Enhancement of cryopreservation with intracellularly permeable zwitterionic polymers

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MATERIALS & METHODS

Materials

Methacryloyl chloride, 2-(methylthio)ethanol, *n*-butyl amine, and 2,2'-azobis(isobutyronitrile) (AIBN) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan); fluorescein *o*-acrylate, triethyl amine (TEA) and 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT) were purchased from Sigma-Aldrich (St. Louis, MO, USA); tris(2-carboxyethyl)phosphine hydrochloride and 3-(4,5-dimethylthial-2-yl)-2,5-diphenyltetrazalium bromide (MTT) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); dichloromethane (DCM), dimethyl sulfoxide (DMSO), methanol and hydrogen peroxide (30%) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan); and 3-((3-acrylamidopropyl)dimethylammonio)propane-1-sulfonate was supplied by Osaka Organic Chemical Industry, Ltd (Osaka, Japan). All the reagents were used as received without further purification.

Characterization

^{1}H NMR

^{1}H NMR was measured using a Bruker Avance III 400. The deuterated solvent used was purchased from Sigma-Aldrich. The NMR data were analysed using Topspin 3.5 software.

Fourier-transform infrared spectrum (FT-IR)

FT-IR was measured using JASCO FT/IR-6X in attenuated total internal reflection mode. Measurements were performed with 16 scans per spectrum and a resolution of 4 cm^{-1}.

Size exclusion chromatography

The molecular weights and distributions (polydispersity index; PDI) of the polymers were determined by gel permeation chromatography (GPC; BioSep-s2000, Phenomenex, Inc., CA, USA) using a Waters Alliance HPLC system. A 0.1 M sodium bromide aqueous solution was used as the mobile phase (flow rate, 1 mL/min) and pullulan as the standard.

Methods

Synthesis of 2-(methylthio)ethyl methacrylate (MTEMA)

TEA (4.46 g, 44.1 mmol) and 2-(methylthio)ethanol (3.64 g, 39.5 mmol) were added to 50 mL of DCM and kept at 0 °C. Then, methacryloyl chloride (4.60 g, 44.0 mmol) was added dropwise and the mixture was allowed to react for 10 h. After completion of the reaction, the precipitates were

filtered and washed thrice with a saturated aqueous sodium hydrogen carbonate solution. They were then washed with saturated saline and dehydrated using magnesium sulphate. The solvent was removed using an evaporator and dried under a vacuum (5.71 g, yield 52.0 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ –6.13 (s, 1H, H_{cis}), 5.59 (s,1H, H_{trans}), 4.33 (t, 2H, –O–CH_2), 2.77 (t, 2H, –CH_2–S–), 2.17 (s, 3H, –S–CH_3), 1.95 (s, 3H, –CH_3).

Synthesis of 2-(methysulfinyl)ethyl methacrylate (MSEMA)

MTEMA (4.0 g, 25 mmol) was cooled to 0 °C in an ice bath, and N_2 was bubbled through for 30 min. Then, hydrogen peroxide water (3.12 g, 27.5 mmol) was added to the mixture dropwise to raise its temperature back to 25 °C for reaction to proceed for 24 h, following which 50 mL distilled water was added to terminate the reaction. The product was then extracted with DCM thrice, and the collected organic layers were removed by evaporation and dried under vacuum (3.44 g, yield 78.3 %). ^{1}H NMR ($CDCl_3$, 400 MHz): δ –6.15 (s, 1H, H_{cis}), 5.64 (s, 1H, H_{trans}), 4.59 (m 2H, –O–CH_2), 3.07 (m, 2H, –CH_2–SO–), 2.67 (s, 3H, –S–CH_3), 1.96 (s, 3H, –CH_3).

Synthesis of zwitterionic polymer

Poly(SPB) was prepared by dissolving SPB (5.57 g, 20 mmol) in DDMAT (72.9 mg, 0.2 mmol), and AIBN (6.57 mg, 0.04 mmol) in 40 mL of a 1:3 water/methanol solution. When synthesizing copolymers with MSEMA, MSEMA was added to the reaction mixture at 10, 20, and 30 % of the total polymer content. For example, when the introduction rate was 10 %, SPB (5.01 g, 18.0 mmol), MSEMA (0.352 g, 2.0 mmol), DDMAT (72.9 mg, 0.2 mmol), and AIBN (6.57 mg, 0.04 mmol) were dissolved in 40 mL of 1:3 water/methanol solution. N_2 bubbling was performed for 1 h, and the mixture was reacted at 70 °C for 6 h. Samples were subsequently taken for ^{1}H NMR analysis. Monomer conversion was determined using vinyl protons near 5.8 ppm and 6.2 ppm based on the methylene peak of the polymer near 2.9 ppm. After completion of the reaction, the sample was dialysed against methanol and water for 24 h (MWCO of 3.5 kDa, Spectra/Por® 3 Dialysis Membrane, Spectrum Labs, Inc., Rancho Dominguez, CA, USA) and freeze-dried. The chemical structure of the compound was confirmed by ^{1}H NMR spectroscopy, using D_2O as the solvent.

