Electronic Supplementary Material (ESI) for Chemical Communications

Electronic supporting information

Dually Cationic and Anionic pH/Temperature-Sensitive Injectable Hydrogels and Potential Application as Protein Carrier †

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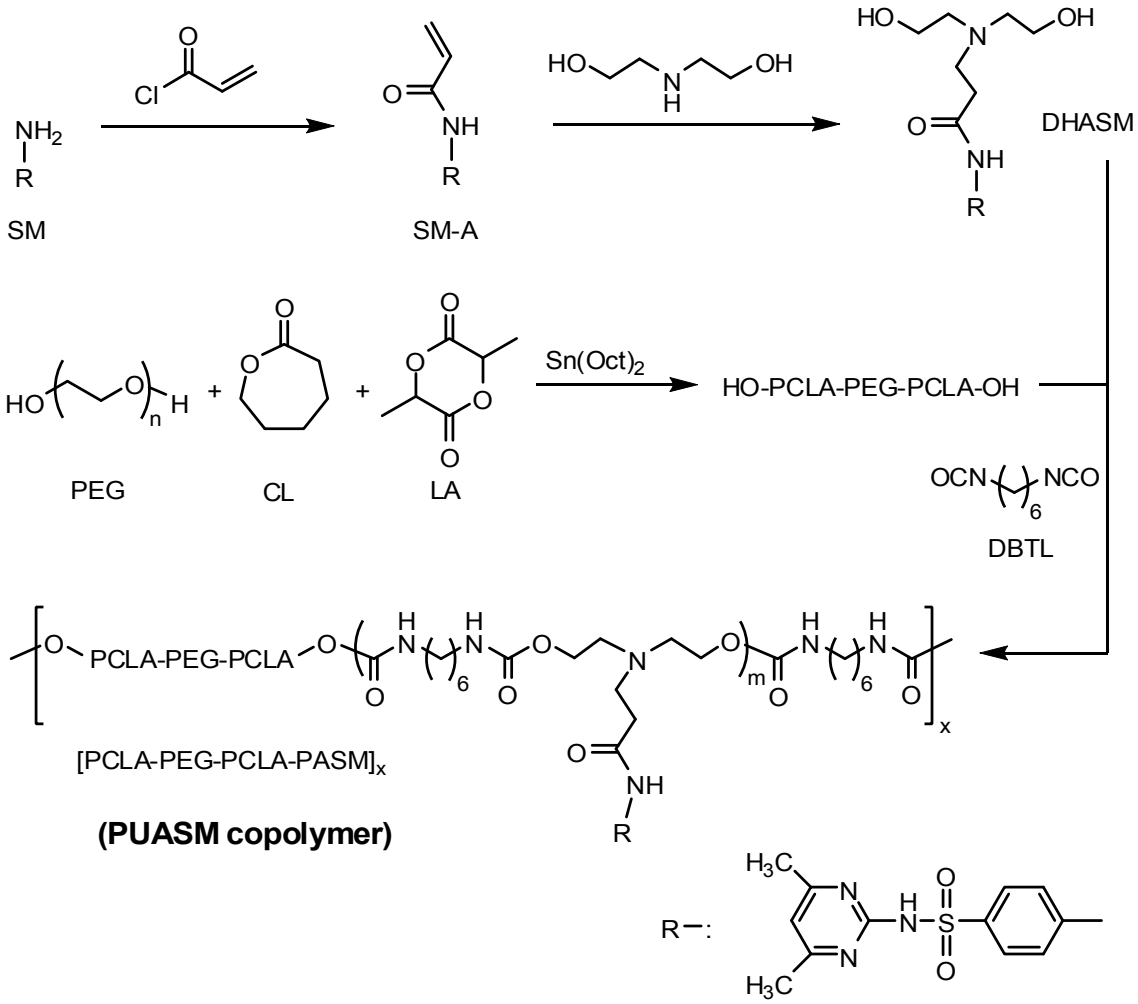
1. Experimental

