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Enzyme-Induced Formation of Thermoreversible Micellar Gels from Aqueous Solutions of Multiresponsive Hydrophilic ABA Triblock Copolymers

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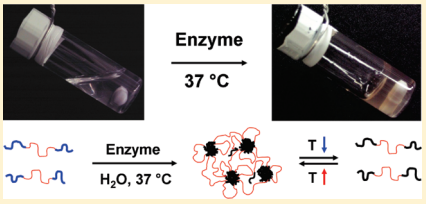
Supporting Information

ABSTRACT: We report on the synthesis of thermo- and enzyme-responsive hydrophilic ABA triblock copolymers, poly(ethoxydi(ethylene glycol) acrylate-*co*-4-((dihydroxyphosphoryl)oxy)butyl acrylate)-*b*-poly(ethylene oxide)-*b*-poly(ethoxydi(ethylene glycol) acrylate-*co*-4-((dihydroxyphosphoryl)oxy)butyl acrylate) (P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA)), and the enzyme-induced formation of thermoreversible micellar gels from their moderately concentrated aqueous solutions at 37 °C. PDEGEA is a thermosensitive water-soluble polymer with a lower critical solution temperature (LCST) at 9 °C in water. The block copolymers were prepared by atom transfer radical polymerization of DEGEA and 4-((di-*tert*-butoxyphosphoryl)oxy)butyl acrylate and subsequent removal of *tert*-butyl groups. To seek optimal conditions for enzymatic gelation of aqueous solutions of triblock copolymers, a study of dephosphorylation of a random copolymer P(DEGEA-*co*-OPBA) by acid phosphatase in water at 37 °C was carried out. The time for the solution to turn cloudy was found to decrease with the decrease of pH from 5.48 to 4.70 and level off from pH 4.39 to 4.23. The cleavage of phosphate groups made the polymer less hydrophilic and decreased the LCST from above to below 37 °C. Therefore, pH 4.4 was selected to conduct the enzyme-induced gelation of 7.9 wt % aqueous solutions of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA). The gelation processes were monitored by rheological measurements; the sol−gel transition temperature decreased and the gel strength increased with the increase of reaction time. The gels formed were thermoreversible; lowering temperature converted the gels to free-flowing liquids. From ^{1}H and ^{31}P NMR spectroscopy analysis, the degree of dephosphorylation was high. The formation of three-dimensional micellar network gels stemmed from the thermosensitive properties of the resultant dephosphorylated triblock copolymers, which was confirmed by a dynamic light scattering study. At a slightly higher pH (4.67), the enzyme-induced gelation was significantly slower, consistent with the observation of the effect of pH on dephosphorylation of the random copolymer by acid phosphatase.



INTRODUCTION

Polymer hydrogels have been intensively investigated for biomedical applications including contact lenses, sustained or triggered release of drugs and biomolecules, cell culture, and tissue engineering.[1−9] While chemically cross-linked polymer gels are being widely used and continuously evaluated, there is a growing interest in stimuli-responsive block copolymer aqueous micellar gels.[10−21] These physically cross-linked or jammed micellar gels can be more advantageous for some applications because of the in situ gelation of a liquid precursor induced by environmental changes, allowing, e.g., for minimally invasive administration by injection via syringe and needle. A notable example of injectable gel drug delivery systems, reported by Jeong et al., was based on aqueous solutions of block copolymers that underwent thermo-induced sol−gel transitions.[12] The polymer solutions were loaded with a model drug in the sol state at an elevated temperature. Upon subcutaneous injection at the body temperature, the solutions formed gels instantaneously that subsequently acted as matrices for sustained release of drug molecules.

In general, there are two types of stimuli-responsive block copolymer aqueous micellar gels: three-dimensional network gels, in which one block, e.g., the central block of an ABA triblock copolymer, forms bridges among micellar cores of other blocks,[13−16] and physically jammed micellar gels, in which discrete spherical micelles of block copolymers are jammed and packed into an ordered structure.[10,11,17] While the second type of gels typically form at a polymer concentration of ∼20 wt %, the critical gelation concentration (CGC) of ABA triblock copolymers with stimuli-sensitive outer blocks is significantly lower. For example, Kirkland et al. reported that a 7.5 wt % aqueous solution of poly(*N*-isopropylacrylamide)-*b*-poly(*N*,*N*-dimethyacrylamide)-*b*-poly(*N*-isopropylacrylamide) formed a free-standing gel upon heating.[13] We previously reported that the CGC of a thermosensitive ABA triblock copolymer in water was between 3 and 4 wt %.[14]

Of particular interest to us are block copolymer aqueous micellar gels that can respond to two or more external stimuli. Such gels would offer greater design flexibility and more advantages compared with those that respond to only one external stimulus. There have been a number of reports on multiresponsive aqueous polymer

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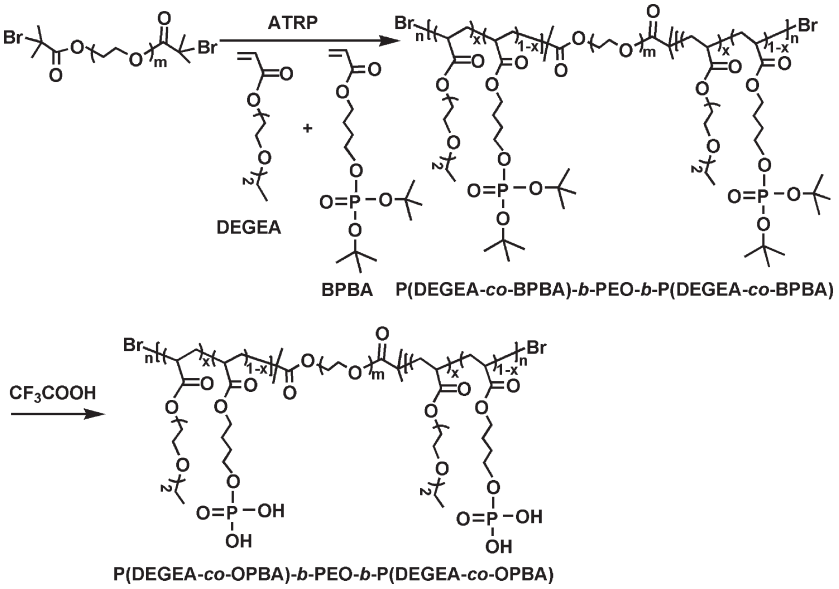
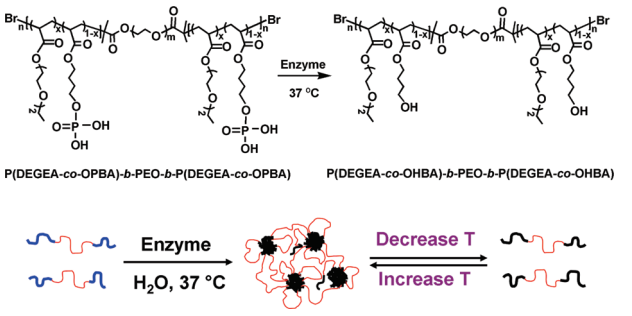
Scheme 1. Synthesis of Thermo- and Enzyme-Responsive ABA Triblock Copolymer P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA)



micellar gels.[18−21] For example, Li et al. reported the synthesis of thermo- and redox-sensitive ABA triblock copolymers and demonstrated the thermally and biochemically induced sol−gel transitions of aqueous solutions of these copolymers.[18a] Temperature- (*T*-) and pH-sensitive polymer micellar gels are probably the most studied and used multiresponsive gels.[19,20] The block copolymers used in these studies were usually prepared by either growing pH-sensitive blocks from or introducing carboxylic acid or other pH-responsive groups to the chain ends of an ABA triblock copolymer that could form thermoreversible gels in water (e.g., PEO-*b*-PPO-*b*-PEO).[19] Other types of multiblock copolymers were also employed.[20]

We previously reported thermo- and light- or pH-sensitive aqueous micellar gels of ABA triblock copolymers in which a small amount of stimuli-responsive functional groups was incorporated and randomly distributed in the thermosensitive outer blocks.[14,21] The LCST of the thermosensitive blocks can be modified by applying a second external stimulus. While we have shown that aqueous solutions of such triblock copolymers can undergo multiple sol−gel−sol transitions in response to environmental changes and the phase transitions can be well controlled, from a biological perspective, it is extremely attractive to use enzymes, a class of highly efficient and specific biocatalysts, to induce the gelation of aqueous solutions or develop enzyme-responsive hydrogels. One can imagine that such enzyme-responsive systems can be advantageous in some situations as they can respond to biological cues, e.g., the overexpression of specific enzymes for certain tissues or diseases. Nature actually uses enzymatic gelation to regulate the self-assembly of biomolecules in cytosol to locally switch part of the cell between the sol and gel states to control cell focal adhesion and migration.[22,23] Inspired by nature and motivated by biological perspectives, there have been many reports in the literature on enzyme-induced formation of small molecule hydrogels and covalently cross-linked polymer gels as well as enzyme-responsive cross-linked hydrogels.[23−34] For example, Xu et al. used kinase and phosphatase to control phosphorylation and dephosphorylation of a small molecule

Scheme 2. Dephosphorylation of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) by Acid Phosphatase and Schematic Illustration of Enzymatically Induced Formation of Thermoreversible Aqueous Micellar Network Gel



hydrogelator and to regulate the formation of supramolecular hydrogels.[24] Anseth et al. reported human neutrophil elastase-responsive poly(ethylene glycol) hydrogels for controlled release.[27]

