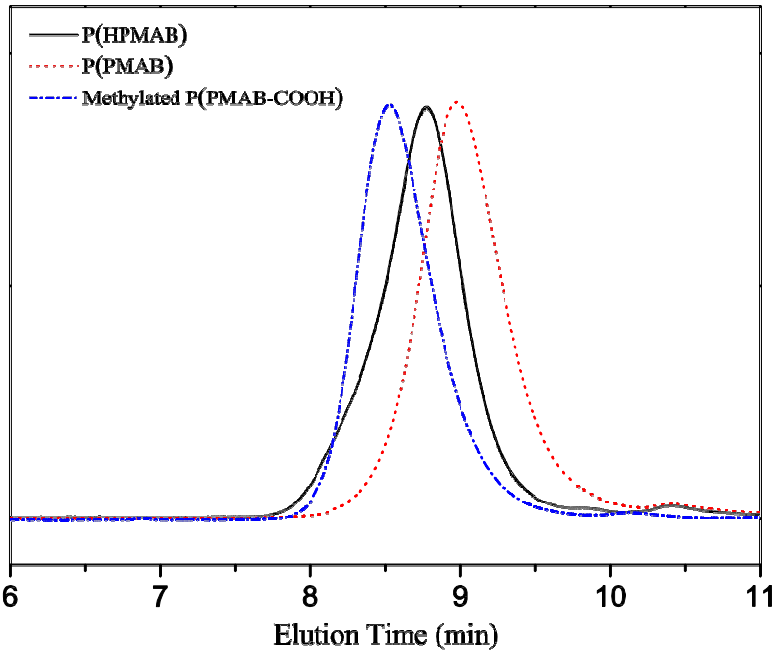


Figure 3 GPC traces of homopolymers.

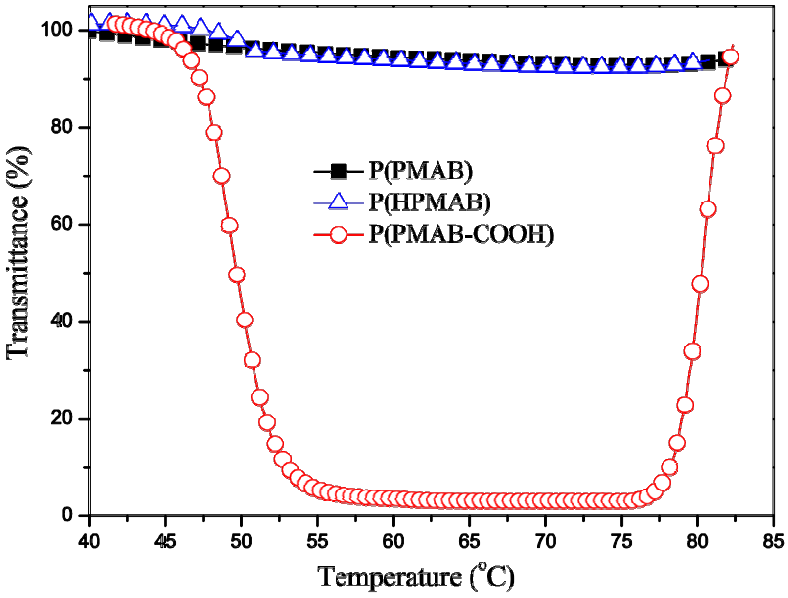


Figure 4 Temperature dependence of the transmittance of P(PMAB), P(HPMAB) and P(PMAB-COOH) at the concentration of 1.0 mg/mL in water.