1.1. Materials

Poly(ethylene glycol) (PEG, M_n 1,750) was provided by ID Biochem Inc. (Seoul, Korea). PEG (M_n 2,000), sulfamethazine (SM), acryloyl chloride (AC), anhydrous acetone, diethanolamine (DEA), anhydrous *N*,*N*-dimethylformamide (DMF), D,L-lactide (LA), ε-caprolactone (CL), stannous 2-ethylhexanoate ($Sn(Oct)_2$), dibutyltin dilaurate (DBTL), 1,6-diisocyanato hexamethylene (HDI), and phosphate buffer saline (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Human growth hormone (hGH) was provided from LG chemicals (Seoul, Korea). Sodium hydroxide (NaOH), hydrochloric acid (HCl), diethyl ether and n-hexane were all products of Samchun Co. (Seoul, Korea). All other reagents were of analytical grade and used without further purification.

1.2. Synthesis of biodegradable $[PCLA\text{-}PEG\text{-}PCLA\text{-}PASM]_x$ block copolymers (PUASM copolymers)

The PUASM block copolymers were synthesized by the polyaddition of the isocyanate groups of HDI with the hydroxyl groups of a synthesized dihydroxyl amino sulfamethazine monomer (DHASM), and of triblock poly(ε-caprolactone-lactide)-poly(ethylene glycol)-poly(ε-caprolactone-lactide) (PCLA-PEG-PCLA) in DMF, in the presence of DBTL as a catalyst (Scheme S1). The PCLA-PEG-PCLA triblock copolymers were synthesized and characterized as in the previous report.



Scheme S1: Synthetic scheme of PUASM block copolymers

1.2.1. Synthesis of monomer DHASM.

The DHASM was synthesized by Michael-addition reaction between the secondary amine groups of DEA and the vinyl groups of sulfamethazine-acrylated (SM-A), which was synthesized similarly the previous procedure. The detail reaction to synthesize the monomer DHASM is as follows: SM-A and DEA were placed in a 250 mL two-neck round-bottom flask at a molar ratio of 1/1. Anhydrous DMF was added to dissolve the reactants at a concentration of 10 wt%. The flask was then placed in a 50 ^{o}C oil-bath with a constant stir rate for 12 h. After 12 h, the reaction solution was concentrated under reduced vacuum and precipitated in an excess of n-hexane. The precipitation was repeated two times and DHASM was filtered and dried under vacuum at room temperature for 48 h prior to use.

1.2.2. Synthesis of copolymer PUASM.

The synthesis of PUASM block copolymers is as follows (PUASM-2): triblock PCLA-PEG-PCLA (M_n 5860) (1.00 mmol), DHASM (5.00 mmol) and DBTL (0.002 g) were dried in a 250 mL two-neck round-bottom flask under vacuum at 80 ^oC for 2 h. The temperature was then decreased to 70 ^oC and the vacuum was replaced by dried nitrogen followed by the addition of 90 mL anhydrous DMF. After the reactants completely dissolved, HDI (6.00 mmol) was added and the reaction was carried out for 3 h. Finally, the reaction solution was concentrated under reduced vacuum and precipitated in an excess of diethyl ether. The precipitated copolymer was filtered and dried under vacuum at room temperature for 48 h. The final yield was approximately 90%. The synthesized PUASM block copolymers were characterized using ^1H NMR and gel permeation chromatography (GPC).

1.3. Characterization

A Varian Unity-500 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) was used to record the NMR spectra using D_2O or $CDCl_3$ as the solvents. The molecular weights of the copolymers and their distributions were measured using GPC (Futecs NS2001, Futecs Co., Ltd., Dae Jeon, Korea) with a refractive index detector (Shodex RI-101, Showa Denko K.K., Kanagawa, Japan) and three Styragel (KF-803, KF-802.5 and KF-802, Showa Denko K.K., Kanagawa, Japan) columns in series, at a flow rate of 1.0 mL/min (eluent: $CHCl_3$; 35 °C). PEG standards (Waters Corp., Mainz, Germany) were used for calibration.