Synthesis of fluorescence-modified polymers

Each polymer (0.76 μmol) was dissolved in 1 M NaCl aqueous solution, and tris(2-carboxyethyl)phosphine hydrochloride (17.4 μmol) was added. *N*-butyl amine (0.76 mmol) was added, reacted at room temperature for 2 h, dialysed for 7 d (MWCO of 3.5 kDa), and freeze-dried. The obtained polymer (6.08 μmol) was dissolved in PBS containing a small amount of tris(2-carboxyethyl)phosphine hydrochloride. Fluorescein *o*-acrylate (18.2 μmol) was dissolved in DMSO, mixed with the polymer solution, and allowed to react at room temperature for 10 h in the dark. Following the completion of the reaction, the solution was dialysed for 7 days (MWCO of 3.5 kDa) and then freeze-dried.

Cell culture

Mouse fibroblasts (L929; American Type Culture Collection, Manassas, VA, USA) were prepared in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % foetal bovine serum (FBS). Cells were cultured in an incubator at 37 °C with 5 % CO_2. Passaging was performed when the cells exceeded 80 % of the bottom of the dish. For passaging, the cells were detached by treatment with trypsin solution (0.25 % [w/v] trypsin containing 0.02 % [w/v] ethylenediaminetetraacetic acid in PBS).

Cryopreservation

The polymer solution was dissolved in an aqueous 0.3 M NaCl aqueous solution to 10 w/w%. The solution was filter-sterilised through a 0.22-μm filter. Then, 1×10^6 L929 cells were suspended in 1 mL of the polymer solution and stored at –80 °C; cooling was conducted without a controlled cooling rate. After permeation of the cells with the polymer, 1×10^6 permeated-L929 cells were seeded in a 35 mm dish and cultured for 24 h. Then, the medium was removed and 1 mL of polymer solution was added to the culture, which was incubated at 4 °C for 10 min. After washing thrice with PBS, the cells were treated with trypsin and collected by centrifugation (1000 rpm). They were then suspended in 1 mL of the polymer solution and stored at -80 °C in the same manner. After 24 h, the cells were thawed by immersing the samples in a water bath at 37 °C. The cell suspension was then diluted 10-fold with DMEM and centrifuged at 1000 rpm for 4 min. The cells were then suspended in a small amount of DMEM and viability was determined by staining them with trypan blue. Cell viability and recovery were determined using the following formula (Eq. 1).

$$\text{Recovery rate}\ (\%) = \frac{\text{Living cell}}{\text{Frozen cell}} \times 100 \qquad \text{(Eq. 1)}$$

In addition, to examine cell proliferation ability after thawing, 1.0×10^4 cells were seeded in a 24-well plate, and the number of cells was counted after 1, 3, 5, and 7 days.

Cytotoxicity test

L929 cells were dispensed into 96-well plates at 1×10^3 cells/100 μL and cultured in an incubator for 3 days. Samples were prepared by dissolving in DMEM so that the concentrations of the polymer and DMSO ranged between 0 and 20 %, followed by filter sterilisation. After culturing, 100 μL of each prepared sample was dispensed and further incubated again in an incubator for 2 days. MTT was dissolved to 0.3 mg/mL in DMEM without FBS, and 100 μL of the solution was dispensed and incubated for 3 h. Subsequently, all solutions were removed from the plate, 100 μL of DMSO was dispensed, and the absorbance was measured at λ = 540 nm using a microplate reader (Infinite 200 PRO M Nano, Tecan).

Confocal Microscopy

For fluorescence observation, 1×10^6 L929 cells were seeded in a 35-mm glass-bottom dish and cultured for 24 h. Then, the medium was removed, 1 w/w% fluorescence-modified polymer solution was added dropwise, and the mixture was incubated at 4 °C for 10 min. After removing the polymer solution and washing with PBS thrice, the cells were treated with PBS again and observed under a confocal laser microscope (FV1000D, Olympus).

