Supporting Information

Thermoresponsive hydrogels based on phosphorylated star-shaped copolymer: mimicking the extracellular matrix for in situ bone repair

Wei Wu,[ab] Zaifu Lin,[a] Yanpeng Liu,[a] Xinyuan Xu,[a] Chunmei Ding[a] and Jianshu Li*[a]

[a] Dr. W. Wu, Z. Lin, Dr. Y. Liu, Dr. X. Xu, Dr. C. Ding, Prof. Dr. J. Li
College of Polymer Science and Engineering
State Key Laboratory of Polymer Materials Engineering
Sichuan University
Chengdu, 610065, China
*E-mail: jianshu_li@scu.edu.cn

[b] Dr. W. Wu
Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory For Vascular Implants, Bioengineering College
Chongqing University
Chongqing, 400030, China

Experimental Section

Materials: *tert*-Butyl acrylate (*t*BA, 98%), *O*-phosphorylethanolamine (PEA, 97%), *N,N′*-dicyclohexyl carbodiimide (DCC, 97%), α-bromoisobutyryl bromide (BiBB), 4-dimethylaminopyridine (DMAP) and *N*-hydroxysuccinimide (NHS, 97%) were purchased from TCI. *N*-Isopropylacrylamide (NIPAM, 97%) and *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 98%) were obtained from J&K. Tris(2-(dimethylamino)ethyl)amine (Me_6TREN, 99%) and diethyl-meso-2,5-dibromo adipate (DEDBA, 98%) were bought from Alfa Aesar. Trifluoroacetic acid (TFA, 99%), pentaerythritol (98%), CuBr, CuCl and other solvents were purchased from Kelong Reagent Co. Ltd. Dialysis bag, low-glucose dulvecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS), minimum essential medium alpha medium (*α*-MEM), penicillin, streptomycin, calcium colorimetric assay kit and BCA protein assay kit were purchased from Baoxin Biotechnology Co. Ltd. The incubation medium L-DMEM and *α*-MEM both contained 10 wt% of FBS, 100 unit/mL penicillin and 100 μg/mL of streptomycin. CuBr and CuCl were washed with acetic acid and ethanol in turn and dried in vacuum before use. NIPAM was purified by recrystallization two times from n-hexane, dried in vacuum, and then stored at 0 °C before use. *t*BA was rinsed with 5% NaOH aqueous solution and dried over $CaCl_2$, then distilled under reduced pressure, and stored at 0 °C. All of other solvents used in the syntheses were freshly distilled. Ultrapure water with a resistivity of 18.2 MΩ·cm was used throughout.

Synthesis of star-PAA(PEA)-PNIPAM: To synthesize the tetrafunctional initiator (4Br, Fig. S1, Supporting Information), pentaerythritol (1 g, 7.34 mmol) and triethylamine (TEA, 7.5 mL, 53.96 mmol) were firstly dissolved in anhydrous tetrahydrofuran (THF, 15 mL) under the ice bath. Then, after dropwise adding BiBB (5.95 mL, 48.14 mmol), DMAP (5 mg, 0.041 mmol) was subsequently added. The mixture reaction solution was stirred at room temperature for 24 h. The white precipitate was removed by filtration, and the solvent was removed by reduced pressure rotary evaporation. The crude product was dissolved using small amount of

N,N-dimethylformamide (DMF), and the final solution was dialyzed against water for 2 days. Finally, the white precipitate was filtered out and further dried to obtain the pure product 4Br (5.21 g, yield: 96.57%).