1.4. Sol-gel phase transition measurement

The sol (flow)-gel (non-flow) phase transition of the copolymer in aqueous solution was determined using the tube inversion method. Briefly, the copolymer was dissolved in phosphate buffered saline solution (PBS) at pH~9 in a 4 mL vial (10 mm diameter) at the given concentrations for 4 h. The pH was adjusted to designed values (IQ240 pH meter, Hach Co., CO, USA) using 5 N NaOH and 5 N HCl solutions at 0 ^oC and the samples were stabilized at 2 ^oC overnight. The sample vials containing approximately 0.5 mL of the copolymer solution were placed in a water-bath and

heated slowly from 0 °C to 65 °C. The samples were equilibrated for 10 min at 2 °C intervals. The sol-gel transition was determined by inverting the vials.

1.5. Rheological measurement

A dynamic mechanical analyzer (Bohlin Rotational Rheometer, Malvern Instruments Ltd., WR14, UK) was used to determine the change in the viscosity of the copolymer aqueous solutions. Oscillation mode with a controlled stress of 0.4 Pa and a frequency of 1 rad/s was performed. The heating rate was 1 °C/min. A copolymer solution in PBS was placed between a 20 mm diameter plate and a 100 mm diameter plate with a gap of 250 μm and the test was performed.

1.6. Zeta potential measurement

To confirm the dual ionic properties, the zeta potentials of synthesized amphoteric PUASM, cationic PAE-based (PAE-PCL-PEG-PCL-PAE:1250-1500-1650-1500-1250), anionic OSM-based (OSM-PCLA-PEG-PCLA-OSM:1140-1550-1750-1550-1140) copolymers in deionized water were compared. The zeta potential of the copolymer solutions was measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., WR14, UK) at room temperature. The copolymer solutions were prepared in deionized water at a concentration of 2.0 mg/mL with different pH values and performed the tests within an hour after preparation.

1.7. *In vitro* degradation

The *in vitro* degradation of the PUASM hydrogels was examined. Briefly, 4 mL vials containing 0.5 mL hydrogels at pH 7.4 were incubated at 37 °C for 15 min to form gels. Subsequently, 1 mL of PBS at pH 7.4 was added and the sample vials were incubated at 4 or 37 °C. At a predetermined times, the samples were collected, frozen and lyophilized. The GPC was performed to determine the molecular weight of degraded copolymer.

1.8. *In vitro* cytotoxicity of PUASM copolymer hydrogels

In vitro cytotoxicity of copolymer hydrogels was examined according to the ISO/EN 10993 Part 5 Guidelines. These guidelines prescribe the use of the Dulbecco's modified Eagle's medium (DMEM) extraction test to assess the possible toxic effects of the components released from the

medical polymers during extraction. Different amounts (10-300 mg/mL) of copolymer were extracted at 37 ^{o}C for 24 h using the DMEM (Invitrogen Corporation, CA, USA) culture medium as the extraction fluid. After incubation, the extracts were filtered (0.2 μm pore size; Advantec MFS, Inc. CA, USA) and 1mL of each extract was added to the L929 fibroblast cells (Korean Cell Line Bank, Seoul, Korea), which had been seeded in 24-well plates. Fresh DMEM was used as a negative control. After 48 h incubation, the cell viability and proliferation were determined using an MTT assay. Briefly, 100 μL of fresh growth medium containing 50 μg MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the cells were incubated at 37 ^{o}C for 4 h. The absorbance at 570 nm measured using SpectraMax® M5 Microplate Reader (Molecular Devices, Inc., CA, USA) was directly proportional to the number of living cells. The percentage survival relative to the mock-treated cells (100 % survival) was calculated.

1.9. *In vivo* experiments

Male Sprague-Dawley (SD) rats (Hanlim Experimental Animal Laboratory, Seoul, Korea) were used for the *in vivo* experiments. The rats (5-6 weeks old, average body weight 200 g) were handled in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23, revised 1985). To examine the injectability and *in vivo* gelation of PUASM hydrogel, an aqueous solution (200 μL, 25 wt%) at pH 6.8 or 8.0 was subcutaneously injected into the back of the SD rats. After 5 min, the rats were sacrificed and the gels morphology was observed. For *in vivo* hGH release experiments, 200 μL hGH-loaded copolymer solution (hGH 10 mg/mL, 25 wt% PUASM-2) or 200 μL hGH solution (10 mg/mL) was injected into the SD rats (5 rats/group). At predetermined time points, blood samples were taken from the tail vein of the rats and centrifuged to obtain the sera, which were stored at -21 ^{o}C until assayed. The hGH concentration in the sera was analyzed using a commercial immunoenzymetric assay kit (hGH-EASIA, DIAsource ImmunoAssays S.A, Belgium).