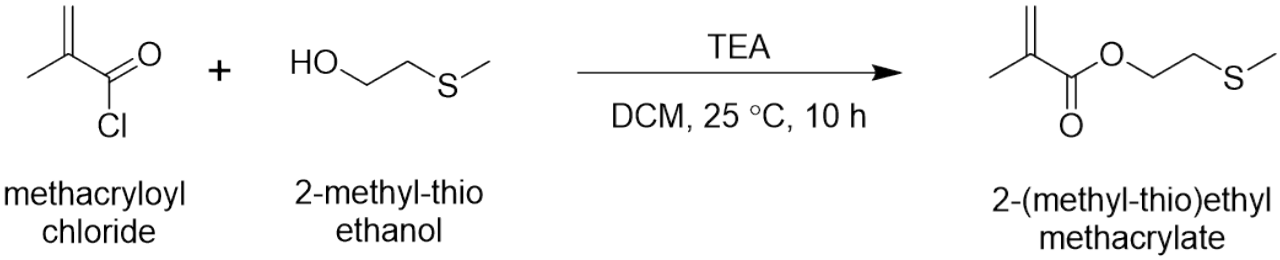
Observation of counter diffusion

To observe the counter diffusion, 1×10^6 L929 cells were seeded in a 35 mm dish and cultured for 24 h. Subsequently, the medium was removed, 1 w/w% of the fluorescence-modified polymer solution was added dropwise, and the mixture was incubated at 4 °C for 10 min. After washing thrice with PBS, the cells were trypsinised and collected by centrifugation. The harvested cells were suspended in 10 mL of medium, and 1 mL of the suspension was plated on a 35-mm glass-bottom dish and incubated at 37 °C. Fluorescence observations were performed every 10 min using BZ-X800 (Keyence, Osaka, Japan).

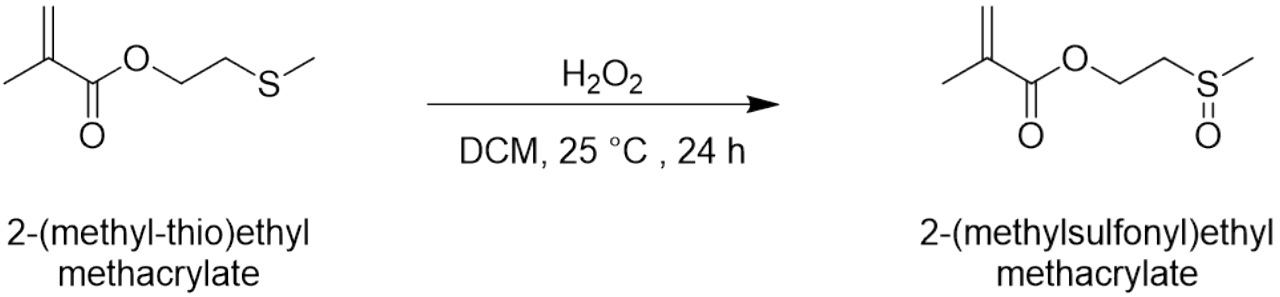
Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed using the EXTAR SII-6200 (Seiko Instruments, Japan). After cooling from 5 °C to –50 °C at a cooling rate of 1 °C/min under a nitrogen flow of 50 mL/min, the temperature was maintained for 5 min and then heated to 20 °C at 1 °C/min.

