Supplemental Information

A nanocomposite approach to develop biodegradable thermogels exhibiting excellent cell-compatibility for injectable cell delivery

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Experimental Section

Synthesis of PLGA-PEG-PLGA copolymers

PLGA-PEG-PLGA was synthesized through bulk ring-opening copolymerization of DL-lactide (LA) and glycolide using tin 2-ethylhexanoate as a catalyst. Briefly, under a nitrogen atmosphere, PEG3k (2250 mg, 0.75 mmol), DL-lactide (1944 mg, 13.5 mmol), glycolide (783 mg, 6.75 mmol), and tin 2-ethylhexanoate (12 mg, 30 μmol) were placed into a glass flask. The mixture was dried under vacuum overnight. The flask was then purged with nitrogen and sealed in vacuo. The sealed flask was placed in an oil bath at 150 ºC for 6 hours. The purification of the reaction mixture was performed by the reprecipitation method using chloroform as a solvent and diethyl ether as a non-solvent, and dried under vacuum overnight to give flake of PLGA-PEG3k-PLGA. The average number of molecular weight (M_n) and the polydispersity indexes (M_w/M_n) of these copolymers were determined by gel permeation chromatography (GPC) (detector: RI, standard: PEG; eluent: DMSO). The molecular compositions of PEG, DL-LA, and GA in the obtained copolymers were estimated from ^{1}H-NMR measurements (JEOL, ECA-500, solvent: $CDCl_3$).

Characterizations

The critical micelle concentration (CMC) values of PLGA-PEG-PLGA and PLGAPEG-PLGA/LP hybrids in water were determined using pyrene as a fluorescence probe at 20 ºC. Pyrene partitioned preferentially in the hydrophobic core of aggregates (micelles) and changed the photophysical properties of the micelles under investigation. Pyrene was first dissolved in acetone and then added to water to a concentration of 740 μM. Acetone was subsequently removed by reducing the pressure and stirring for more than 12 h at 20 ºC. The concentration of the copolymers and LP in sample solution was varied from 0.00001 to 0.1

wt% and 0.0001 to 0.01 wt%, respectively. The fluorescence emission spectra of pyrene at 335 nm and 338 nm in the presence of copolymer and LP were measured at room temperature using a fluorescence spectrophotometer (JASCO, FP-6200) at a fixed emission wavelength of 390 nm. The average diameters of nanostructures of PLGA-PEG-PLGA/LP hybrids in water were measured by dynamic light scattering (DLS, ZETASIZER NanoSeries ZEN-3600, MALVERN). The hybrid solutions were prepared by mixing of copolymer solution (0.1 wt%) and LP solution (from 0.001 to 0.1 wt%) at room temperature, and then DLS measurements of the samples were performed with different temperature from 20 to 50 ºC. DLS measurements of LP or copolymer solutions were also performed with the same temperature condition as the hybrids. The storage moduli (G') and loss moduli (G'') of the hybrid solutions, copolymer, and LP solutions were measured by rheometer (HAAKE, Thermo HAAKE RS600) at from 10 to 45 ºC. The sample solutions were quickly applied between parallel plates of 25 mm in diameter with a gap of 1.0 mm using syringe, and the plates were heated with rates of 1.0 ºC/min. The data were collected under a controlled stress (4.0 dyn/cm^2) and a frequency of 1.0 rad/s. FTIR spectrometry was performed using a FTIR-6300A (JASCO) spectrometer. All spectra were recorded using a transmission mode with a wavelength range of 600–4000 cm^{-1}.The sample solutions were placed onto hydrophilized TEM grid and frozen carefully to maintain the nanostructures composed of PLGA-PEG-PLGA, LP, and water. Then, the frozen samples were measured by cryo-TEM.

Thermo-responsive behavior

Certain amounts of polymers were dissolved in acetone and the predetermined volume of pure water was added to the acetone solution, and then the acetone was completely evaporated to prepare aqueous solutions of polymers with certain concentrations. LP aqueous solution was added to the solution to prepare PLGA-PEG-PLGA/LP composites. The temperature-responsive sol-gel transition of the hybrid solutions was determined by a test tube inverted method. The aqueous solutions of each sample were placed in glass vial and then the vial was immersed in a water bath at a designated temperature for 2 minutes. The transition temperatures were determined by flow (sol)-no flow (gel) criterion when the vial was inverted using a temperature increment of 1 ºC per step. The sample was regarded as a gel when no flow was kept for 1 min after the vial was inverted.