2. Results

2.1. Synthesis and characterization amphoteric PUSAM copolymers

The PUASM block copolymers were synthesized by the polyaddition of the isocyanate groups of 1,6-diisocyanato hexamethylene (HDI) with the hydroxyl groups of a synthesized dihydroxyl amino sulfamethazine monomer (DHASM) and of triblock poly(ε-caprolactone-lactide)-poly(ethylene glycol)-poly(ε-caprolactone-lactide) (PCLA-PEG-PCLA) copolymers in *N*,*N*-dimethylformamide (DMF) in the presence of dibutyltin dilaurate (DBTL) as a catalyst (Scheme S1). Fig. S1 shows ^{1}H NMR spectra with labeled protons of the synthesized triblock PCLA-PEG-PCLA (II), sulfamethazine-acrylated (SM-A), DHASM and PUASM copolymers. The signals at 3.65 (a), 5.20 (b) and 2.30 (d) ppm in Fig. S1a were assigned to the typical protons of PEG (-C*H*$_2$-C*H*$_2$-O-), LA (-C*H*(CH$_3$)-) and CL (-CO-C*H*$_2$-CH$_2$-) of the PCLA-PEG-PCLA triblock copolymer. The segment ratio of PEG, LA and CL blocks in triblock PCLA-PEG-PCLA copolymer were calculated using the peak area of a, b and d. Signals at 2.01 (b) and 5.6-6.4 (e, e1, e2) ppm were respectively assigned to the methylene protons of sulfamethazine (SM) (C*H*$_3$-) and protons of vinyl group (-C*H*=C*H*$_2$) (Fig. S1b), indicating the successful conjugation of acryloyl chloride (AC) into SM. Signals at 2.45 (e) and 2.82 (f) ppm in Fig. S1c were assigned to the protons of formed methylene groups (–CO-C*H*$_2$-C*H*$_2$-N=) and the signals at 2.60 (g) and 3.58 (h) ppm were assigned to the protons of methylene groups (=N-C*H*$_2$-C*H*$_2$-OH) of coupled diethanolamine (DEA) in the DHASM. Fig. S1d shows ^{1}H NMR spectrum of the PUASM-2 copolymer. The presences of PEG, LA and CL were respectively confirmed by the signals at 3.65, 5.20 and 2.30 ppm. The signal at 3.15 (i) ppm was the first methylene protons of HDI (-NH-C*H*$_2$-). The signals at 2.55 (e), 6.58 (a), 7.72 (d) and 8.08 (c) ppm confirmed the presence of DHASM in the final copolymer. The fraction of poly(amino sulfamethazine) (PASM) blocks in the final copolymer were calculated using the peak area of e (PASM) and PEG in Fig. S1d. The ^{1}H NMR results confirmed the successful synthesis of the PCLA-PEG-PCLA triblock, SM-A, DHASM and PUASM copolymer. The detailed characteristics of the synthesized PUASM copolymers are listed in Table S1.