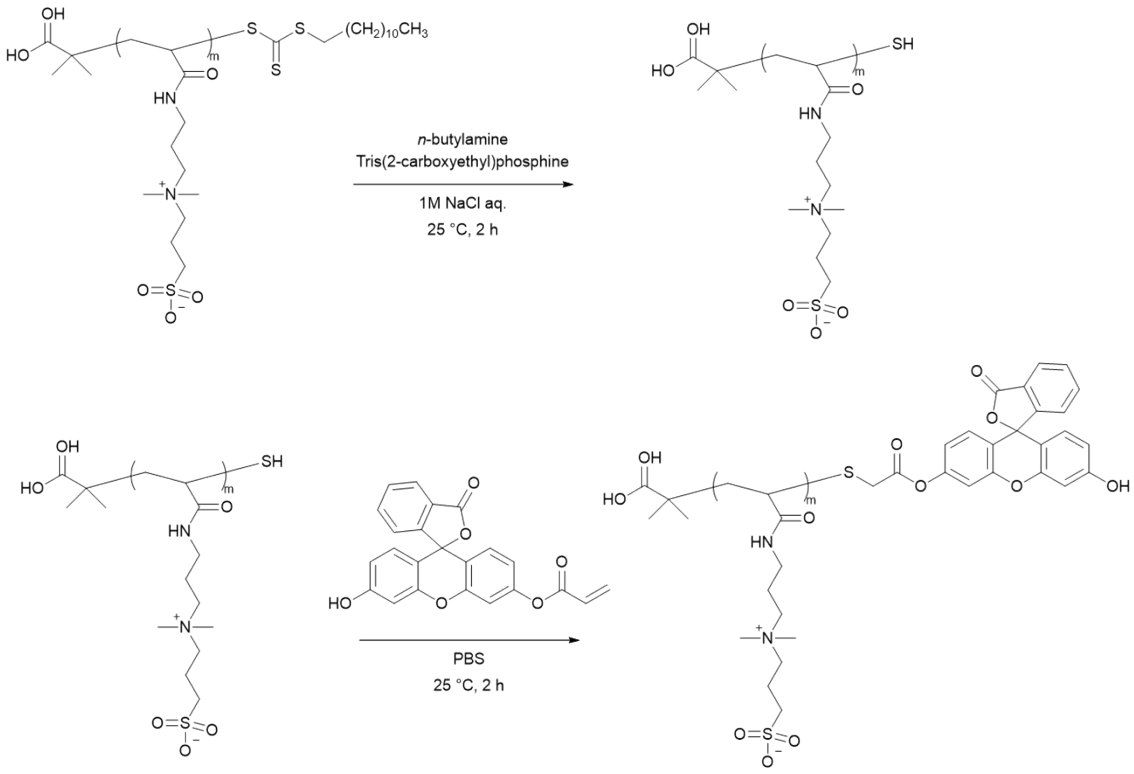
SUPPLEMENTARY FIGURES AND TABLES



Scheme S1 Synthesis of MTEMA.



Scheme S2 Synthesis of MSEMA.



Scheme S3 Synthesis of fluorescence-modified polymers.