Here we present the synthesis of thermo- and enzyme-responsive hydrophilic ABA triblock copolymers (Scheme 1) and the enzyme-induced formation of thermoreversible three-dimensional micellar network gels from their aqueous solutions at the physiological temperature, 37 °C. We utilize the enzyme-catalyzed dephosphorylation,[24,35] a common biochemical reaction, in our gel design; a small amount of hydrophilic phosphate groups is incorporated in a random distribution fashion into the thermosensitive outer blocks of ABA triblock copolymers. The enzymatic dephosphorylation decreases the LCST of thermosensitive blocks from above to below the experimental temperature, resulting in the formation of a thermoreversible three-dimensional network (Scheme 2). The multiresponsive triblock copolymers, poly(ethoxydi(ethylene glycol) acrylate-*co*-4-((dihydroxyphosphoryl)oxy)butyl acrylate)-*b*-PEO-*b*-poly(ethoxydi(ethylene glycol) acrylate-*co*-4-((dihydroxyphosphoryl)oxy)butyl acrylate)

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(P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA)), were synthesized by atom transfer radical polymerization (ATRP) of a mixture of DEGEA and 4-((di-*tert*-butoxyphosphoryl)oxy)butyl acrylate (BPBA) from a difunctional PEO macroinitiator and subsequent removal of *tert*-butyl groups (Scheme 1). PDEGEA is a thermosensitive polymer with a LCST of 9 °C in water,[21b,36] which belongs to a new class of thermosensitive polymers with a short oligo(ethylene glycol) pendant from each repeat unit.[37] We show that 7.9 wt % aqueous solutions of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) undergo gelation in the presence of acid phosphatase, an enzyme that catalyzes dephosphorylaton, at a constant temperature, 37 °C, and the gels formed are thermoreversible. To the best of our knowledge, this is the first report on the formation of physically cross-linked micellar gels of block copolymers in water induced by an enzyme at the physiological temperature. Compared with chemically cross-linked hydrogels, thermoreversible micellar gels have many advantages; a notable one is the facile removal.

EXPERIMENTAL SECTION

Materials. 1,4-Butanediol (99%), di-*tert*-butyl diisopropylphosphoramidite (95%), tetrazole solution (~0.45 M in acetonitrile), and acid phosphatase (Type II, from potato, lyophilized powder, 1.55 units/mg solid) were purchased from Aldrich and used as received. Acryloyl chloride (96%, Alfa Aesar) was used as received. *N*,*N*,*N*′,*N*′,*N*″-Pentamethyldiethylenetriamine (PMDETA, Aldrich), ethyl 2-bromoisobutyrate (EBiB, Aldrich), and anisole (99%, Acros) were distilled over calcium hydride under reduced pressure. CuBr (98%, Aldrich) was stirred in glacial acetic acid, filtered, and washed with absolute ethanol and diethyl ether. The purified CuBr was then dried in high vacuum and stored in a desiccator. Poly(ethylene oxide) (HO−PEO−OH, MW = 20 000 g/mol, Aldrich) was end-functionalized via the reaction with 2-bromoisobutyryl bromide as described in a previous publication,[14] yielding a difunctional PEO macroinitiator, Br−PEO−Br. Trifluoroacetic acid (99%, Acros), H_2O_2 solution (30%, Fisher Scientific, stabilized with sodium stannate), $Na_2S_2O_5$ (99%, Acros), citric acid (99%, Acros), and citric acid trisodium salt (99%, Acros) were used as received. All other chemicals were purchased from either Aldrich or Fisher/Acros and used without further purification.

General Characterization. Size exclusion chromatography (SEC) was carried out at room temperature using PL-GPC 20 (an integrated GPC system from Polymer Laboratories, Inc.) with a refractive index detector, one PLgel 5 μm guard column (50 × 7.5 mm), and two PLgel 5 μm mixed-C columns (each 300 × 7.5 mm, linear range of molecular weight from 200 to 2 000 000 according to Polymer Laboratories, Inc.). The data were processed using Cirrus™ GPC/SEC software (Polymer Laboratories, Inc.). THF was used as the carrier solvent at a flow rate of 1.0 mL/min. Standard polystyrenes with narrow polydispersity indexes (Polymer Laboratories, Inc.) were used for calibration. The ^{1}H (300 MHz), ^{13}C (75 MHz), and ^{31}P (121 MHz) NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer. The mass spectrometry analysis was performed at the Mass Spectrometry Center in the Department of Chemistry at the University of Tennessee, Knoxville, TN, using a JEOL (Peabody, MA) AccuTOF-D time-of-flight mass spectrometer with a DART (direct analysis in real time) ionization source.

Synthesis of 4-Hydroxybutyl Acrylate (OHBA). 1,4-Butanediol (26.9 g, 0.299 mol) and triethylamine (10.9 g, 0.108 mol) were dissolved in dichloromethane (75 mL) in a 250 mL three-necked round-bottom flask. The flask was then placed in an ice/water bath and the mixture was stirred with a magnetic stir bar under nitrogen atmosphere. A solution of acryloyl chloride (6.92 g, 0.0765 mol) in dichloromethane (20 mL) was added dropwise into the flask. The mixture was allowed to warm to room temperature and stirred overnight. The precipitate was filtered off and the solvent was then removed by a rotavapor. Diethyl ether was added and the mixture was washed twice with a saturated aqueous solution of sodium bicarbonate and then once with water. The organic layer was dried over anhydrous sodium sulfate overnight. After the removal of sodium sulfate, the solution was concentrated using a rotavapor. The crude product was purified by column chromatography using hexanes/ethyl acetate (v/v, 1:1) as eluent. The pure product was obtained as a nearly colorless liquid. ^{1}H NMR ($CDCl_3$): δ (ppm), 6.38 (d, 1H, C*H*H=CH), 6.09 (dd, 1H, CHH=C*H*−), 5.80 (d, 1H, CH*H*=CH−), 4.17 (t, 2H, −COOCH_2−), 3.67 (m, 2H, −CH_2OH), 1.77−1.60 (m, 4H, −CH$_2$CH_2CH_2CH$_2$−), 1.46 (s, 1H, −O*H*); ^{13}C NMR ($CDCl_3$): δ (ppm) 166.30, 130.67, 128.34, 64.30, 62.07, 28.95, 24.99. MS (ESI+): 145.09 ([M + H$^+$]).

Synthesis of 4-((Di-*tert*-butoxyphosphoryl)oxy)butyl Acrylate (BPBA). 4-Hydroxybutyl acrylate (1.13 g, 7.85 mmol) and di-*tert*-butyl diisopropylphosphoramidite (3.08 g, 0.0111 mol) were dissolved in dichloromethane (50 mL) in a 250 mL three-necked round-bottom flask. The flask was placed into an ice/water bath and a solution of tetrazole in acetonitrile (0.45 M, 20 mL, 9.0 mmol) was added dropwise into the flask. The mixture became hazy white in appearance. The reaction mixture was allowed to warm to room temperature and stirred overnight. The flask was again placed in an ice/water bath and a 30% aqueous solution of hydrogen peroxide (5 mL) was added into the flask in a dropwise fashion. The mixture became clear after the addition of H_2O_2. The reaction was kept at 0 °C for additional 3 h. A 10 wt % aqueous solution of $Na_2S_2O_5$ (30 mL) was slowly added into the reaction mixture to quench unreacted hydrogen peroxide (Caution! noxious fumes). The organic layer was separated, washed with water, and then dried over anhydrous sodium sulfate. The product was isolated by column chromatography using hexanes/ethyl acetate (1:1) as eluent. After drying in vacuum, the pure product was obtained as a colorless liquid (1.72 g, yield: 65.2%). ^{1}H NMR ($CDCl_3$): δ (ppm), 6.37 (d, 1H, C*H*H=CH−), 6.08 (dd, 1H, CHH=C*H*−), 5.79 (d, 1H, CH*H*=CH−), 4.16 (t, 2H, −COOCH_2−), 3.96 (m, 2H, −CH_2OP−), 1.74 (m, 4H, −CH$_2$CH_2CH_2CH$_2$−), 1.47 (s, 18H, −OC(CH_3)$_3$); ^{13}C NMR ($CDCl_3$): δ (ppm) 166.13, 130.60, 128.36, 82.07, 81.98, 66.13, 63.92, 30.28, 30.23, 29.81, 29.75, 26.86, 24.94. MS (ESI+): 337.18 ([M + H$^+$]).