Cell viability in polymer sol

L929 mouse fibroblast cell line was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% fetal calf serum in a humidified 5% CO_2 at 37 ºC. L929 cells (1.0×10^4 cells) were seeded on 96-well plate with 200 μL of DMEM and incubated for 1 day at 37 ºC. After incubation, DMEM was completely removed and 200 μL of sample solutions ($P_{1.5k}$, P_{3k}, or P_{3k}/LP) were added to each well. After predetermined periods, sample solutions were removed and rinsed with PBS, and then 0.4% of trypan blue solution was added to each well to investigate cell viability in the sample solutions.

Hemolysis assay

EDTA stabilized blood samples (3 mL) were centrifuged at 1600 rpm for 5 minutes and red blood cells (RBCs) were obtained after removing the blood plasma. Precipitated RBC pellet was washed five times with 6 mL of PBS and RBCs were dispersed in 25 mL of PBS. 0.2 mL of RBCs was placed in plastic vials and 0.8 mL of polymer sample ($P_{1.5k}$ or $P_{1.5}$k/LP) solutions in PBS at different concentrations was added. Also, positive and negative control samples were incubated at different temperatures for 2 hours. Samples were slightly shaken once for every 30 minutes to re-suspend the RBCs and polymer nanostructures. After incubation, RBC were precipitated at 1600 rpm and 200 μL of supernatants was transferred to a 96-well plate to measure the absorbance of released hemoglobin, from damaged RBCs, with a microplate reader at 570 nm. Absorbance at 650 nm was recorded as references. Hemolysis percentages of the RBCs were calculated using the following formula;

Hemolysis (%) = (absorbance of polymer samples – absorbance of negative control) / (absorbance of positive control – absorbance of negative control)

Percent hemolysis values were calculated from three separate experiments.

Cell viability on polymer gel

200 μL of sample solutions ($P_{1.5k}$ or P_{3k}/LP) were added to glass bottom multi-well cell culture plate and incubated for 1 hour at 37 ºC to allow gel formation. 100 μL of L929 cell suspensions (1.0×10^5 cells/well) were place onto the gels and incubated for 3 hours. Calcein-AM and propidium iodide (PI) solutions were added to the well and incubated for 30 minutes to allow cell stain. Cells cultured on the gels were washed gently with PBS three times and then observed by fluorescence microscope (KEYENCE BIOREVO BZ-9000) to visualize cell viability on the gels.

Cell encapsulation

L929 cells cultured in tissue culture polystyrene (TCPS) were harvested by usual trypsin treatment, and the cells were suspended with DMEM, and centrifuged to give a pellet of L929 cells. The pellet was re-suspended with DMEM, and Calcein-AM was added to the suspension and incubated for 30 minutes for live cell labeling. The pellet (1.0×10^5 cells) of labeled cells was re-suspended with 100 μL of PLGA-PEG-PLGA solution followed by 100 μL of LP solution, and the resultant cellular suspension was seeded onto glass bottom multi-well cell culture plate, then incubated at 37 ºC for 1 hour to form L929 cells encapsulated hydrogels. Then, 300 μL of DMEM was added onto the hydrogels and incubated at 37 ºC for 4 hours. PI solutions were added into the dish and incubated at 37 ºC for 30 minutes. The hydrogels were washed with 300 μL of PBS three times and observed by fluorescence microscope to visualize cell viability in the hydrogels.