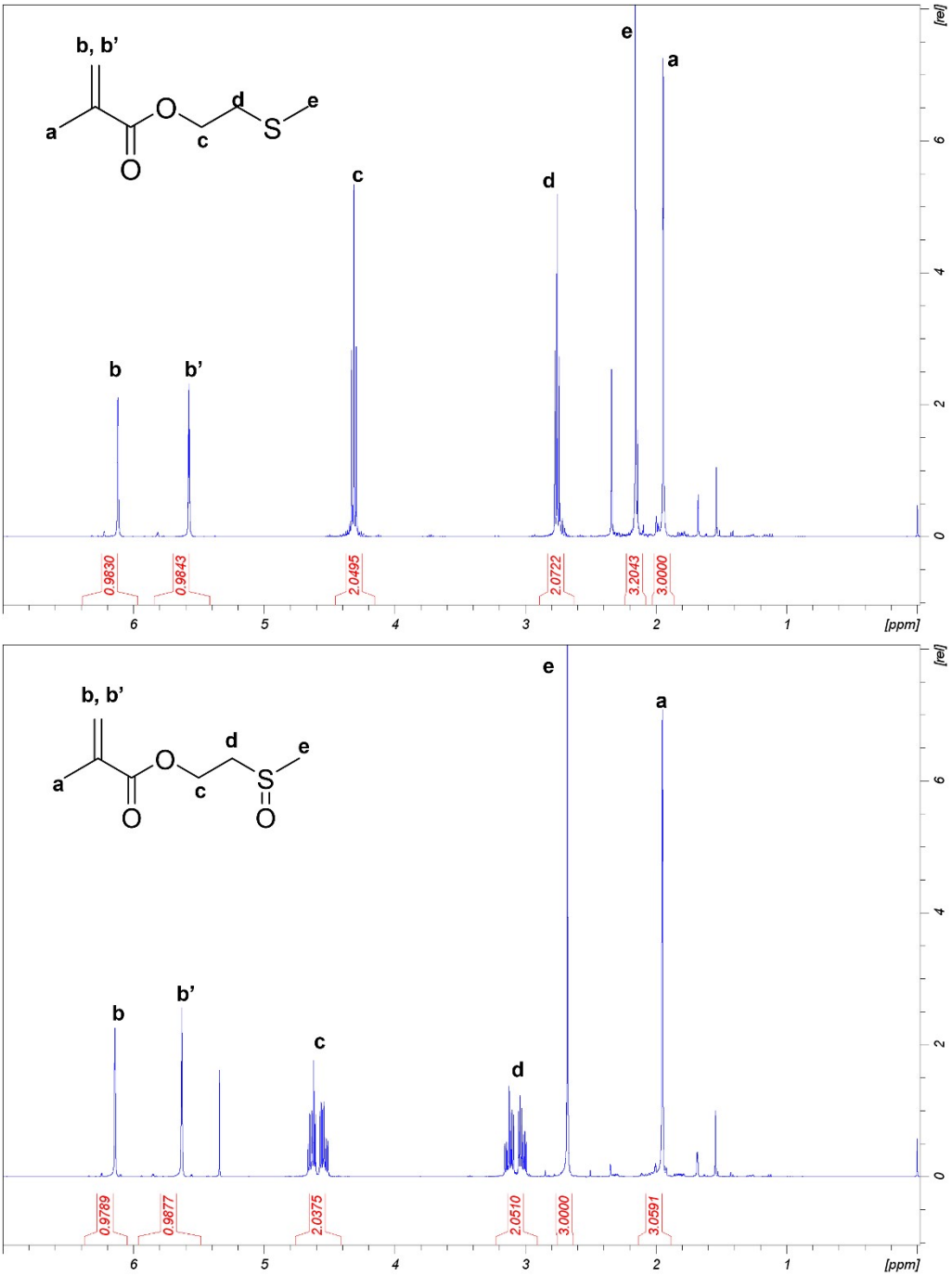


Fig. S1 ^{1}H NMR spectra of MTEMA and MEMA. Top: MEMA, Bottom: MTEMA.

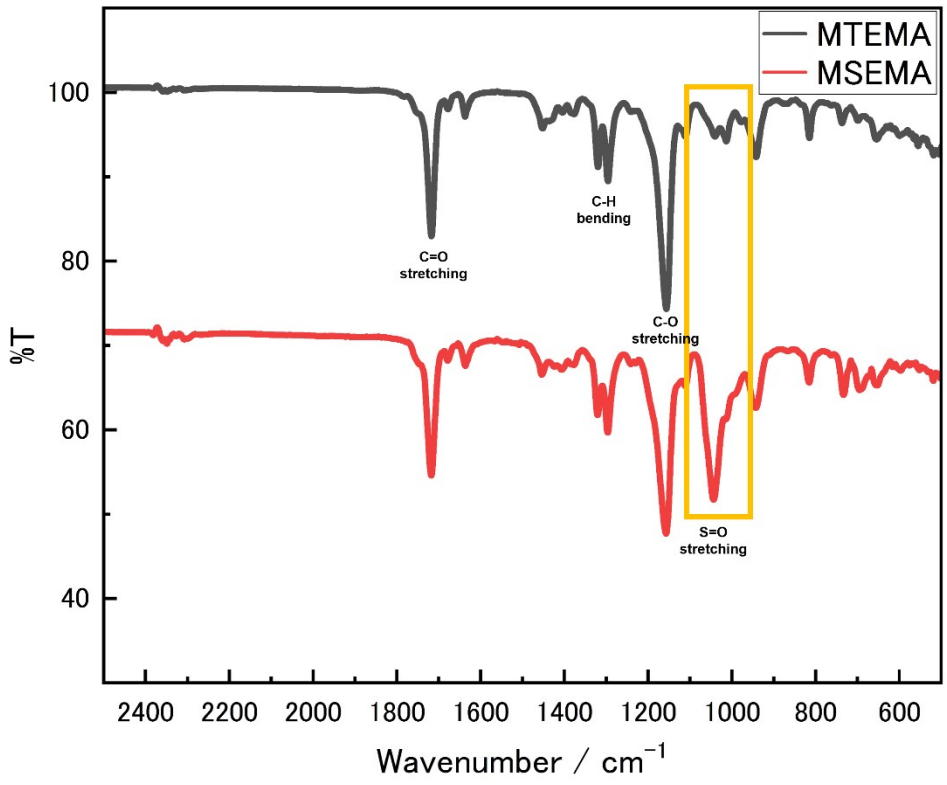


Fig. S2 FT-IR spectra of MTEMA and MSEMA.