The star macroinitiator (star-P*t*BA-Br) was synthesized by ATRP using 4Br as an initiator, *t*BA as the monomer and PMDETA as the ligand. The brief synthesis process was described as follows: Under nitrogen atmosphere and ice bath, tetrafunctional initiator 4Br (14.6 mg, 0.02 mmol), PMDETA (33 μL, 0.16 mmol), *t*BA (6.96 mL, 48 mmol) were dissolved in anisole (2.78 mL). After deoxygenating by three inflate-pump cycles with nitrogen and further purging with nitrogen for 30 min, fresh CuBr (23 mg, 0.16 mmol) was added under nitrogen flow, and then the flask was sealed and placed in an oil bath at 65 °C. Once CuBr was added, the systems turned light-green and gradually deepened along with the reaction. After 24 h reaction, excess THF was poured in to stop the polymerization. The resulting solution was then stirred overnight and passed through an alumina column to remove the catalyst. After removal of solvent, the concentrated solution was dropped into a 10-fold-excess water/methanol (1:1) mixture for product precipitation. The star macroinitiator star-P*t*BA-Br was obtained by filtration and drying under vacuum (4.0 g, yield: 65.09%).

In the next step, star-P*t*BA-PNIPAM was also synthesized by ATRP. Star-P*t*BA-Br (1 g, 0.005 mmol), Me_6TREN (109 μL, 0.4 mmol), the second monomer NIPAM (4.56 mL, 40 mmol) were dissolved in DMF (7.6 mL). After deoxygenating by three inflate-pump cycles with nitrogen and further purging with nitrogen for 30 min, fresh CuCl (40 mg, 40 mmol) was added under nitrogen flow, and then the flask was sealed and placed in an oil bath at 35 °C. After 48 h reaction, DMF was poured in to stop the polymerization and the resulting solution was then stirred overnight. The final solution was dialyzed against water for 3 days (molecular weight cut-off 8000-14000 Da). Finally, the product star-P*t*BA-PNIPAM was obtained through lyophilization (2.0 g, yield: 40%).

Star-P*t*BA-PNIPAM (1.8 g, 7.6 mmol *t*BA) was firstly dissolved in dichloromethane

(DCM, 30 mL). Then, TFA (10.8 mL, 146 mmol) was added in the solution and the mixture solution was stirred at 35 °C for 24 h. Then, most of the volatiles and solvent were removed by a rotavapor. The obtained oily mixture was dropped into 10-fold-excess water. After filtration, the hydrolysate was dried under vacuum to obtain the star-PAA-PNIPAM (1.1 g, yield: 80.53%).

Star-PAA-PNIPAM (0.8 g, 11.1 mmol) and NHS (1.532 g, 13.32 mmol) was dissolved in DMF (15 mL). DCC (4.584 g, 22.22 mmol) dissolved in another 10 mL of DMF was then added dropwise at 0 °C. The reaction mixture was stirred at room temperature for 24 h, followed by filtration and concentration. Then, NaOH was gradually added into the mixture of methanol (25 mL) and PEA (1.565 g, 11.1 mmol) until to form a clear solution, which was poured into the above reaction mixture, and stirred at room temperature for 48 h. Then, the solvent was evaporated, and water (30 mL) was added to dissolve the product. Further separation and purification involves centrifugation (10000 rpm, 20 min), filtration (0.45 μm), dialysis (molecular weight cut-off 8000-14000 Da). The final product star-PAA(PEA)-PNIPAM was obtained through lyophilization (1.3 g, 54.97%).

Chemical structure characterizations: Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured on a Bruker AV II-400 MHz spectrometer to provide the main structure information of polymers. Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet 6700 FT-IR spectrometer using dispersion of polymer powders in potassium bromide (KBr) pellets. The number-average molecular weights (*M*n), weight-average molecular weights (*M*w), and polydispersity index (PDI) were obtained on Gel permeation chromatography (GPC, HLC-8320GPC EcoSEC, Tosoh), which were performed with two columns of TSK gel Super AWM-HSuper equipped with a UV/Vis detector. DMF was used as eluent at a flow rate of 0.4 mL/min at 40 °C. Near-monodispersed poly(methyl methacrylate) (PMMA) standards were used for calibration.