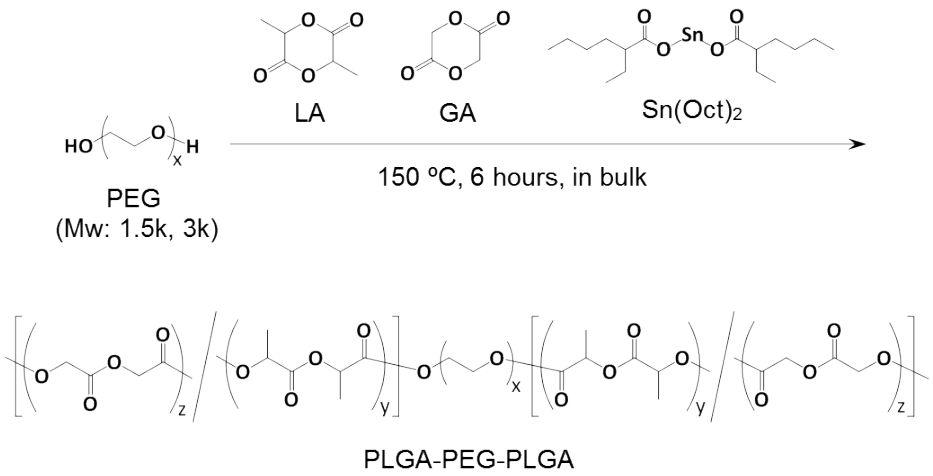
In vivo gel formation

All of the animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH. The animal experiments were also carried out in strict accordance with the guidelines for animal experiments at the Konan University. 200 μL of $P_{3k}3L0.9$ aqueous solution loading L929 cells (5.0×10^5 cells) was injected carefully into the back of mouse (BALB/cCrSlc, 11.4 week old male) by a syringe with a 26-gauge needle. After 2 hours, mouse was sacrificed and injected site was carefully cut open and a gel was taken out from the site.

Table S1. Characterization of PLGA-PEG-PLGA copolymers

sample	DP of LA [a]	DP of GA [a]	M_w of PEG	Total M_w [b]	M_w/M_n [c]
PLGA-PEG$_{1.5k}$-PLGA ($P_{1.5k}$)	8.2	4.1	1,500	4,810	1.18
PLGA-PEG$_{3k}$-PLGA (P_{3k})	7.9	4.0	3,000	6,200	1.13

[a] Estimated by ^{1}H-NMR. [b] Estimated by following equation; M_w of copolymer = M_w of PEG + 2 × (M_w of PLGA). [c] Estimated by GPC (eluent: DMSO, standard: PEG).



Scheme S1. Synthesis of PLGA-PEG-PLGA copolymers.

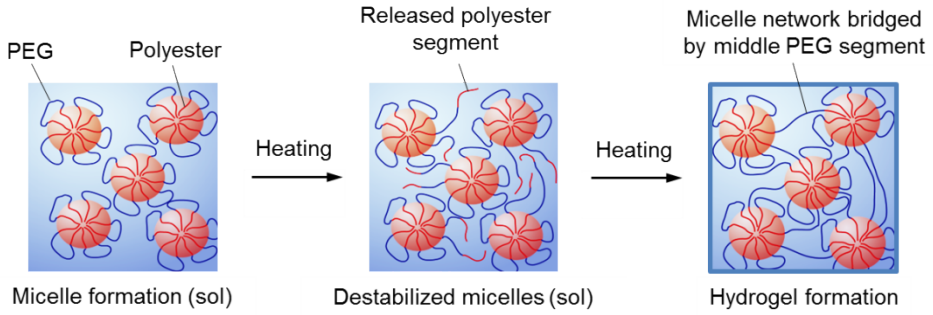


Figure S1. Schematic illustration for thermo-gelation of aqueous PEG-polyester block copolymer solution.

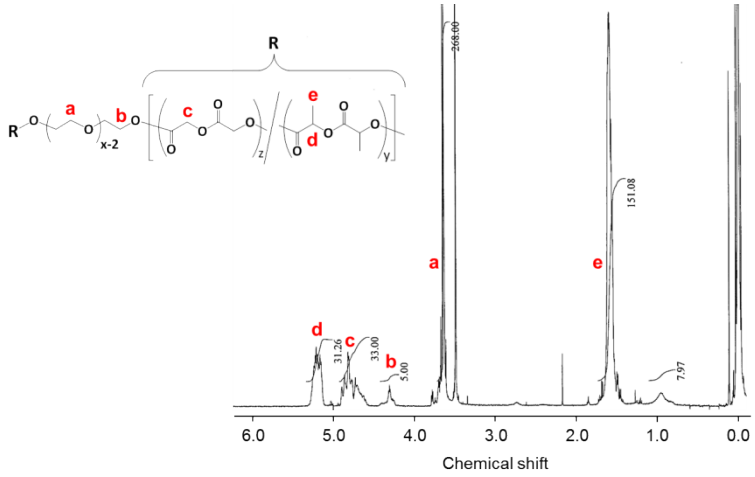


Figure S2. ^{1}H-NMR spectrum of P_{3k} measured in $CDCl_3$.