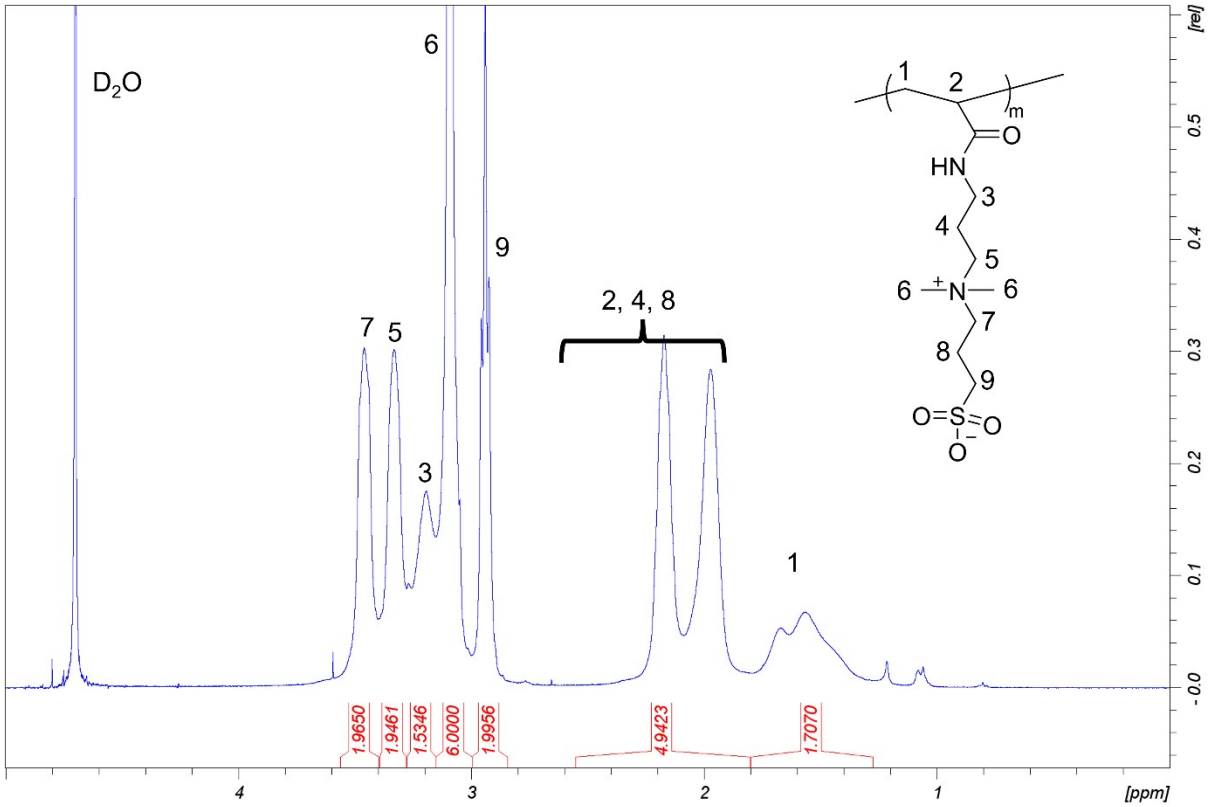


Fig. S3 ^{1}H NMR spectra of poly(SPB).