Synthesis of P(DEGEA-*co*-BPBA) by Atom Transfer Radical Polymerization. CuBr (11.3 mg, 0.0788 mmol), ethoxydi(ethylene glycol) acrylate (DEGEA, 2.03 g, 10.8 mmol), 4-((di-*tert*-butoxyphosphoryl)oxy)butyl acrylate (BPBA, 0.351 g, 1.04 mmol), ethyl 2-bromoisobutyrate (EBiB, 10.6 mg, 0.0543 mmol), anisole (2.43 g), and *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine (29.5 mg, 0.170 mmol) were added into a 25 mL two-necked flask. After the mixture was degassed by three freeze−pump−thaw cycles, the flask was placed into a 90 °C oil bath. The reaction was monitored by both SEC and ^{1}H NMR spectroscopy analysis. After the polymerization proceeded for 175 min, the flask was removed from the oil bath and the solution was diluted with THF. The mixture was passed through an Al_2O_3/silica gel column using THF as eluent. The solution was concentrated via rotary evaporation and then precipitated three times in hexanes/diethyl ether (v/v = 80: 20, 100 mL). A nearly colorless polymer was obtained. SEC analysis results (polystyrene standards): $M_{n,SEC}$ = 12 000 g/mol; polydispersity index (PDI) = 1.14. The degree of polymerization (DP) of the polymer was calculated from the monomer conversion and the monomer-to-initiator ratio. The peaks located in the range of 3.9−4.4 ppm, which were from −CH_2OOC− of DEGEA and BPBA and −CH_2OP− from BPBA were used as the internal standard. The conversion was calculated from the integral values of the peaks located at 5.6−6.0 ppm (CH*H*=CH− from both BPBA and DEGEA monomers) at t = 0 and 175 min. The calculated DP was 82. The molar ratio of DEGEA and BPBA units in the copolymer was 100: 13.3, determined from the ^{1}H NMR spectrum using the peaks located at 3.9−4.4 ppm, which were from −CH_2OOC− of DEGEA and BPBA units and −CH_2OP− from BPBA units, and

1.2 ppm, which was the methyl peak of DEGEA units. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.19 (−COOCH_2− of DEGEA units), 4.05 (−COOCH_2- of BPBA units), 3.98 (−CH_2OP of BPBA units), 3.85−3.20 (−COO-$CH_2CH_2OCH_2CH_2OCH_2CH_3$ of DEGEA units), 2.32 (−CH_2CHC-OO- of both DEGEA and BPBA units), 2.05−1.36 (−CH_2CHCOO− of both DEGEA and BPBA units), 1.70 (−$COOCH_2CH_2CH_2CH_2$OP of BPBA units), 1.47 (s, −C(CH_3)$_3$ of BPBA units), and 1.20 (t, −OC-H_2CH_3 of DEGEA units). ^{31}P NMR ($CDCl_3$): δ (ppm), −9.71.

Synthesis of P(DEGEA-*co*-OPBA). P(DEGEA-*co*-BPBA) (0.200 g) was dissolved in methylene chloride (5 mL) in a 50 mL round-bottom flask, followed by addition of trifluoroacetic acid (TFA, 2.0 g). After the reaction mixture was stirred at room temperature for 48 h, the volatiles were removed by a rotary evaporator. The residue was dissolved in methylene chloride (25 mL) and the volatiles were evaporated again by a rotavapor. This process was repeated an additional two times to remove as much trifluoroacetic acid as possible. The polymer was purified by precipitation three times in hexanes/diethyl ether (100: 20, v:v) and then once in hexanes (100 mL). After drying under high vacuum, the polymer was obtained as a clear sticky solid with a yield of 91% (182.3 mg). The removal of *tert*-butyl groups of P(DEGEA-*co*-BPBA) was evidenced by the disappearance of the *tert*-butyl peak at 1.47 ppm in the ^{1}H NMR spectrum and the shift of the ^{31}P peak from −9.71 to 1.02 ppm in the ^{31}P NMR spectra. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.18 (−COOCH_2− of DEGEA units), 4.06 (−COOCH_2− and −CH_2OP of OPBA units), 3.85−3.20 (−$COOCH_2CH_2OCH_2CH_2OCH_2CH_3$ of DEGEA units), 2.32 (−CH_2CHCOO− of both DEGEA and OPBA units), 2.05−1.30 (−CH_2CHCOO− of both DEGEA and OPBA units), 1.74 (−COO-$CH_2CH_2CH_2CH_2$OP of OPBA units), and 1.18 (t, −OCH_2CH_3 of DEGEA units). ^{31}P NMR ($CDCl_3$): δ (ppm), 1.02.

Synthesis of P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA). Below is a typical procedure for the synthesis of P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA). CuBr (14.6 mg, 0.102 mmol), difunctional PEO macroinitiator Br-PEO-Br (0.864 g, 0.043 mol), DEGEA (3.237 g, 17.22 mmol), BPBA (0.610 g, 1.81 mmol), and anisole (3.486 g) were weighed into a 25 mL two-necked flask, followed by the injection of PMDETA (18.8 mg, 0.109 mmol) via a microsyringe. The mixture was degassed by three freeze−pump−thaw cycles. The polymerization was started by placing the flask into a 90 °C oil bath. After 110 min, the flask was removed from the oil bath and the mixture was diluted with THF. The copper catalyst was removed by passing the solution through a short neutral aluminum oxide/silica gel column with THF as eluent. The polymer solution was then concentrated via rotary evaporation and precipitated in hexanes/ethyl ether (v/v = 80: 20, 100 mL). Size exclusion chromatography analysis results (polystyrene standards): $M_{n,SEC}$ = 49800 g/mol and the polydispersity index (PDI) = 1.12. ^{1}H NMR spectroscopy analysis showed that the numbers of DEGEA and BPBA units in the triblock copolymer were 143 and 28, respectively. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.18 (−COOCH_2− of DEGEA units), 4.04 (−COOCH_2− of BPBA units), 3.97 (−CH_2OP of BPBA units), 3.90−3.30 (−CH_2CH_2O− of PEO block and −$COOCH_2CH_2OCH_2CH_2OCH_2CH_3$ of DEGEA units), 2.32 (−CH_2CHCOO− of both DEGEA and BPBA units), 2.05−1.36 (−CH_2CHCOO− of both DEGEA and BPBA units), 1.70 (−$COOCH_2CH_2CH_2CH_2$OP of BPBA units), 1.47 (s, −C(CH_3)$_3$ of BPBA units), and 1.19 (t, −OCH_2CH_3 of DEGEA units). ^{31}P NMR ($CDCl_3$): δ (ppm), −9.71.

Synthesis of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA). P(DEGEA-*co*- BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA) (0.987 g, $M_{n,SEC}$ = 49800 g/mol, PDI = 1.12) was dissolved in dry dichloromethane (10 mL) in a 20 mL scintillation vial, followed by the addition of trifluoroacetic acid (3.813 g). The reaction mixture was stirred at room temperature for 48 h. The volatiles were then removed using a rotavapor. The residue was dissolved in dichloromethane (20 mL) and the volatiles were evaporated again by a rotavapor. This process was repeated an additional two times to remove as much trifluoroacetic acid as possible. The polymer was then dissolved in THF (5 mL), precipitated in hexanes/diethyl ether (v/v = 80: 20, 100 mL) three times, and dried in high vacuum. The removal of the *tert*-butyl groups was confirmed by ^{1}H NMR spectroscopy analysis; the *tert*-butyl peak located at 1.47 ppm disappeared. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.18 (−COOCH_2− of DEGEA units), 4.06 (−COOCH_2− and −CH_2OP of OPBA units), 3.90−3.30 (−CH_2CH_2O− of PEO block and −$COOCH_2CH_2OCH_2CH_2OCH_2CH_3$ of DEGEA units), 2.32 (−CH_2CHCOO- of both DEGEA and OPBA units), 2.05−1.36 (−CH_2CHCOO− of both DEGEA and OPBA units), 1.74 (−$COOCH_2CH_2CH_2CH_2$OP of OPBA units), and 1.19 (−OCH_2CH_3 of DEGEA units). ^{31}P NMR ($CDCl_3$): δ (ppm), 1.02.

Preparation of 0.5 wt % Aqueous Solution of P(DEGEA-*co*-OPBA) and Study of pH Dependence of Cloud Point of P(DEGEA-*co*-OPBA) in Water. A 0.5 wt % aqueous solution of P(DEGEA-*co*-OPBA) was made by dissolving the random copolymer (33 mg, dried in high vacuum) in a 50 mM aqueous citrate buffer. The vial was then placed into the water bath of a Fisher Scientific Isotemp refrigerated circulator. The temperature was gradually increased from 8 °C. At each temperature, the sample was allowed to equilibrate for 20 min. When the solution turned cloudy, the temperature was recorded as the cloud point. The pH of the solution was then adjusted by using either 1.0 M KOH or 1.0 M HCl and the cloud point was determined by visual examination as described above.

Preparation of 0.5 wt % Aqueous Solutions of P(DEGEA-*co*-OPBA) and Study of pH Effect on Acid Phosphatase-Catalyzed Dephosphorylation. P(DEGEA-*co*-OPBA) was added into a preweighed 100 mL round-bottom flask and dried under high vacuum in a 60 °C oil bath for 3 h. The mass of the dried polymer was 91.5 mg. A 50 mM aqueous citrate buffer with pH of 5.10 (18.235 g) was added into the flask and the mixture was sonicated in an ultrasonic ice/water bath to dissolve the polymer. The resultant homogeneous polymer solution was distributed roughly equally into six glass vials with an inner diameter of 12 mm. The solutions were then adjusted to desired pH values by injecting either 1.0 M KOH or 1.0 M HCl aqueous solution via a microsyringe. An aqueous solution of acid phosphatase with a concentration of 1.99 wt % was made by dissolving 14.2 mg of acid phosphatase in deionized water (0.698 g). A calculated amount of the enzyme solution was added into each vial via a microsyringe; the enzyme-to-polymer mass ratio for all solutions was 7.5:100. The vials were then placed into a 37 °C water bath. The time at which a solution turned cloudy was recorded as the clouding time. For the samples with pH of 4.23 and 5.48, the polymers were isolated at the clouding times and the ^{1}H NMR spectra were recorded. Below is the ^{1}H NMR data of the polymer isolated from the experiment at pH = 4.23. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.18 (−COOCH_2− of both DEGEA and OHBA units), 3.85−3.30 (−$COOCH_2CH_2OCH_2CH_2OCH_2CH_3$ of DEGEA units and −CH_2CH_2OH of OHBA units), 2.32 (−CH_2CHCOO− of both DEGEA and OHBA units), 2.08−1.29 (−CH_2CHCOO− of both DEGEA and OHBA units, and −$COOCH_2CH_2CH_2CH_2$OH of OHBA units), and 1.19 (−OCH_2CH_3 of DEGEA units).