Rheometric studies: Star-PAA(PEA)-PNIPAM with different concentrations was

dissolved in PBS solution. The pH of the solution was adjusted to be neutral as tested by pH meter. Rheometric measurements were performed with a MARS Ⅲ rheometer (HAAKE corporation) with a 35 mm parallel plate and gap of 200 μm. Precautions were taken to minimize water evaporation. The linear viscoelasticity regimes were determined by the strain tests from the stain of 0.001 to 0.5 at several frequencies at 20 and 50 °C, respectively. An optimum frequency of 0.8 Hz was chosen, and an appropriate strain of 0.4 was determined within the corporate linear viscoelasticity regimes. The measuring temperatures range from 20 to 50 °C with a heating rate of 2 °C /min.

In vitro mineralization experiments: To carry out the mineralization test in simulated body fluid (SBF), a dialysis bag (molecular weight cut-off 8000-14000 Da) was loaded with star-PAA(PEA)-PNIPAM aqueous solution (2.0 w/v% in PBS, 2 mL) and immersed in 100 mL of SBF in a polyethylene beaker (100 mL, diameter 5 cm, height 5.6 cm, supplier: Haihong, Chengdu) at 37 °C. SBF was replaced by preheated fresh solution every day. The conventional SBF was used: NaCl (7.996 g/L), $NaHCO_3$ (0.350 g/L), KCl (0.224 g/L), $K_2HPO_4 \cdot 3H_2O$ (0.228 g/L), $MgCl_2 \cdot 6H_2O$ (0.305 g/L), HCl (1 mol/L, 40 mL), $CaCl_2$ (0.278 g/L), Na_2SO_4 (0.071 g/L), and tris(Hydroxymethyl)aminomethane (Tris, 6.057 g/L). The concentration and pH of SBF were Na^+ 142, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO^{3-} 4.2, HPO_4^{2-} 1.0, SO_4^{2-} 0.5 mmol/L and 7.25, which was almost equal to those of human blood plasma. After incubation for 8 weeks, the dialysis bag was soaked in 1 L of water (37 °C) for 12 h to remove the free ion. After that, liquid nitrogen was used to freeze the sample, which was then lyophilized to obtain the mineralized gels.

Scanning electron microscope (SEM, Hitachi S-450, 20 kV, Japan) was used to examine the surface of the mineralized gels. The samples were sputter-coated with gold before observation.

X-ray diffraction analysis (XRD, Dmax 1400, 40 kV, 110 mA, Japan) was performed to examine the formed mineral crystal and the orientation degree of crystallinity.

For Ca^{2+} absorption determination in cell culture medium, the sterilized star-PAA(PEA)-PNIPAM was firstly dissolved in L-DMEM medium to obtain a final concentration of 20 mg/mL and stored at 0 °C over night. Polymeric L-DMEM medium (0.3 mL) was added into the sterile PP plastic tube, and sealed with dialysis membrane (molecular weight cut-off 300000). After that, tube was carefully inverted to a 24-well plate with 3 mL L-DMEM medium each well. Finally, the plate was sealed to prevent medium losses and incubated at 37 °C in an incubator shaker (30 rpm). At pre-determined interval time, the external buffers (50 μL) of dialysis tube were collected and replaced by same volume fresh buffers.

The Ca^{2+} absorption amount from L-DMEM by star-PAA(PEA)-PNIPAM hydrogels was measured by calcium colorimetric assay kit. 10 μL of the collected buffer and 40 μL of ultrapure water were added to assay kit, then 90 μL of chromogenic reagent and 60 μL of calcium assay buffer were subsequently added. After being incubated for 10 min under dark at room temperature, the absorbance values were measured at a wavelength of 575 nm using a KHB ST-360 microplate reader (Shanghai Kehua). The residual Ca^{2+} concentration of L-DMEM was calculated using the pre-established calibration curve.