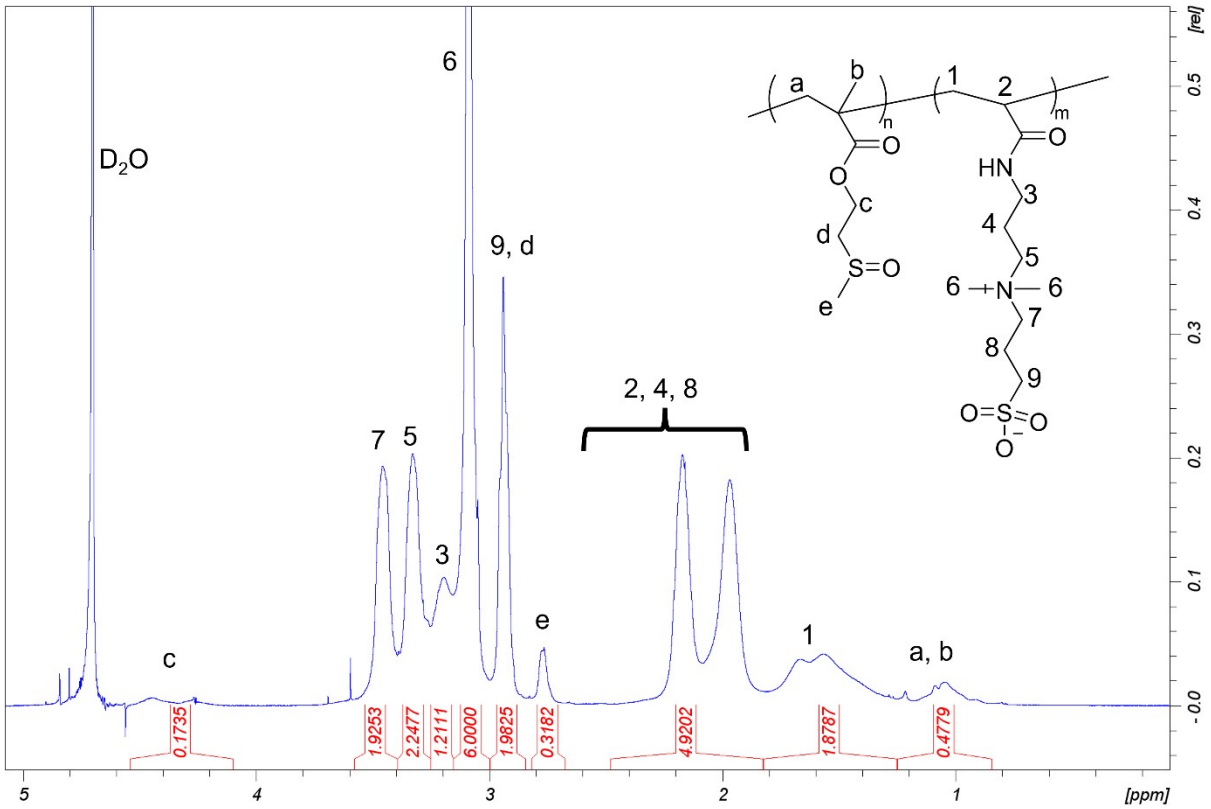


Fig. S4 ^{1}H NMR spectra of poly(SPB/MSEMA10).

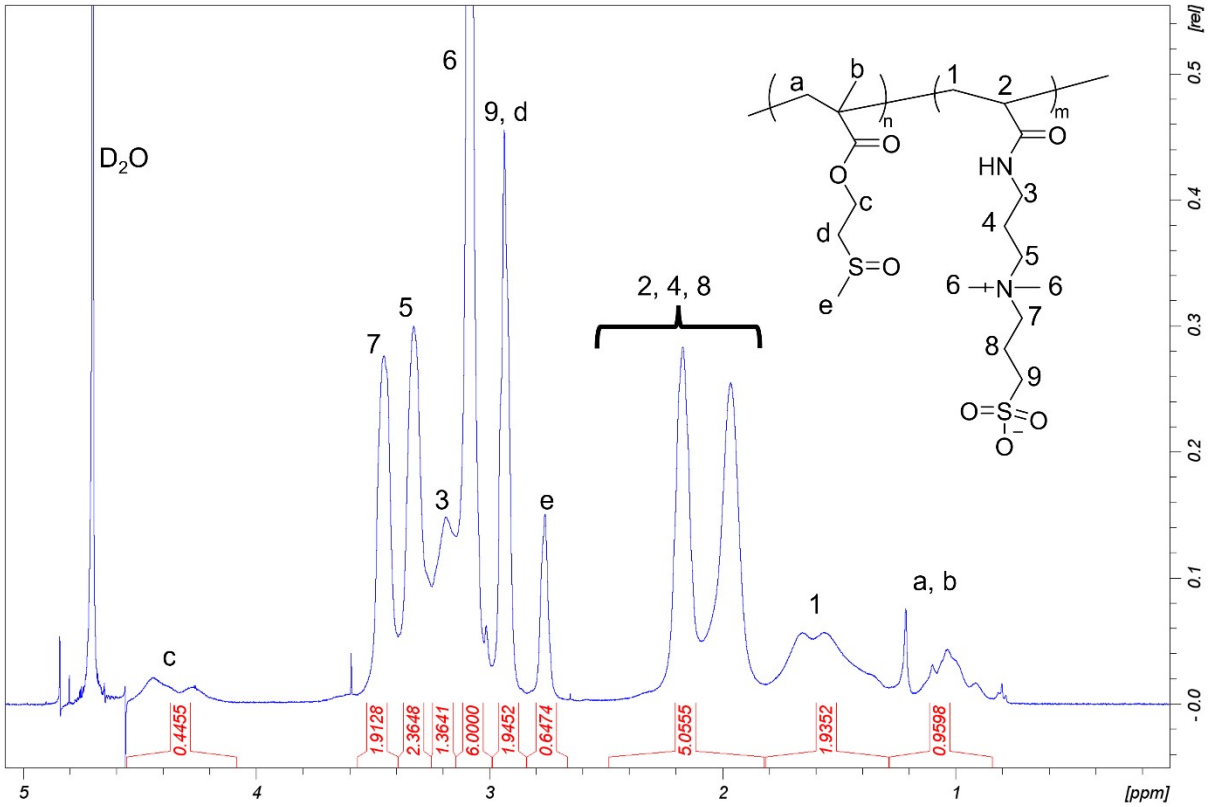


Fig. S5 ^{1}H NMR spectra of poly(SPB/MSEMA20).