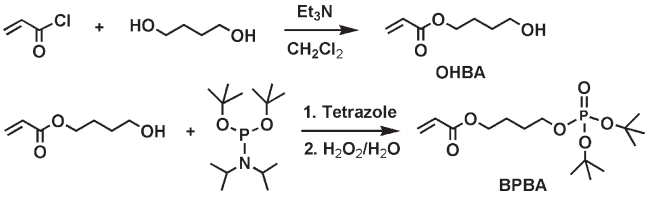
Preparation and Enzyme-Induced Gelation of 7.9 wt % Aqueous Solutions of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA). Below is a typical procedure for an enzyme-induced gelation experiment. P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (**ABA-1**) was added into a preweighed 3.7 mL vial. The vial was placed into a larger flask and dried under high vacuum in a 60 °C oil bath for 3 h. The mass of the dried polymer was 0.115 g. The polymer was then dissolved in a 50 mM aqueous citrate buffer with a pH value of 4.90 (1.335 g) and the pH of the solution was adjusted to 4.40 by using a 1.0 M HCl solution. After acid phosphatase (5.60 mg) was added, the vial was capped tightly and placed into a 37 °C water bath (t = 0 min). The mixture was stirred with a magnetic stir bar. After the solution became viscous and the

stir bar did not move freely, aliquots were begun to take at various time intervals for rheological measurements. Each time, the vial was removed from the 37 °C water bath and placed into an ice/water bath. After a 90 μL aliquot was withdrawn, the vial was sealed and immediately placed back into the 37 °C water bath. The reaction was stopped after the rheological measurements showed no change in the sol−gel transition temperature. The resultant polymer was isolated for ^{1}H NMR spectroscopy analysis. ^{1}H NMR ($CDCl_3$): δ (ppm), 4.18 (−COOCH_2− of both DEGEA and OHBA units), 3.90−3.30 (−CH_2CH_2O− of PEO block, −COOCH$_2$CH_2OCH_2CH_2OCH_2CH$_3$ of DEGEA units, and −CH$_2$CH_2OH of OHBA units), 2.32 (−CH$_2$CHCOO− of both DEGEA and OHBA units), 2.00−1.34 (−CH_2CHCOO− of both DEGEA and OHBA units, and −COOCH$_2$CH_2CH_2CH$_2$OH of OHBA units), and 1.19 (−OCH$_2$CH_3 of DEGEA units).

Rheological Measurements. Rheological experiments were conducted using a rheometer from TA Instruments (Model TA AR2000ex). A cone−plate geometry with a cone diameter of 20 mm and an angle of 2° (truncation 52 μm) was employed; the temperature was controlled by the bottom Peltier plate. In each measurement, 90 μL of a polymer solution was loaded onto the plate by a micropipet. The solvent trap was filled with water and a solvent trap cover was used to minimize water evaporation. Dynamic storage (G') and loss moduli (G'') of a polymer solution were measured by oscillatory shear experiments performed at a frequency of 1 Hz in a heating ramp at a heating rate of 3 °C/min. The frequency dependences of G' and G'' of a polymer solution at selected temperatures were obtained by frequency sweep tests. A strain amplitude of γ = 0.2% was used in all dynamic tests to ensure that the deformation was within the linear viscoelastic regime.

Dynamic Light Scattering Study of 0.02 wt % Aqueous Solution of P(DEGEA-*co*−OHBA)-*b*-PEO-*b*-P(DEGEA-*co*−OHBA). The thermo-induced micellization at a concentration of 0.02 wt % of P(DEGEA-*co*−OHBA)-*b*-PEO-*b*-P(DEGEA-*co*−OHBA), obtained from the enzymatic dephosphorylation of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), was studied by dynamic light scattering (DLS). The 0.02 wt % aqueous solution of P(DEGEA-*co*−OHBA)-*b*-PEO-*b*-P(DEGEA-*co*-OHBA) was prepared by diluting the corresponding 7.9 wt % polymer solution after the enzymatic dephosphorylation with a 50 mM aqueous citrate buffer. The pH value of the solution was maintained at 4.40. The DLS measurement was conducted with a Brookhaven Instruments BI-200SM goniometer equipped with a PCI BI-9000AT digital correlator, a temperature controller, and a solid-state laser (model 25-LHP-928−249, λ = 633 nm) at a scattering angle of 90°. The polymer solution was filtered into a borosilicate glass tube with an inner diameter of 7.5 mm using a 0.2 μm hydrophilic PTFE filter. The glass tube was then sealed with a PE stopper. The sample was placed into the cell holder of the light scattering instrument and gradually heated. At each temperature, the solution was equilibrated for 20 min prior to data recording. The time correlation functions were analyzed with a Laplace inversion program (CONTIN).

Scheme 3. Synthesis of 4-((Di-*tert*-butoxyphosphoryl)oxy)butyl Acrylate (BPBA)



RESULTS AND DISCUSSION

Synthesis of Monomer 4-((Di-*tert*-butoxyphosphoryl)oxy)-butyl Acrylate (BPBA). BPBA was synthesized via a two-step procedure as shown in Scheme 3. 4-Hydroxybutyl acrylate (OHBA) was prepared first through the reaction of acryloyl chloride with a large excess of 1,4-butanediol. The phosphorylation of OHBA was conducted using 1.4 equiv of di-*tert*-butyl diisopropylphosphoramidite in the presence of 1.1 equiv of tetrazole, followed by oxidation with a 30% aqueous solution of hydrogen peroxide. The pure product was isolated by column chromatography using hexanes/ethyl acetate (1:1, v/v) as eluent and the molecular structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy as well as mass spectroscopy analysis.

Preparation of Thermo- and Enzyme-Responsive Hydrophilic ABA Triblock Copolymers and a Random Copolymer. The thermo- and enzyme-responsive hydrophilic ABA triblock copolymers, P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), were prepared according to the procedure illustrated in Scheme 1. The precursor polymers, P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA), were synthesized by ATRP of a mixture of DEGEA and BPBA from a difunctional PEO macroinitiator with a molecular weight of 20000 g/mol at 90 °C using CuBr/PMDETA as catalyst. Figure 1A shows the SEC traces of PEO macroinitiator and an ABA triblock copolymer P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA) (**ABA-1-P** in Table 1). The peak shifted to the high molecular weight region and remained narrow, though there was a small shoulder peak on the front side, which was likely from the

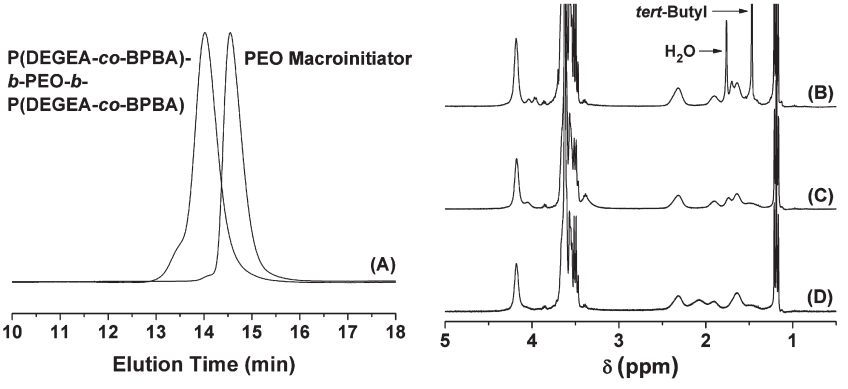


Figure 1. (A) Size exclusion chromatography traces of PEO macroinitiator and an ABA triblock copolymer P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA) (**ABA-1-P**) and ^{1}H NMR spectra of (B) **ABA-1-P**, (C) **ABA-1**, and (D) the dephosphorylated triblock copolymer obtained from the enzymatic gelation experiment of **ABA-1**. $CDCl_3$ was used as solvent in ^{1}H NMR spectroscopy. The sharp peak at 1.76 ppm in part B is from water.

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Table 1. Characterization Data for Multiresponsive ABA Triblock Copolymers P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), Random Copolymer P(DEGEA-*co*-OPBA), and their Precursor Polymers

polymers[a]	$M_{n,SEC}$ (g/mol)[b]	PDI[b]	numbers of DEGEA and BPBA (or OPBA) units[c]
ABA-1-P	49 800	1.12	143, 28
ABA-1	NA	NA	143, 28
ABA-2-P	58 900	1.11	187, 45
ABA-2	NA	NA	187, 45
R-1-P	12 000	1.14	72, 10
R-1	NA	NA	72, 10

[a] **ABA-1** and **-2** are thermo- and enzyme-responsive ABA triblock copolymers P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), which were obtained by the removal of *tert*-butyl groups of **ABA-1-P** and **ABA-2-P** (P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA)), respectively. [b] The number-average molecular weights ($M_{n,SEC}$) and polydispersity indexes (PDI) were measured by SEC using polystyrene calibration. [c] The numbers of DEGEA and BPBA (or OPBA) units in the copolymers were calculated from ^{1}H NMR spectra.