Cell experiments: The cytotoxicity of star-PAA(PEA)-PNIPAM was evaluated by standard MTT assay against bone marrow stromal cells (BMSCs) and L929 rat fibroblasts, respectively. Bone marrow stromal cells (BMSCs) were cultured to the third generation. Cells were seeded in a 96-well plate at an initial density of 10^4 cells/well and cultured in 100 μL pre-prepared α-MEM, supplemented with 10% heat-inactivated FBS for 24 h. The culture media was replaced with 100 μL of fresh culture media containing serial dilutions of star-PAA(PEA)-PNIPAM copolymers, and cells were incubated for another 24 h. Then, MTT reagent (in 10 μL PBS, 5 mg/mL) was added to each well. After 5 h, the media was drawn off carefully, and 100 μL DMSO was added into each well. The absorbance values were measured at a wavelength of 492 nm using a KHB ST-360 microplate reader (Shanghai Kehua). The experiments using L929 rat fibroblasts were also the same as above. The relative cell viability (%) was calculated using

the following Equation:

$$\text{Cell viability (\%)} = \frac{A_{test}}{A_{control}} \times 100\%$$

where A_{test} and $A_{control}$ are the mean absorbance value of treatment group and the mean absorbance value of control group (without star-PAA(PEA)-PNIPAM), respectively. Each concentration had 6 parallel samples.

For cell proliferation assay, the sterilized star-PAA(PEA)-PNIPAM (120 mg) was firstly dissolved in L-DMEM medium (6 mL) to obtain a final concentration of 20 mg/mL. The L-DEMEM medium was heated at 37 °C to form polymeric hydrogel, and another 6 mL of L-DEMEM medium was added. After 12 h, the color of upper medium changed from red to yellow, and the color of hydrogel changed from colorless to red. To reach the pH/ionic balance between hydrogel and culture medium, the culture medium changed every 2 days until the color of culture medium did not change to yellow. Then, 6 mL of the polymeric L-DMEM solution (sol state) was added to the PP plastic tube containing 1.8×10^5 cells of BMSCs (3×10^4 cells/mL), and the resuspended BMSCs was added to 12-well plate to obtained BMSCs loaded hydrogel by further culturing for 2 h. At the same time, BMSCs incubated without polymeric hydrogel was used as the control group. Culture medium was changed every 2 days using 1 mL of fresh culture. After 1, 3, 5, 7 days cell culture, 200 μL/well of cell counting kit-8 (CCK-8) working solution was added and further incubated for another 2 h. Finally, 100 μL of CCK-8 containing culture medium was collected to measure the optical densities (OD) values at 450 nm, and subsequently the cell proliferation was calculated according the OD values.

Animal experiments: The *in vivo* experiments were permitted by the Animal Research Committee of the University. The sterilized star-PAA(PEA)-PNIPAM was dissolved into α-MEM culture medium to obtain the polymeric concentration of 20 mg/mL. 0.75 mL of polymeric liquid was subcutaneously injected into the left and right side back of SD rat (200 ± 5 g), respectively. SD rats were fed standard with enough food and water. After 1, 2, 3, 4, 6, 8

weeks fed, SD rats were sacrificed and subcutaneous tissue surrounding the injected hydrogel was preserved in 4% formaldehyde (diluting by PBS) at 4 ℃. Then, the subcutaneous tissues were washed with water, dehydrated in alcohol (30%, 50%, 70%, 80%, 90%, 100%, in sequence), clearing in xylene, and finally embedded using paraffin.

Hematoxylin-eosin (H&E) staining: After deparaffinating with xylene for 3 times and washing with 95% alcohol and water, tissues were stained by hematoxylin for 5 min. Then, the stained tissues were washed with water to remove excessive hematoxylin, differentiated in 1% acid alcohol and subsequently washed with water, bluing in 0.2% ammonia water for 20 min and washed with water. After that, tissues were further stained by 0.5% eosin for 3 min. The eosin stained tissues were washed with water, dehydrated in alcohol (80%, 90%, 95%, 100%, in sequence), clearing in xylene for 5 min, mounted with xylene based mounting medium. Finally, images were taken using an optical microscope.