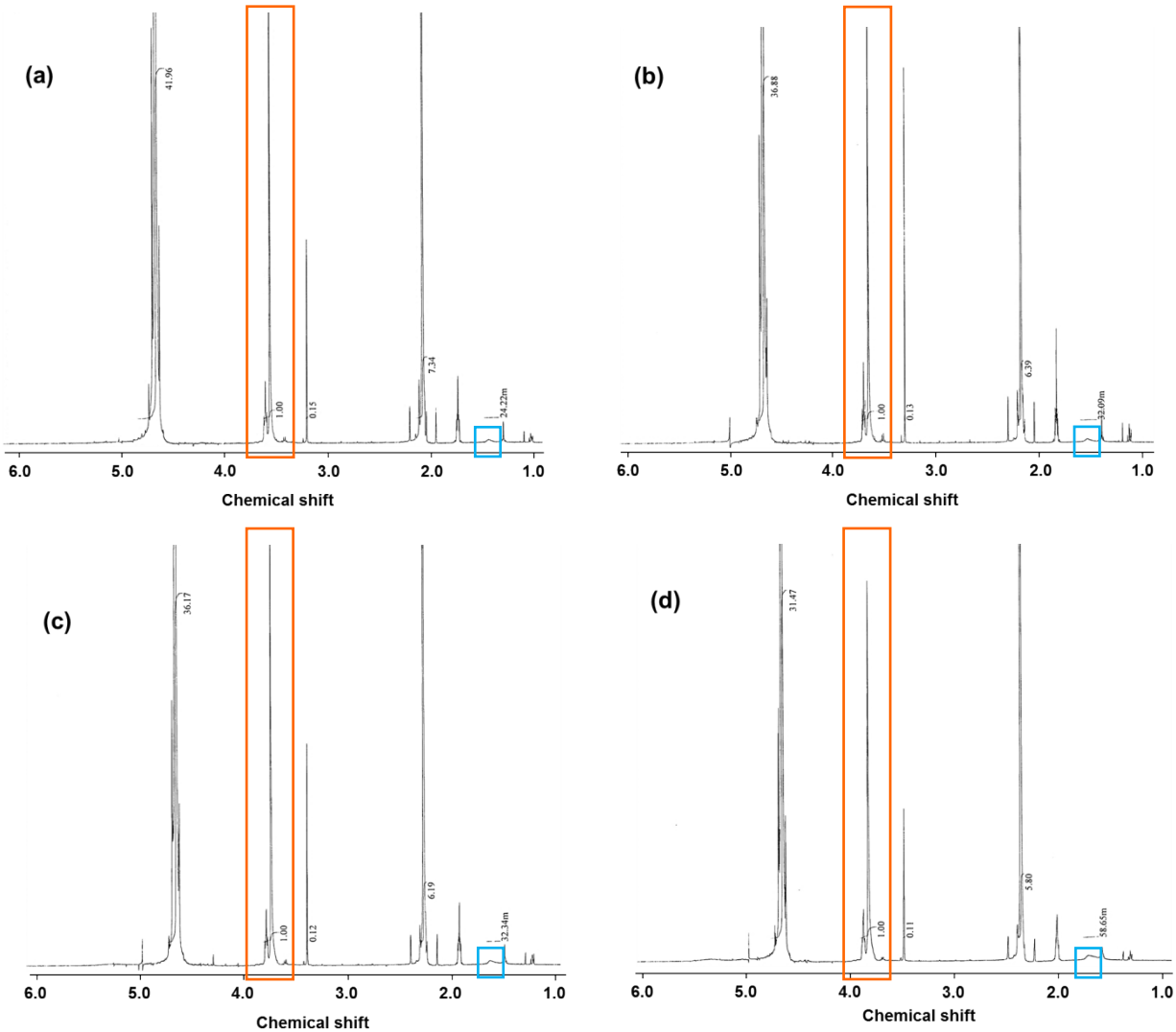


Figure S3. ^{1}H-NMR spectra of P_{3k} in D_2O at (a) 20 ºC, (b) 30 ºC, (c) 40 ºC, and (d) 50 ºC.