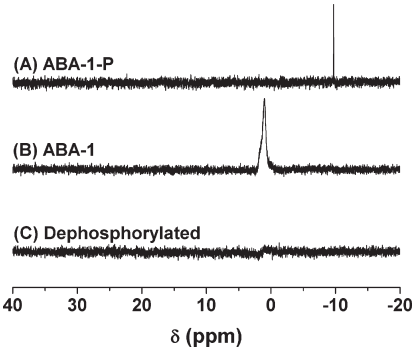

Figure 2. ^{31}P NMR spectra of (A) P(DEGEA-*co*-BPBA)-*b*-PEO-*b*-P(DEGEA-*co*-BPBA) (**ABA-1-P**), P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (**ABA-1**), and (C) the dephosphorylated triblock copolymer obtained from the enzymatic gelation experiment of **ABA-1**.

product of the coupling reaction. The number-average molecular weight ($M_{n,SEC}$) and polydispersity index (PDI) of **ABA-1-P** were 49.8 kDa and 1.12, respectively (relative to polystyrene standards). The numbers of DEGEA and BPBA units in the copolymer were determined from ^{1}H NMR spectrum (Figure 1B) using the peaks located at 4.36−3.90 ppm (−C*H*$_2$OCO of DEGEA units, −C*H*$_2$OCO and −C*H*$_2$OP− of BPBA units), the peaks at 3.90−3.30 ppm (−C*H*$_2$C*H*$_2$O− of PEO block and −COOCH$_2$-C*H*$_2$OC*H*$_2$C*H*$_2$OC*H*$_2$CH$_3$ of DEGEA units), and the peaks at 1.35−1.00 ppm (−C*H*$_3$ of DEGEA units). They were 143 and 28, respectively. The *tert*-butyl groups in the copolymer were then cleaved using trifluoroacetic acid (TFA), yielding the targeted thermo- and enzyme-responsive ABA triblock copolymer P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA). Figure 1C shows the ^{1}H NMR spectrum of **ABA-1**; the removal of *t*-butyl groups was evidenced by the disappearance of the *t*-butyl peak in the ^{1}H NMR spectrum. TFA is known not to affect other ester bonds.[37f] Parts A and B of Figure 2 display the ^{31}P NMR spectra of **ABA-1-P** and **ABA-1**, respectively. The ^{31}P peak shifted from −9.7 ppm to +1.0 ppm, also indicating the successful removal of *tert*-butyl groups.

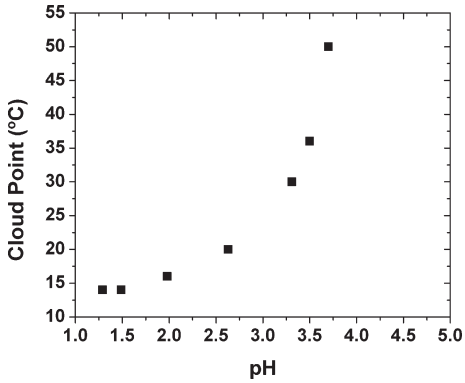

Figure 3. pH dependence of cloud point of P(DEGEA-*co*-OPBA) (**R-1**) in a 50 mM citrate buffer at a concentration of 0.5 wt %. The cloud points were determined by visual examination.

Two ABA triblock copolymers with slightly different molecular weights and compositions were synthesized and used in this work. The characterization data for these copolymers and their precursors are summarized in Table 1. To seek optimal conditions for enzymatic gelation of moderately concentrated aqueous solutions of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), we synthesized a random copolymer P(DEGEA-*co*-OPBA) (**R-1**). Note that all precursor polymers had relatively narrow molecular weight distributions (PDI < 1.15).[38]

pH Dependence of Cloud Point of P(DEGEA-*co*-OPBA). We chose DEGEA as the main component for the thermosensitive blocks to construct thermo- and enzyme-responsive ABA triblock copolymers because the cloud point of PDEGEA in water is 9 °C.[21b] The cleavage of hydrophilic phosphate groups from thermosensitive blocks would yield hydrophobic 4-hydroxybutyl groups (Scheme 2), decrease the LCST of thermosensitive blocks, and induce the gelation of moderately concentrated aqueous polymer solutions at 37 °C, the temperature intended for the enzymatic reaction.

Since phosphoric acid is a weak acid and the cloud point in water of a thermosensitive polymer that contains a small amount of weak acid groups is known to depend on solution pH,[39] we studied the pH dependence of cloud point of a random copolymer P(DEGEA-*co*-OPBA) (**R-1**). The cloud points of aqueous solutions of **R-1** in 50 mM citrate buffers at various pH values were measured (Figure 3). We found that the cloud point of this polymer at pH = 3.70 was 50 °C. When the pH was ≥3.80, no cloud point was observed in the studied temperature range (up to 80 °C). Apparently, the ionization of phosphate groups at pH ≥ 3.80 made the polymer very hydrophilic and caused the LCST to be above 80 °C or disappear. Note that the pK_{a1} of H_3PO_4 is 2.15. With the decrease of pH from 3.70 to 1.49, the cloud point gradually decreased from 50 to 14 °C. No change was observed when the pH was further lowered to 1.29.

pH Effect on Dephosphorylation of Random Copolymer P(DEGEA-*co*-OPBA) at 37 °C. The activity of acid phosphatase in the catalysis of dephosphorylation reaction is known to be sensitive to solution pH.[40] To seek optimal conditions for enzymatic gelation of aqueous solutions of ABA triblock copolymers, we studied how the dephosphorylation of **R-1** by acid phosphatase was affected by solution pH. Six 0.5 wt % aqueous solutions of **R-1** with pH ranging from 4.23 to 5.48 were made in small glass vials using 50 mM citrate buffers, followed by the addition of acid phosphatase (the mass ratio of the enzyme to the copolymer was

7.5: 100 for all samples). The vials were placed immediately in a 37 °C water bath (t = 0 min). When the solution turned cloudy, the time was recorded as clouding time ($t_{clouding}$). We found that the clouding time decreased with the decrease of pH, from 385 min at pH 5.48 to 96 min at pH 4.93, to 41.4 min at pH 4.70, and 7.6 min at pH 4.39 (Figure 4). Further lowering the pH did not change the clouding time much ($t_{clouding}$ = 5.4 min at pH 4.23). Since the cloud point of the copolymer with a very small amount of uncleaved phosphate groups could depend on the solution pH, we performed ^{1}H NMR spectroscopy analysis to look into the degree of the cleavage of phosphate groups at the clouding time. We isolated the polymers from the samples with pH of 4.23 and 5.48 at the clouding times by evaporating water of a small portion of each sample using a stream of nitrogen flow, dried them at 60 °C under high vacuum, and then analyzed them by ^{1}H NMR spectroscopy. The two ^{1}H NMR spectra appeared to be similar;[38] the peak located at 4.06 ppm, which was from $-CH_2OCO-$ and $-CH_2OP-$ of OPBA units in the copolymer, mostly disappeared (the peak of $-CH_2OCO$- shifted to ~4.2 ppm and overlapped with the peak there while the peak of $-CH_2OP$- shifted to ~3.7 ppm and overlapped with the peaks there). This observation suggested that the degrees of the cleavage of phosphate groups at the two pH values were quite similar, though the clouding times were very different.

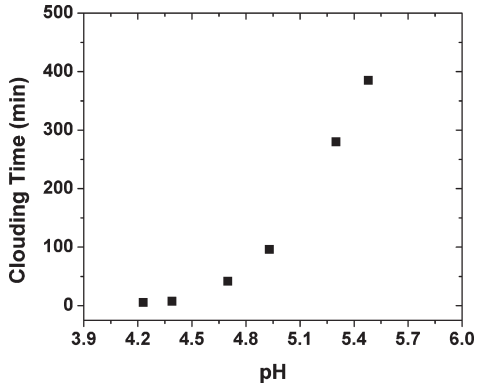

Figure 4. Plot of clouding time, the time for a 0.5 wt % solution of P(DEGEA-*co*-OPBA) (**R-1**) in a 50 mM aqueous citrate buffer in the presence of acid phosphatase at 37 °C to turn cloudy, as a function of solution pH.

Enzyme-Induced Gelation of a 7.9 wt % Aqueous Solution of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (ABA-1) at pH = 4.40 and 37 °C. In light of the observations shown in Figure 4, we chose pH = 4.4 to study the enzymatically induced gelation of a 7.9 wt % aqueous solution of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (**ABA-1**) at 37 °C. The 7.9 wt % aqueous solution of **ABA-1** was made by using a 50 mM citrate buffer and the pH was adjusted to 4.40. A calculated amount of acid phosphatase was added into the polymer solution (the mass ratio of the enzyme to the thermosensitive blocks was 7.5:100, same as that in the enzymatic cleavage experiment of **R-1**). The vial was capped tightly and placed into a 37 °C water bath (t = 0 min). Note that before the addition of the enzyme, the solution exhibited no change in the viscosity upon heating up to 80 °C.

After 1 h, the solution became viscous and the magnetic stir bar did not move freely. The stirrer stopped moving after 2 h. Figure 5 shows the optical pictures of the sample at various times. Evidently, the solution became very viscous after 4.5 h (Figure 5D) and was almost a gel at 6.5 h (Figure 5E). After the reaction proceeded for 8 h, the sample turned into a gel as it remained immobile when tilted or inverted (Figure 5F). The gel was thermoreversible; placing the vial in an ice/water bath transformed the gel into a free-flowing liquid. To quantitatively monitor the gelation process, aliquots were taken at various time intervals (the vial was placed in an ice/water bath to decrease the solution viscosity) and oscillatory shear experiments were conducted at a frequency of 1 Hz in a heating ramp mode at a heating rate of 3 °C/min. A strain amplitude of γ = 0.2% was used for all aliquots to ensure that the measurements were taken in the linear viscoelastic regime.