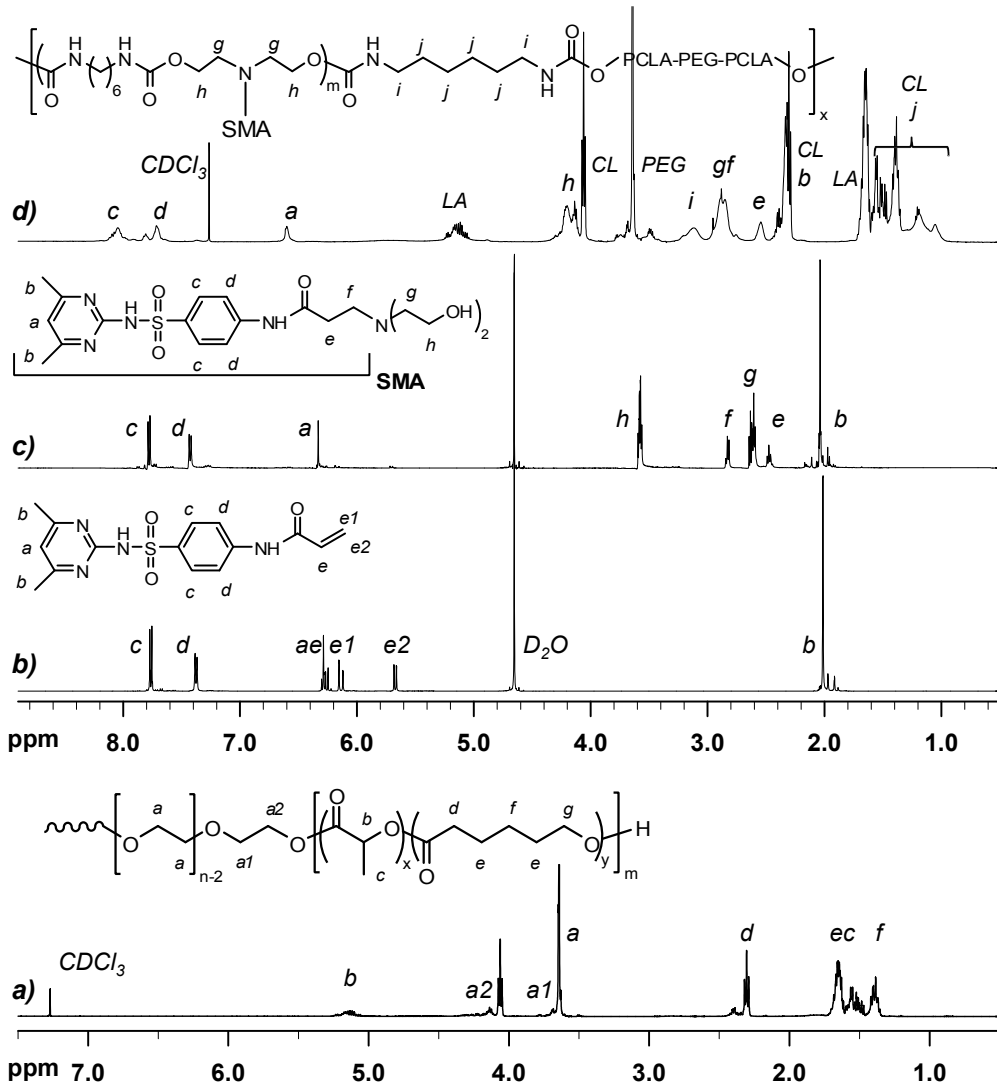


Fig. S1: ^{1}H-NMR spectra of (a) PCLA-PEG-PCLA (II) triblock copolymer (in $CDCl_3$), (b) SM-A (in D_2O), (c) DHASM (in D_2O) and (d) PUASM-2 (in $CDCl_3$).

Table S1. Characteristics of the synthesized PUASM copolymers.

No.	PCLA[b]-PEG[a]-PCLA[b]	PUASM [Tri-PASM[b]]$_x$[c]	M_n[c]	PDI[c]
PUASM-1	1960-1750-1960 (I)	$[5670\text{-}2500]_{1.4}$	11,100	1.78
PUASM-2	1930-2000-1930 (II)	$[5860\text{-}2600]_{1.3}$	10,900	1.79

[a] provided by Sigma-Aldrich and ID Biochem Inc;

[b] calculated from ^{1}H NMR (CL/LA≈1.7, PASM=30.6 wt%);

[c] measured and calculated by GPC.