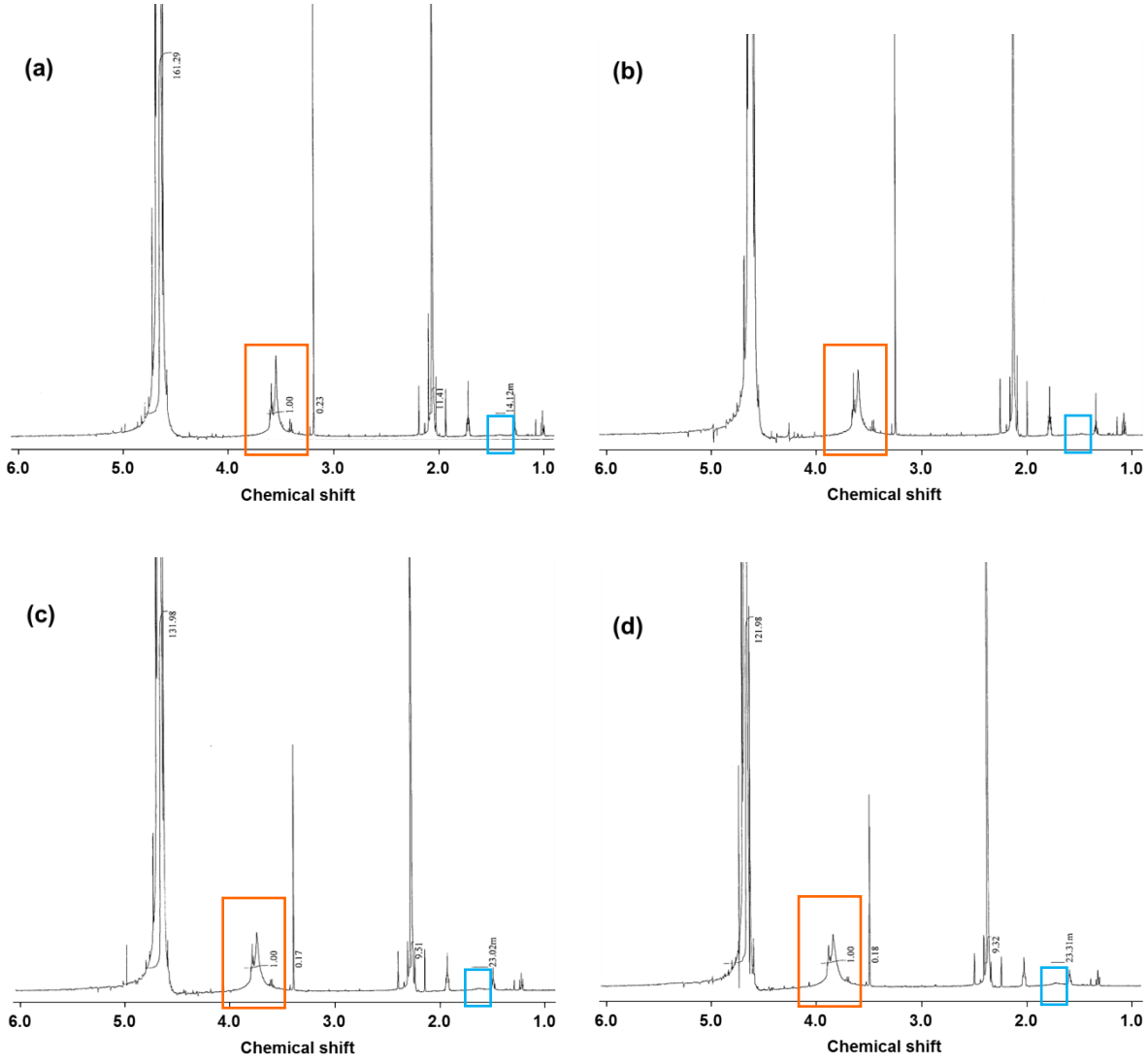


Figure S4. ^{1}H-NMR spectrum of $P_{3k}0.1L0.1$ in D_2O at (a) 20 ºC, (b) 30 ºC, (c) 40 ºC, and (d) 50 ºC.