Figure 6 shows the rheological data for the selected aliquots taken from the sample at 180 (A), 390 (B), 480 (C), and 1314 min (D).[38] Clearly, for all these aliquots, below a certain temperature, the values of dynamic storage modulus G' and loss modulus G'' were small and the data points were quite scattered, indicating that they were liquids.[41] With the increase of temperature, G' and G'' increased and at a certain point G' became larger than G'', suggesting that the aliquots turned into gels. The crossover, $G' = G''$, has been commonly employed as an indicator of the sol−gel transition.[42] Using this simple and convenient method, the sol-to-gel transition temperatures ($T_{sol-gel}$) of the aliquots were determined and a plot of $T_{sol-gel}$ versus time was made (Figure 7A). At the beginning of the experiment, the $T_{sol-gel}$ decreased relatively fast, from 53.1 °C at 82 min to 41.0 °C at 180 min. After that, the

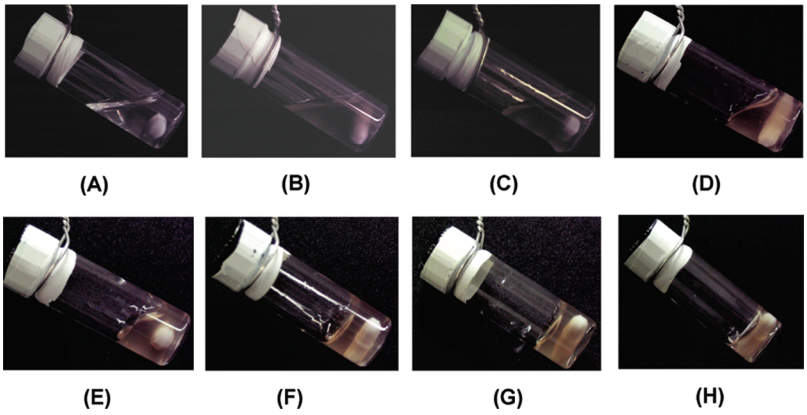

Figure 5. Digital optical pictures of a 7.9 wt % solution of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (**ABA-1**) in a 50 mM aqueous citrate buffer with pH of 4.40 before the addition of acid phosphatase (A) and after the enzymatic reaction at 37 °C for 0 (B), 180 (C), 270 (D), 390 (E), 480 (F), 593 (G), and 1314 min (H).

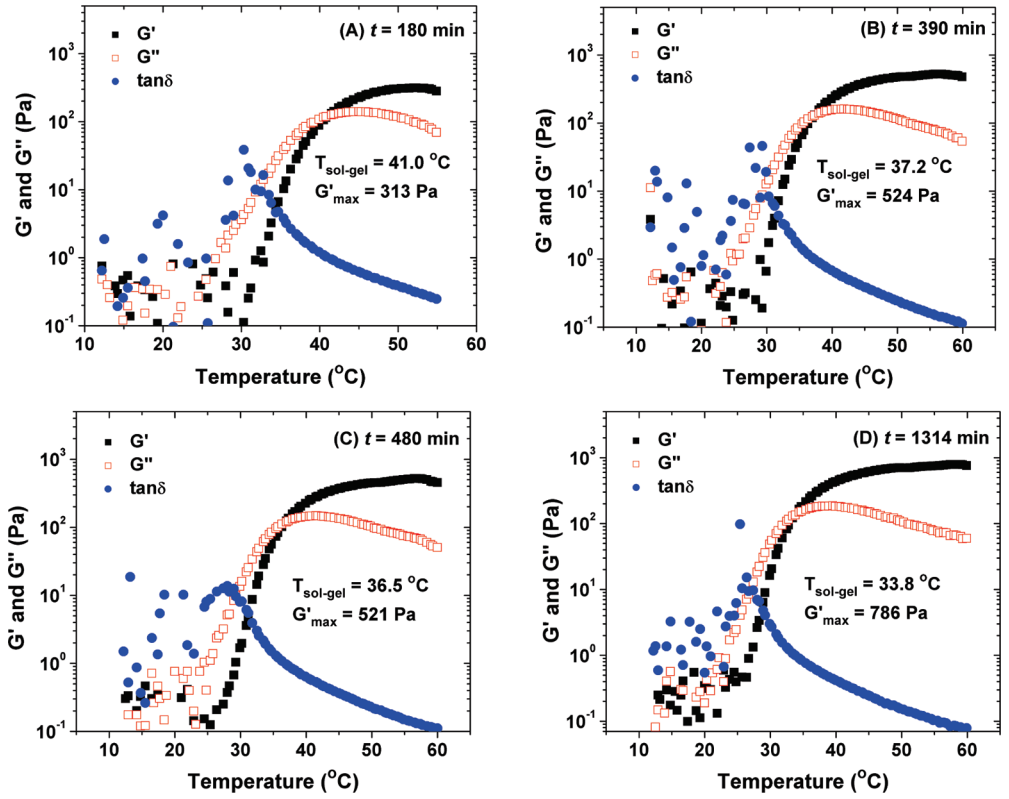

Figure 6. Plot of dynamic storage modulus G' (■), dynamic loss modulus G'' (red □), and tan δ (blue ●) versus temperature for the aliquots taken from a 7.9 wt % solution of P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA) (**ABA-1**) in a 50 mM aqueous citrate buffer with pH of 4.40 in the presence of acid phosphatase at 37 °C after 180 (A), 390 (B), 480 (C), and 1314 min (D). The data were collected from temperature ramp experiments performed by using a frequency of 1 Hz, a strain amplitude of 0.2%, and a heating rate of 3 °C/min.

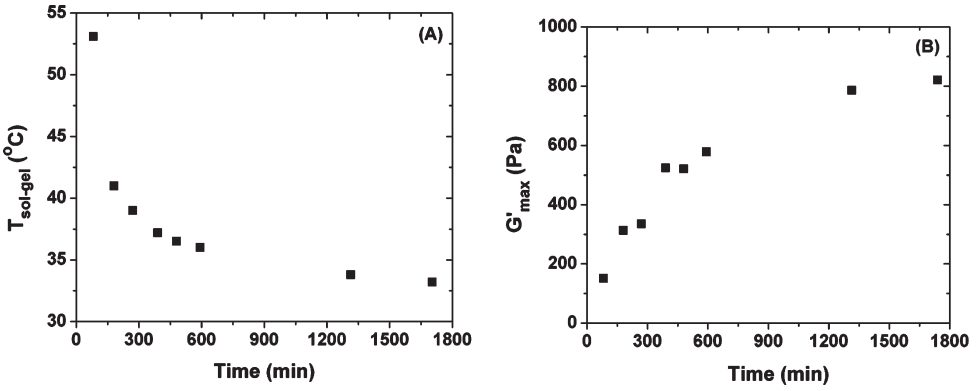

Figure 7. Plot of sol−gel transition temperature ($T_{sol-gel}$) (A) and the maximum value of G' (G'_{max}) (B) versus reaction time. The values of $T_{sol-gel}$ and G'_{max} were obtained from oscillatory shear experiments, which were performed in a heating ramp mode using a frequency of 1 Hz, a strain amplitude of 0.2%, and a heating rate of 3 °C/min.

change became slower; the $T_{sol-gel}$ decreased by only 8 °C from t = 180 to 1740 min. This is reasonable because with the increase of viscosity, the diffusion of acid phosphatase became slower and the access to the remaining phosphate groups on the thermosensitive blocks by the enzyme became more difficult, causing the cleavage reaction to slow down. Note that the $T_{sol-gel}$ at 390 min was 37.2 °C, in agreement with the visual observation that the sample was almost a gel. The experiment was stopped after 1740 min at which only a very small difference in $T_{sol-gel}$ from that at 1314 min was observed.

Figure 7B shows the plot of the maximum value of G' (G'_{max}), obtained from the temperature ramp experiments, versus reaction time. G'_{max} increased with time, from 151 Pa at t = 82 min to 821 Pa at 1740 min. Similar to the trend of $T_{sol-gel}$, the change in the gel strength was faster at the beginning and became slower with the increase of reaction time. We further characterized the final gel sample by frequency sweep experiments (Figure 8). At 25 °C, the solution was a liquid that could flow when tilted. The G' was smaller than G'' in the range from 0.1 to 20 Hz and both exhibited power law dependences on frequency f in the low