Alizarin red S staining: 2% alizarin red S working solution was first prepared and adjusted its pH value to 4.2 using 10% NaOH solution. After being washed with water for 2 min, tissue sections immerged in 2% alizarin red S working solution, and real-timely controlled the strain process by optical microscope until the calcium deposition becoming jacinth. The alizarin red S stained specimens obtained by washing with acetone and acetone/xylene mixture (v/v: 1/1), clearing in xylene, mounting with xylene based mounting medium. Finally, images were taken using an optical microscope.

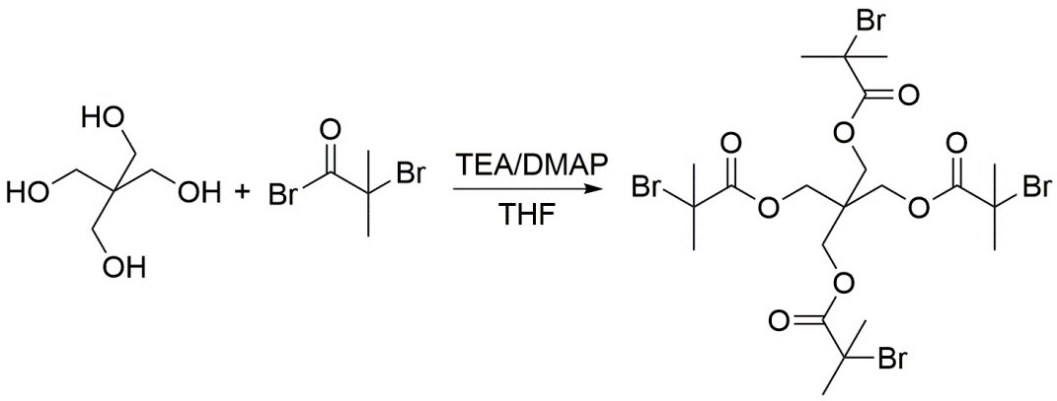

Fig. S1 Synthetic route of tetrafunctional initiator (4Br).

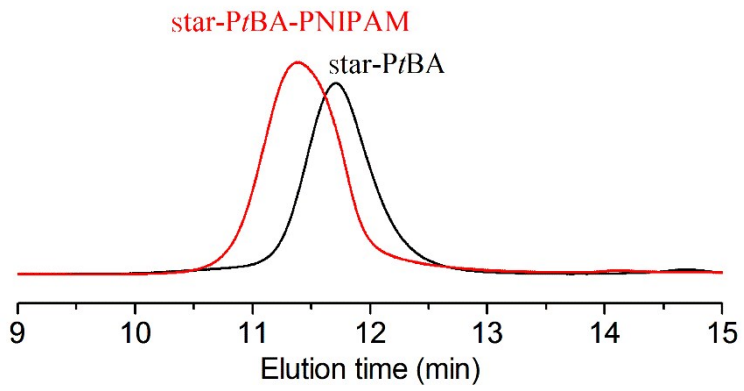

Fig. S2 GPC traces of star-P*t*BA and star-P*t*BA-PNIPAM.