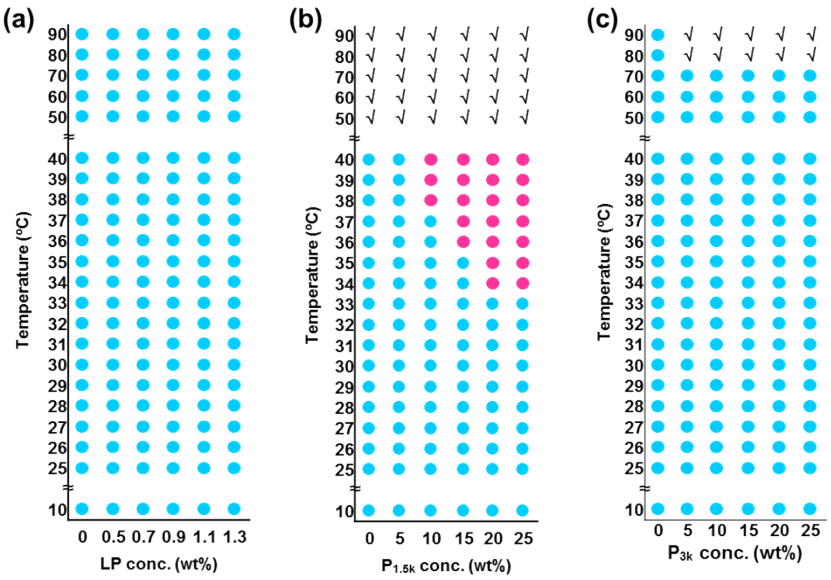


Figure S5. Phase diagram of (a) Laponite: LP, (b) $P_{1.5k}$, and (c) P_{3k} aqueous solutions. ●: sol, ●: gel, √: precipitation.