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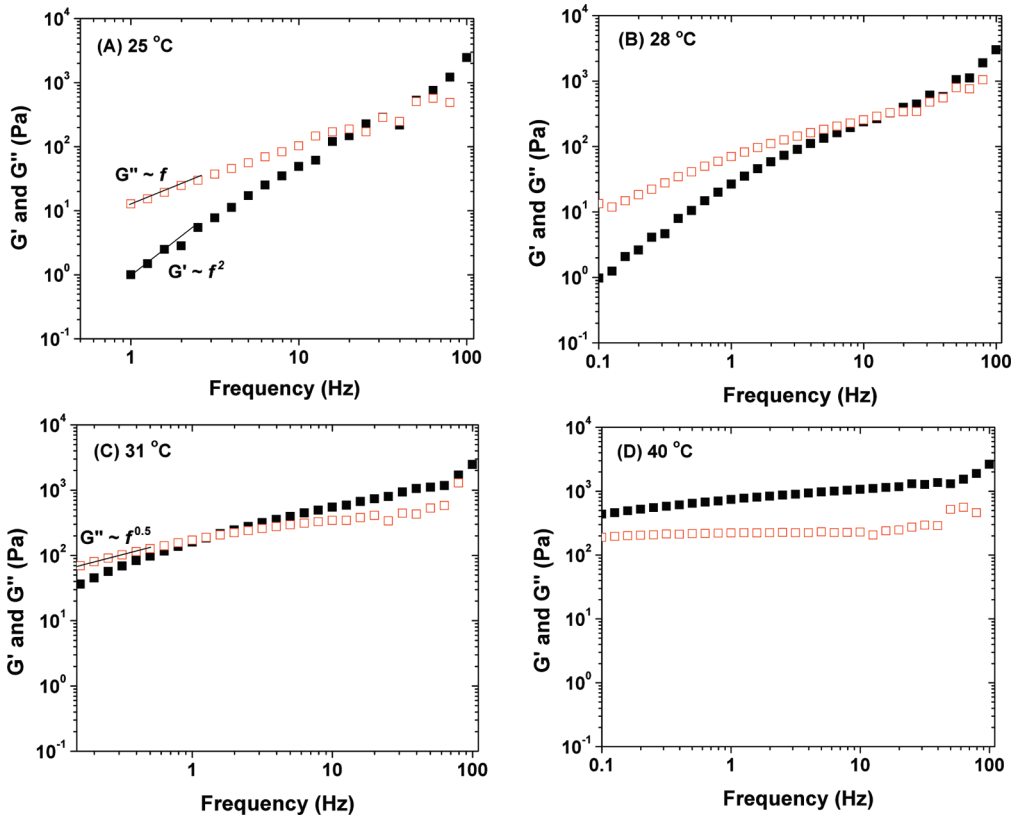

Figure 8. Frequency dependences of dynamic storage modulus G' (■) and loss modulus G'' (red □) at (A) 25, (B) 28, (C) 31, and (D) 40 °C of the final aqueous solution of **ABA-1** with pH of 4.40 after the enzymatic reaction at 37 °C for 1740 min. A strain amplitude of 0.2% was used in all frequency sweep experiments.

frequency region: $G' \sim f^2$ and $G'' \sim f$ (Figure 8A). This is the typical rheological behavior of a viscous liquid.[41] With the increase of temperature, the frequency dependences of G' and G'' evolved (Figure 8B), and at 31 °C, which is close to the $T_{sol-gel}$ (33 °C), G' and G'' were of similar magnitudes in the low frequency region and G'' scaled with $f^{0.5}$ (Figure 8C). This is the signature of the transition between liquid-like and solid-like behavior.[41] At 40 °C, the sample was a free-standing gel. G' was significantly greater than G'' and exhibited a much weaker dependence on frequency (Figure 8D), which is a characteristic of solid-like behavior.[41,42] The plateau modulus G_N of the gel can be obtained from the frequency sweep and it is known that the G_N of a transient gel is a measure of the number density of elastically active polymer chains:

$$G_N = \upsilon k_B T$$

where υ is the number density of elastically active polymer chains (number of elastically active bridging chains per unit volume), k_B is Boltzmann constant, and T is the absolute temperature.[42,43] The G_N is usually evaluated as the G' value at the frequency where G'' exhibits the minimum value, because the increase of G'' at higher frequencies indicates a fast relaxation process separate from the terminal flow process. This method for the determination of G_N is well established for the entangled homopolymer melts and has also been recently used in the study of thermoreversible transient gels.[43] The frequency at which G'' exhibited a minimum value at 40 °C was 12.59 Hz; therefore, the value of G_N was 1103 Pa, close to the modulus in the plateau zone in the heating ramp (821 Pa). If the central block of every polymer chain is elastically active in the gel, a calculation shows that the G_N is 3869 Pa at 40 °C. This means that 28.5% of polymer chains formed effective network strands that could sustain an external stress in the gel at this temperature. The relatively low percentage of polymer chains acting as bridges among micellar cores indicated the presence of the defects in the network, e.g., the loops (two end blocks were located in the same micellar core) and dangling chains (one end block stayed in water instead of in the micellar cores, see Scheme 2).

^{1}H NMR spectroscopy analysis of the polymer isolated after the enzymatic gelation experiment showed that the peak located at 4.06 ppm, which was from $-CH_2OCO-$ and $-CH_2OP-$ of OPBA units in the copolymer, almost disappeared (Figure 1D). This is the same as the dephosphorylation of P(DEGEA-*co*-OPBA) (**R-1**) by acid phosphatase. The ^{31}P NMR spectrum of the dephosphorylated triblock copolymer is presented in Figure 2C. Compared with the ^{31}P NMR spectrum of **ABA-1**, the peak was nearly invisible. Thus, both ^{1}H and ^{31}P NMR spectroscopy analysis suggested that the degree of the cleavage of phosphate groups was high after the enzymatic reaction at 37 °C for 1740 min.

To confirm that the acid phosphatase-induced gelation of the 7.9 wt % aqueous solution of **ABA-1** at 37 °C originated from the LCST behavior of the thermosensitive blocks formed after the cleavage of phosphate groups (i.e., P(DEGEA-*co*−OHBA) blocks), we conducted a dynamic light scattering study of the dephosphorylated triblock copolymer in a 50 mM aqueous citrate buffer with a concentration of 0.02 wt % at the same pH value (4.40).

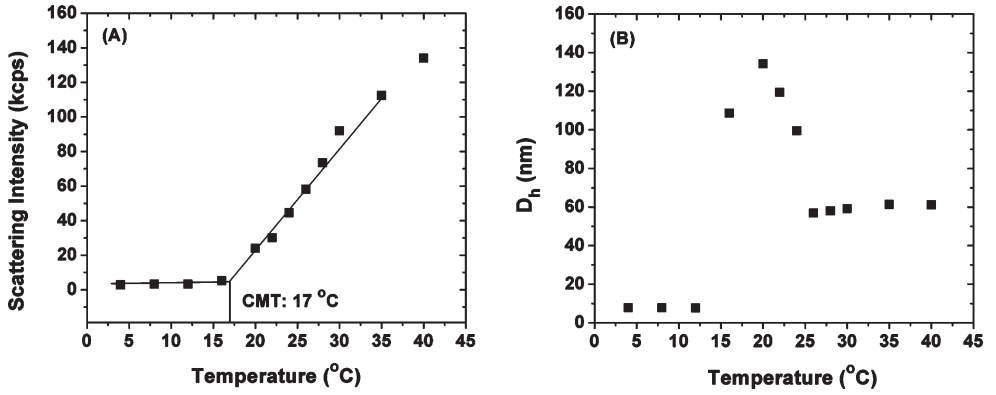

Figure 9. Scattering intensity at scattering angle of 90° (A) and apparent hydrodynamic size D_h (B), obtained from CONTIN analysis, as a function of temperature in a dynamic light scattering study of a 0.02 wt % solution of P(DEGEA-*co*−OHBA)-*b*-PEO-*b*-P(DEGEA-*co*−OHBA) in a 50 mM aqueous citrate buffer with pH of 4.40. The triblock copolymer was obtained after the enzymatic dephosphorylation of **ABA-1** in a 7.9 wt % solution proceeded at 37 °C for 1740 min.

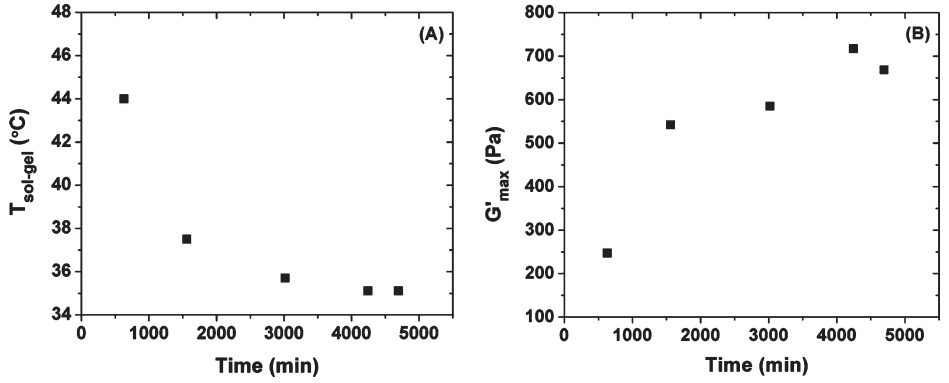

Figure 10. Plot of sol−gel transition temperature ($T_{sol-gel}$) (A) and the maximum value of dynamic storage modulus (G'_{max}) (B) versus reaction time for the enzyme-induced gelation of 7.9 wt % aqueous solution of **ABA-1** at pH 4.67. The values of $T_{sol-gel}$ and G'_{max} were obtained from oscillatory shear experiments, which were performed in a heating ramp mode using a frequency of 1 Hz, a strain amplitude of 0.2%, and a heating rate of 3 °C/min.