The closed-loop gel regions of the PUASM hydrogels can be adjusted by varying the molecular weight of the PEG block or copolymer concentration, as showed in Fig. S2. At fixed block lengths of the PCLA and PASM, the gel region shifted to higher temperatures, as the molecular weight of the PEG block increased from 1,750 to 2,000 (PUASM-1 to PUASM-2) (Fig. S2a). This is similar to the shift of the parent temperature-sensitive PCLA-PEG-PCLA hydrogels as the increased molecular weight of PEG. In addition, the gel regions could be easily expanded by increasing the copolymer concentration (Fig. S2b).

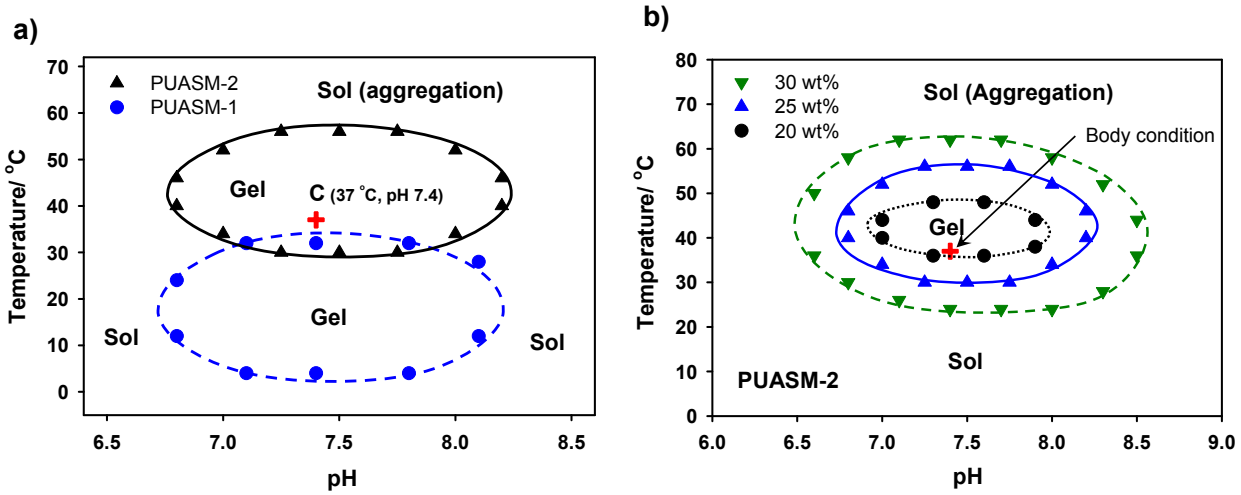


Fig. S2. The closed-loop gel regions of the amphoteric PUASM hydrogels. (a) The gel region shifted to higher temperatures with increasing the molecular weight of PEG (25 wt%). (b) The gel regions could be tailored by a change in the copolymer concentration (PUASM-2).

2.2. *In vivo* gelation

Previous reports showed that pH/temperature-sensitive hydrogel systems could be injected into the body only at mildly basic (i.e., anionic hydrogels) or acidic (i.e., cationic hydrogels) pH. Interestingly, this novel amphoteric PUASM copolymer aqueous solution existed in the free flowing solution at both acidic and basic pH, which would facilitate for simply subcutaneous administration at the both pH conditions. Fig. S3 shows photographs of the gels at five minutes and one week after injection of copolymer solutions at different pH values (e.g., pH 6.8 and 8.0) into the SD rats, presenting injectability of the PUASM solutions at both mildly acidic and basic pH.

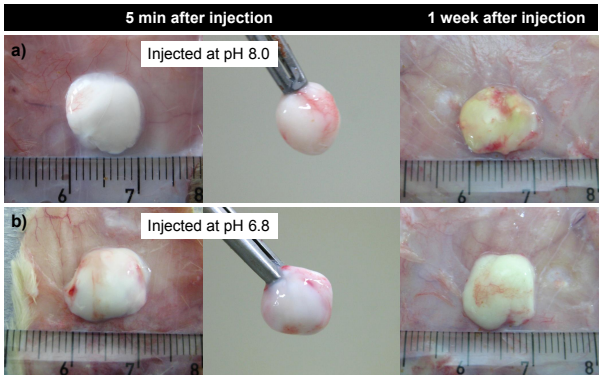


Fig. S3. Photographs of *in vivo* morphology of the 25 wt% PUASM-2 hydrogels at 5 min and one week after the copolymer solutions were injected into SD rats. The injections were performed at room temperature and at pH 8.0 (a) and pH 6.8 (b).