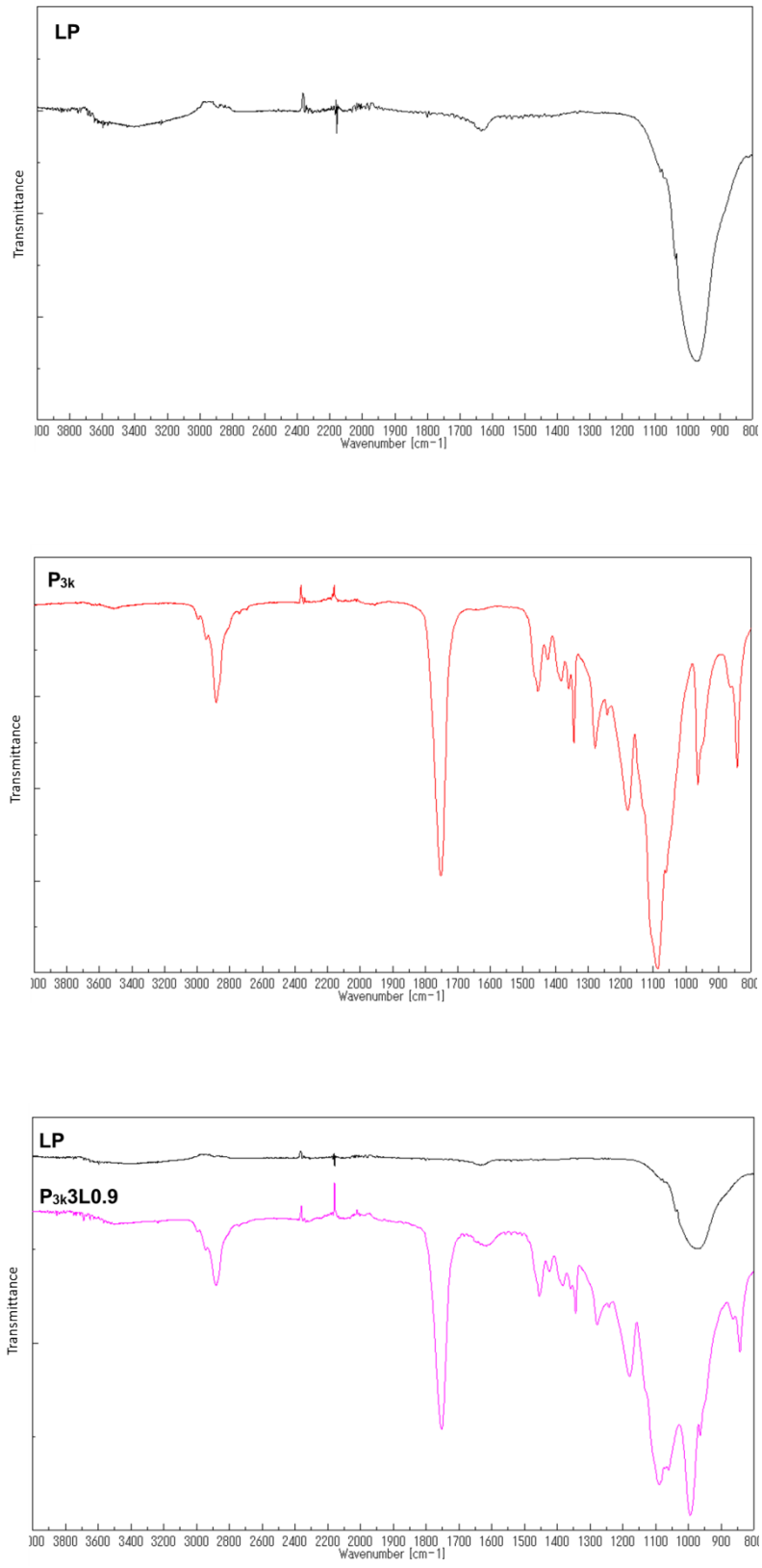


Figure S6. FTIR spectra of LP, P_{3k}, and P_{3k}3L0.9 hybrids.

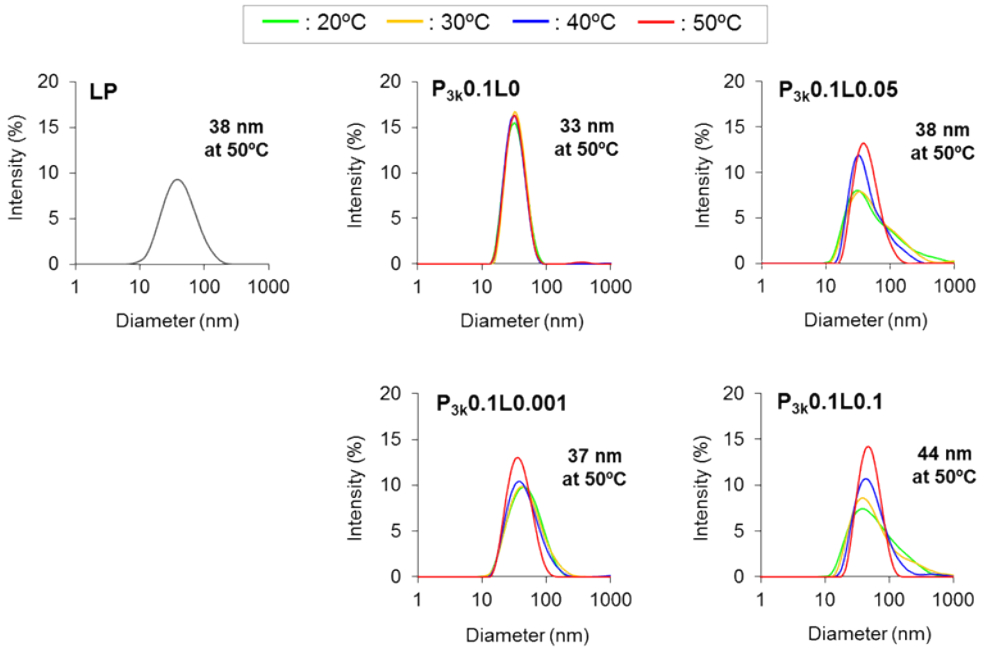


Figure S7. The size and distribution of micelles of P_{3k} in the presence of LP in water (0.1 wt%) as a function of temperature.

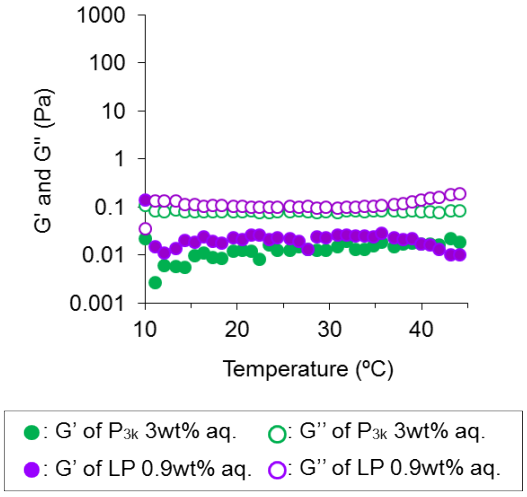


Figure S8. Temperature dependences of storage modulus (G') and loss modulus (G'') of P_{3k} (3 wt%) and LP (0.9 wt%) aqueous solutions.