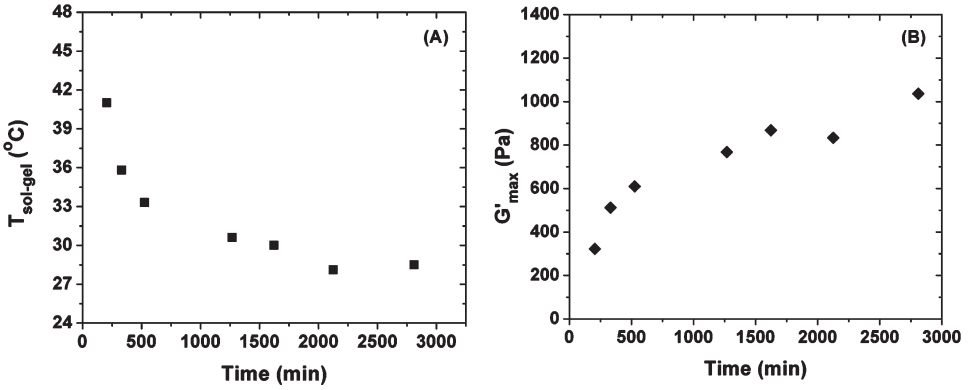

Figure 11. Plot of sol−gel transition temperature ($T_{sol-gel}$) (A) and the maximum value of dynamic storage modulus (G'_{max}) (B) versus reaction time for the enzyme-induced gelation experiment of a 7.9 wt % aqueous solution of **ABA-2** at pH = 4.41. The values of $T_{sol-gel}$ and G'_{max} were obtained from oscillatory shear experiments, which were performed in a heating ramp mode using a fixed frequency of 1 Hz, a strain amplitude of 0.2%, and a heating rate of 3 °C/min.

Figure 9 shows the scattering intensity at scattering angle of 90° and apparent hydrodynamic size, obtained from CONTIN analysis, as a function of temperature. Below 17 °C, the scattering intensity was low and the apparent hydrodynamic size was small (<10 nm), indicating that the polymer dissolved molecularly in the buffer. When the temperature was raised above 16 °C, the scattering intensity began to increase and above 25 °C the hydrodynamic size was around 60 nm. The critical micellization temperature (CMT) determined from the plot of scattering intensity vs temperature was 17 °C. The thermo-induced

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micellization was reversible; lowering the temperature dissociated the micelles. Differently, there was very little change in the scattering intensity of 0.02 wt % aqueous solution of **ABA-1** in the same temperature range.[38] Clearly, in the gelation experiment, the enzymatic cleavage of hydrophilic phosphate groups made the outer blocks less hydrophilic and decreased the LCST. When the LCST became lower than the experimental temperature (37 °C), the triblock copolymer began to self-assemble into micelles and formed a three-dimensional network gel with the PEO blocks forming bridges. Note that the $T_{sol-gel}$ of the final sample was 33 °C, higher than the CMT at the same pH (17 °C). This is understandable because the gelation requires the formation of a three-dimensional network with a sufficient mechanical strength, while the CMT is the temperature at which the thermosensitive P(DEGEA-*co*-OHBA) blocks begin to self-assemble to form micelles in a dilute aqueous solution.

Enzyme-Induced Gelation of 7.9 wt % Aqueous Solution of ABA-1 at pH = 4.67. As shown in Figure 4, the dephosporylation of P(DEGEA-*co*-OPBA) (**R-1**) by acid phosphatase was heavily affected by the solution pH. For example, the clouding time at pH = 4.70 ($t_{clouding}$ = 41.4 min) was 5.4 times that at pH = 4.39 ($t_{clouding}$ = 7.6 min), though the difference in pH values was only 0.31 pH unit. To gain an insight into the effect of pH on the gelation, we carried out an experiment at a slightly higher pH value (4.67) while other conditions remained the same. The gelation was significantly slower (Figure 10A); it took 1560 min for the $T_{sol-gel}$ to decrease to 37.5 °C,[38] while at pH 4.40 the $T_{sol-gel}$ reached 37.2 °C in 390 min, which was 4 times faster. Interestingly, the G'_{max} at the same or similar stage of the gelation process was not heavily dependent on the solution pH. For example, for the aliquot with $T_{sol-gel}$ = 37.5 °C, the G'_{max} was 542 Pa (Figure 10B), essentially identical to that of the aliquot with $T_{sol-gel}$ of 37.2 °C at pH 4.40 (G'_{max} = 524 Pa, Figure 7B). In addition, the highest value of G'_{max} observed in this gelation experiment (717 Pa) was comparable to that at pH = 4.40 (821 Pa).

Enzyme-Induced Gelation of 7.9 wt % Aqueous Solution of ABA-2 at pH = 4.41. We also studied the gelation of a 7.9 wt % solution of **ABA-2** in a 50 mM citrate buffer induced by acid phosphatase under the same conditions (pH = 4.41 and temperature =37 °C). The amount of acid phosphatase with respect to the mass of the thermosensitive blocks of **ABA-2** was also maintained at the same level, that is, 7.5 to 100. Note that **ABA-2** had a slightly higher molecular weight (the $M_{n,SEC}$ of its precursor **ABA-2-P** = 58.9 kDa, PDI = 1.11) and a slightly higher OPBA content (see Table 1). Same as **ABA-1**, the 7.9 wt % aqueous solution of **ABA-2** turned into a micellar gel.[38] Figure 11 shows the plots of $T_{sol-gel}$ and G'_{max} versus reaction time. Compared with **ABA-1**, the gelation was slightly faster; the $T_{sol-gel}$ decreased to 35.8 °C after 331 min. In addition, the $T_{sol-gel}$ of the final sample was lower, 28.5 °C, and the highest value of G'_{max} (1036 Pa) was slightly higher (821 Pa for **ABA-1**). This is likely because the thermosensitive blocks were longer (DP_{total} = 232 in comparison to 171 of **ABA-1**) and the OPBA content was slightly higher. ^{1}H and ^{31}P NMR spectroscopy analysis showed that the degree of the cleavage of phosphate groups was similar to that of **ABA-1** at the end of the gelation experiment.[38] Thus, one can imagine that after dephosporylation the thermosensitive blocks of the triblock copolymer were slightly more hydrophobic due to the slightly higher content of hydrophobic 4-hydroxybutyl acrylate units. This was confirmed by a dynamic light scattering study, which showed that the CMT of the dephosphorylated triblock copolymer at the same pH (4.41) was 13 °C,[38] 4 °C lower than that of the polymer formed from **ABA-1**.

CONCLUSIONS

By incorporating phosphate groups into thermosensitive blocks, we synthesized two thermo- and enzyme-responsive hydrophilic ABA triblock copolymers, P(DEGEA-*co*-OPBA)-*b*-PEO-*b*-P(DEGEA-*co*-OPBA), via ATRP of DEGEA and BPBA from a difunctional PEO macroinitiator and subsequent removal of *tert*-butyl groups of BPBA units. A model study using a random copolymer P(DEGEA-*co*-OPBA) showed that the time for the 0.5 wt % solution to turn cloudy in the presence of acid phosphatase at 37 °C decreased with the decrease of pH from pH 5.48 to 4.39 and leveled off when the pH was further lowed to 4.23. Therefore, we chose pH 4.4 to conduct the enzyme-induced gelation experiments. As expected, a 7.9 wt % aqueous solution of **ABA-1** in the presence of acid phosphatase at 37 °C turned into a gel. Rheological measurements showed that the $T_{sol-gel}$ decreased with the increase of reaction time and reached 37.2 °C at 390 min. The frequency sweep study of the final sample indicated that 28.5% of polymer chains formed effective network strands in the gel at 40 °C. ^{1}H and ^{31}P NMR spectroscopy analysis showed that the degree of the cleavage of phosphate groups was high. The enzymatic dephosphorylation of the triblock copolymer yielded hydrophobic 4-hydroxybutyl groups in the thermosensitive outer blocks and decreased the LCST from above to below the experimental temperature (37 °C), resulting in the formation of a 3-dimentional network gel. This was confirmed by a DLS study of the dephosphorylated triblock copolymer showing that the CMT was 17 °C. In contrast, the gelation at pH = 4.67 took a much longer time, which was in line with the effect of pH on the rate of enzymatic cleavage of phosphate groups in a random copolymer. A similar but slightly faster gelation behavior was observed in the study of **ABA-2** that had a higher molecular weight and a slightly greater OPBA content. Although the gelation in the present work took >5 h for both triblock copolymers, we believe that it can be improved, for example, by selecting a thermosensitive water-soluble polymer with a lower LCST, tuning the phosphate content and the block length of thermosensitive blocks, using a larger amount of enzyme, etc. Since the enzyme-catalyzed reactions are highly specific and efficient, the method reported in this article can be used to design biologically induced micellar gels for potential applications in biomedical areas.

ASSOCIATED CONTENT

Supporting Information. SEC of **ABA-2-P** and ^{1}H NMR spectra of **ABA-2-P**, **ABA-2**, and dephosphorylated triblock copolymer from **ABA-2**, ^{31}P NMR spectra of **ABA-2-P**, **ABA-2**, and dephosphorylated polymer from **ABA-2**, SEC trace of **R-1-P** and ^{1}H NMR spectra of **R-1-P** and **R-1**, ^{31}P NMR spectra of **R-1-P** and **R-1**, ^{1}H NMR spectra of **R-1** and the polymers isolated from the 0.5 wt % aqueous solutions of **R-1** with pH values of 4.23 and 5.48 in the presence of acid phosphatase at the clouding times, additional rheological data for enzymatic gelation experiment of **ABA-1** at pH 4.40, DLS data for 0.02 wt % solution of **ABA-1** with pH of 4.40, rheological data from gelation experiment of **ABA-1** at pH 4.67, rheological data from gelation experiment of **ABA-2**, and DLS data for the dephosphorylated polymer from **ABA-2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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