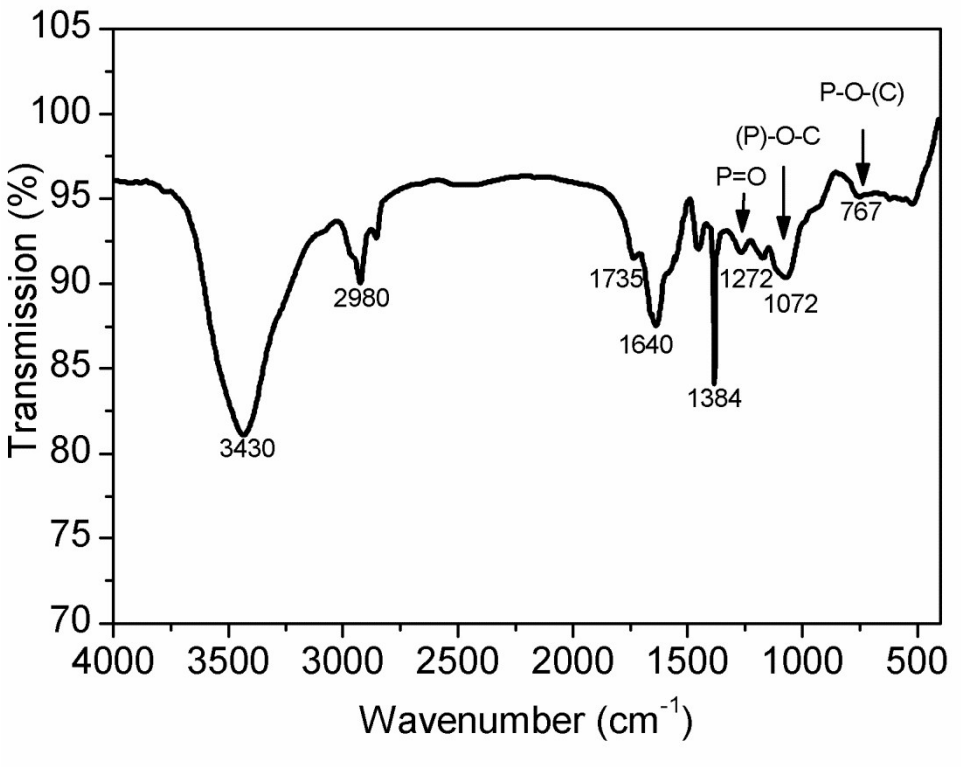

Fig. S3 FT-IR of star-PAA(PEA)-PNIPAM.

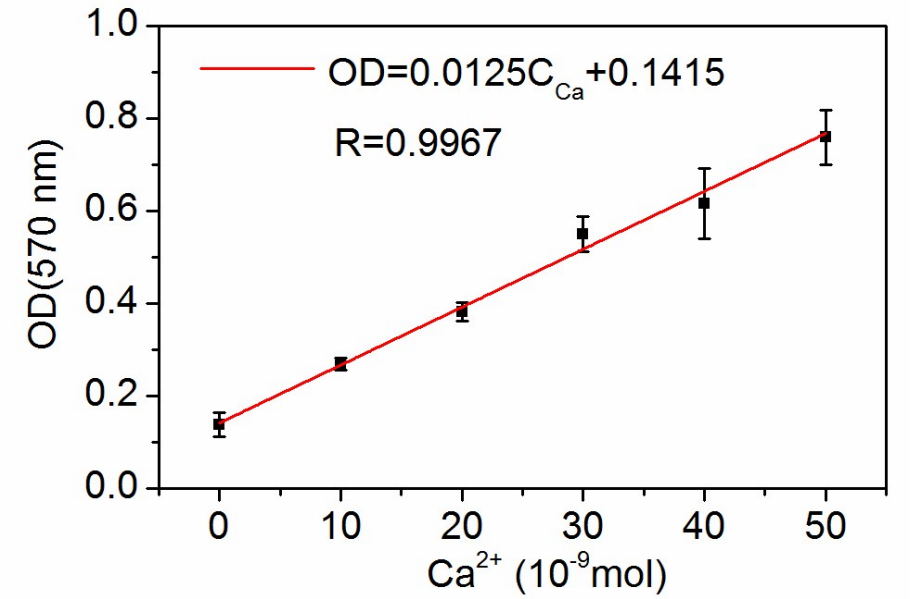

Fig. S4 Standard curve of calcium colorimetric assay kit.

Table S1. Summary of gelation point, and modulus at 37 °C of star-PAA(PEA)-PNIPAM.

Concentrations (wt%)	G′ (Pa)	G″ (Pa)	T_{gel}[a)] (°C)
0.25	0.05	0.06	—
0.5	0.69	0.23	35.9
1.0	22.19	3.23	32.3
2.0	70.66	42.72	29.4

[a)] The temperature at which G' equals G" is designated as the critical gelation temperature T_{gel}.