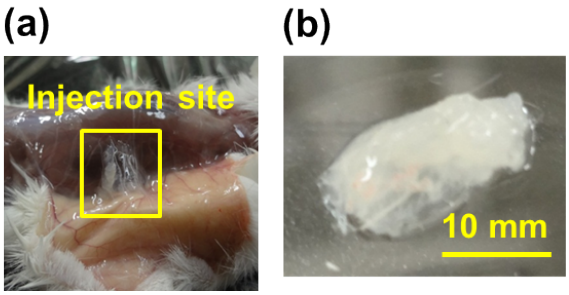


Figure S9. (a) In situ gel formation of 0.2 μL of P_{3k}/LP (P_{3k}3L0.9) solution including L929 cells in the back of mouse (BALB/cCrSlc, 11.4 week old male) after subcutaneous injection. (b) The P_{3k}3L0.9 hybrid gels excised from the mouse after 2 hours. The animal experiments were carried out in strict accordance with the guidelines for animal experiments at the Konan University.

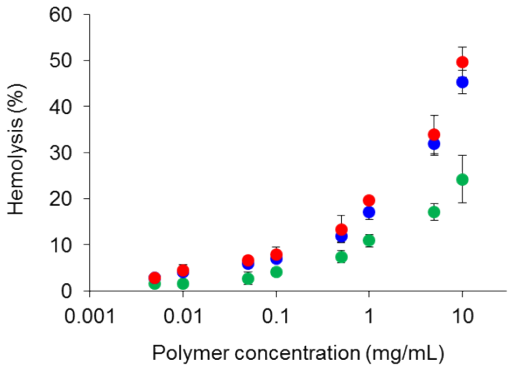


Figure S10. Hemolysis activity of P_{3k} aqueous solutions with different concentration at 20 ºC (●), 30 ºC (●), and 37 ºC (●).

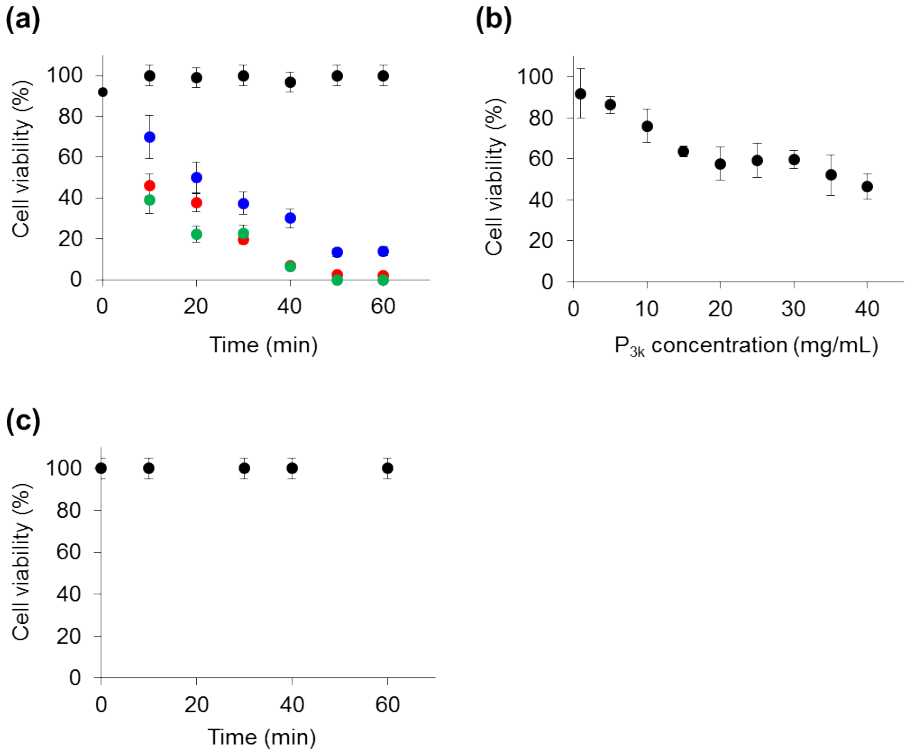


Figure S11. (a) Time course of cell viability of L929 cells after immersion in $P_{1.5k}$ solution at 37 ºC. ●: PBS, ●: 5 wt%, ●: 10 wt%, ●: 15 wt%. (b) Cell viability of L929 cells after immersion in P_{3k} solution at 37 ºC for 10 minutes with different polymer concentrations. (c) Time course of cell viability of L929 cells immersed in $P_{3k}3L0.9$ solution at 37 ºC.