2.3. *In vitro* degradation

In vitro degradation of the PUASM copolymer hydrogels was carried out to confirm their biodegradability. Fig. S4 shows decreases in molecular weights of the parent triblock and PUASM copolymer hydrogels at pH 7.4 and different temperatures. At 4 ^{o}C, both copolymers showed slow degradation rates up to one month. However, at physiological conditions (37 ^{o}C, pH 7.4), both copolymers showed faster degradation rates with gradual decreases in their molecular weights for over one month. The molecular weight of the PUASM copolymer decreased continuously from ~11.0 to ~5.0 kDa by one month. This result confirms the biodegradability of the PUASM copolymers.

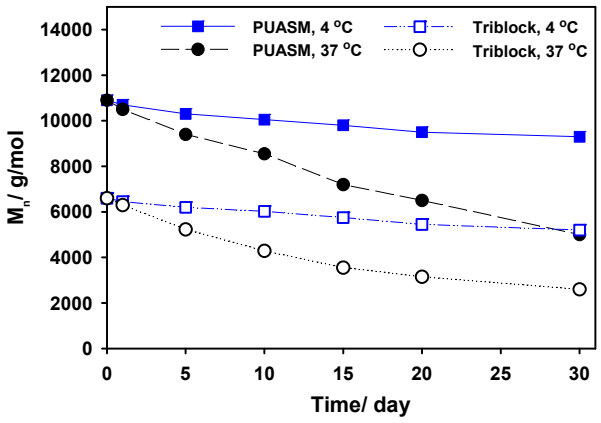


Fig. S4. *In vitro* degradation of the PUASM hydrogels (25 wt% PUASM-2) and its parent PCLA-PEG-PCLA (II) triblock hydrogels (20 wt%) at pH 7.4 and different temperatures.

2.4. *In vitro* cytotoxicity

In vitro cytotoxicity of PUASM hydrogels was examined according to the ISO/EN 10993 Part 5 Guidelines. This test was designed to determine the possible cytotoxic effects of the materials extracted from biomaterials implanted in the body. Various amounts (10-300 mg/mL) of copolymer were incubated in Dulbecco's modified Eagle's medium (DMEM) for 24 h at 37 ^{o}C to obtain the extracts. The fibroblast cells L929 were exposed to the extracts for 48 h and the cytotoxic effects were evaluated. Fresh DMEM was used as a negative control. As shown in Fig. S5, the PUASM hydrogel exhibited low cytotoxicity at high copolymer concentrations. The cell viability in the presence of the hydrogel was greater 90 % up to a copolymer concentration of 300 mg/mL. This result indicates that the PUASM hydrogels can be used as a low cytotoxic material.

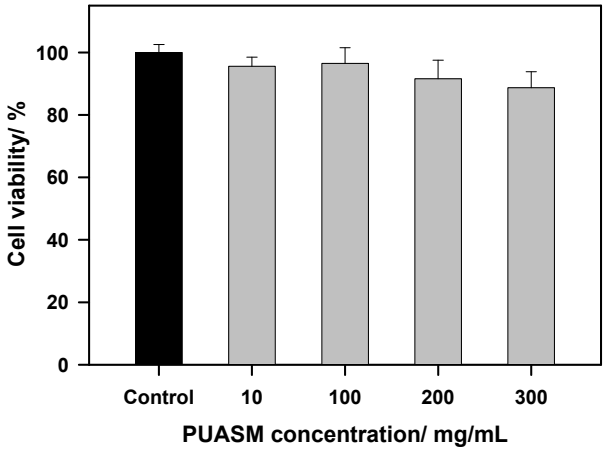


Fig. S5. Viability of L929 fibroblast cells exposed with the PUASM-2 hydrogel (±SD, n = 3).