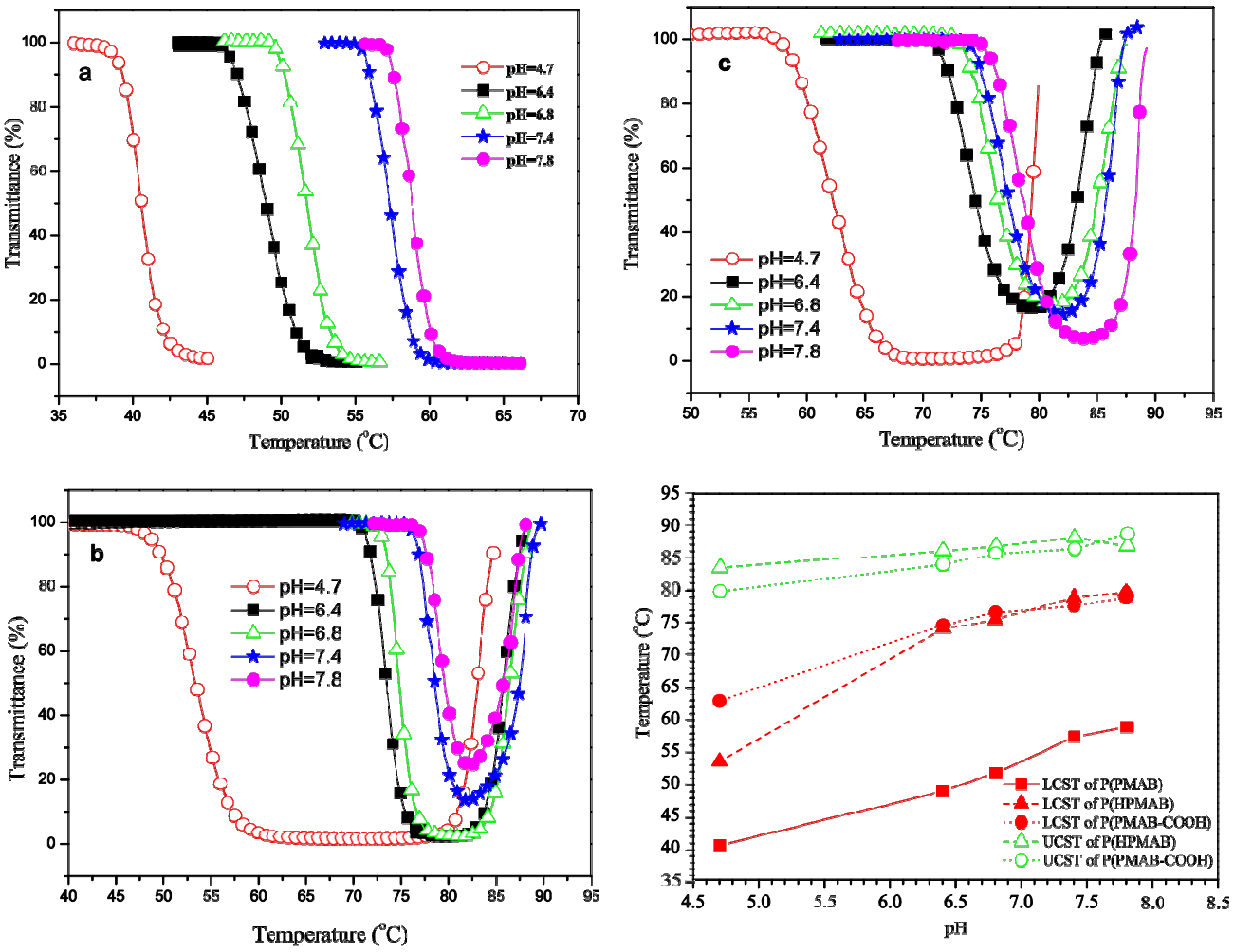


Fig. 5 Temperature dependence of the transmittance of (a) P(HMAB), (b) P(HPMAB) and (c) P(PMAB-COOH) with the concentration of 1.0 mg/mL at different pH in PBS. (d) pH dependence of transition temperatures for three polymers.

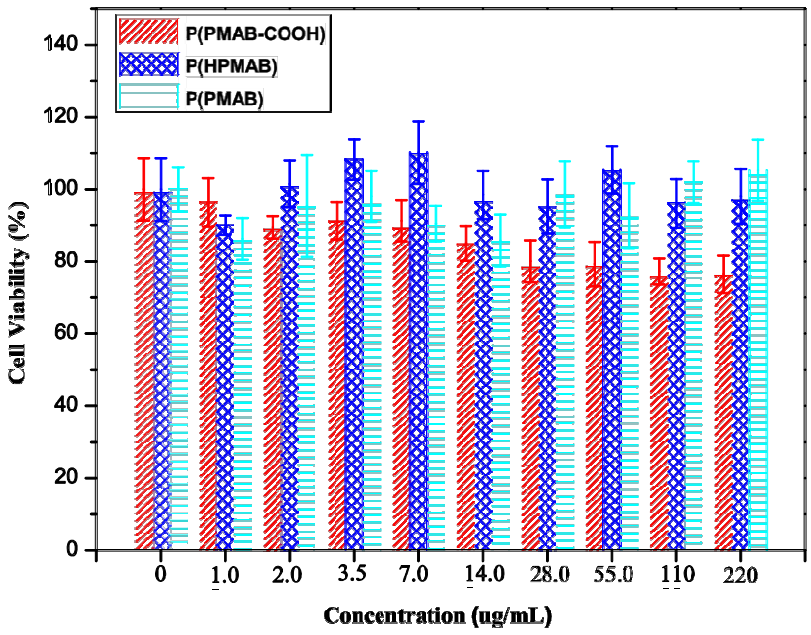
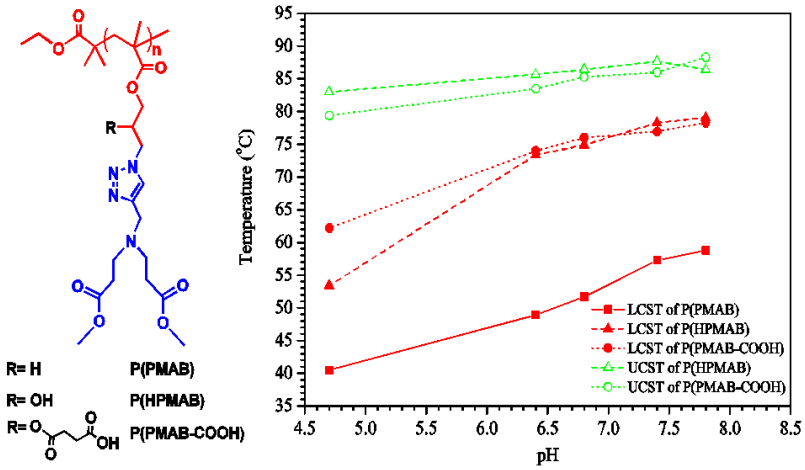


Figure 6. Cytotoxicity of P(PMAB), P(HPMAB) and P(PMAB-COOH) against NIH 3T3 cells.

Graphical abstract

Novel pH-tunable Thermoresponsive Polymers Displaying Lower and Upper Critical Solution Temperatures

Xin Cai, Liang Zhong, Yue Su, Shaoliang Lin, Xiaohua He



Novel pH-tunable thermoresponsive 3-azido-2-hydroxypropyl methacrylate-based polymers displaying LCST and UCST in phosphate buffer solutions were successfully synthesized by ATRP.