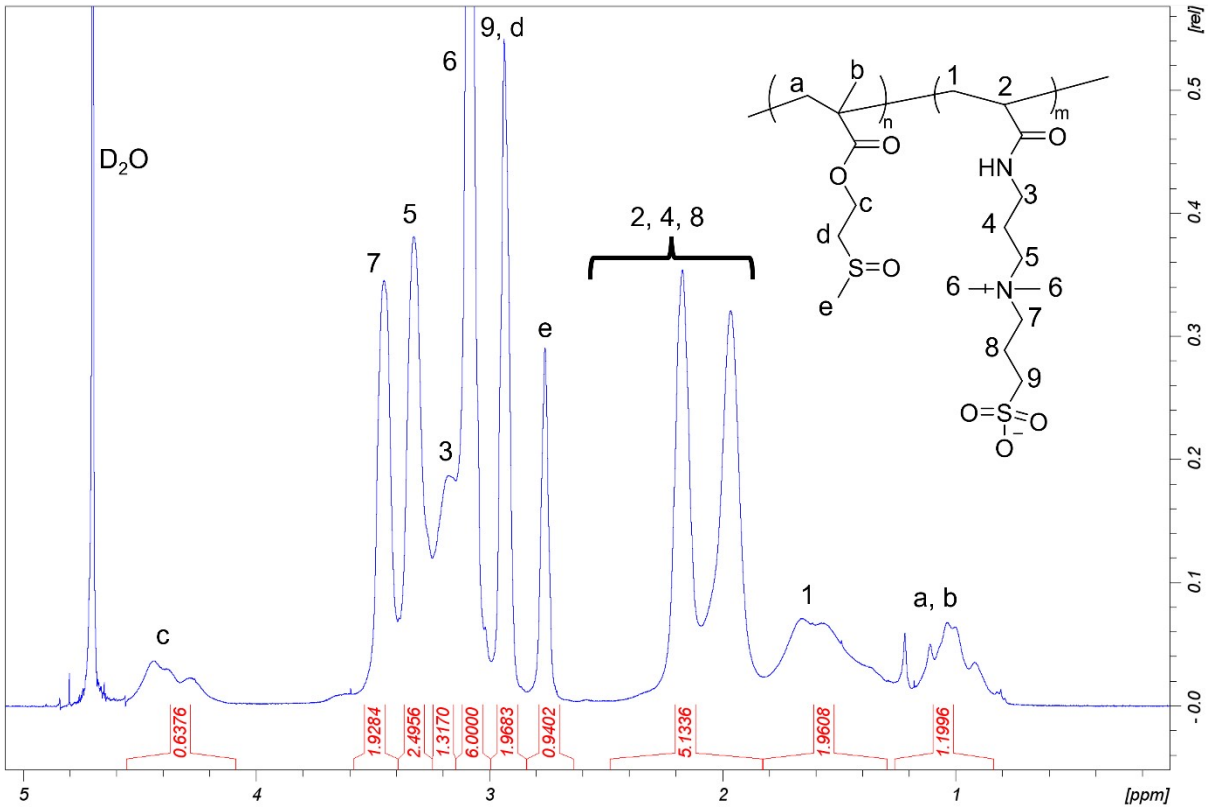


Fig. S6 ^{1}H NMR spectra of poly(SPB/MSEMA30).

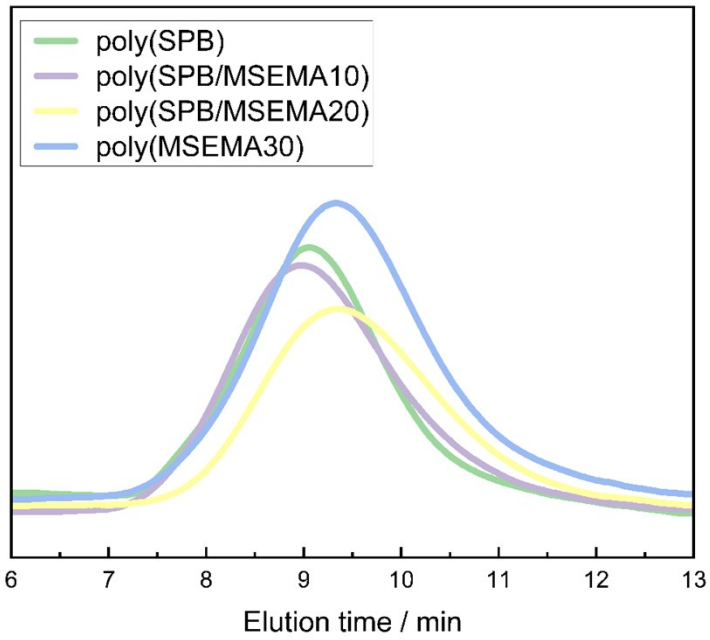


Fig. S7 GPC curve of polymers.

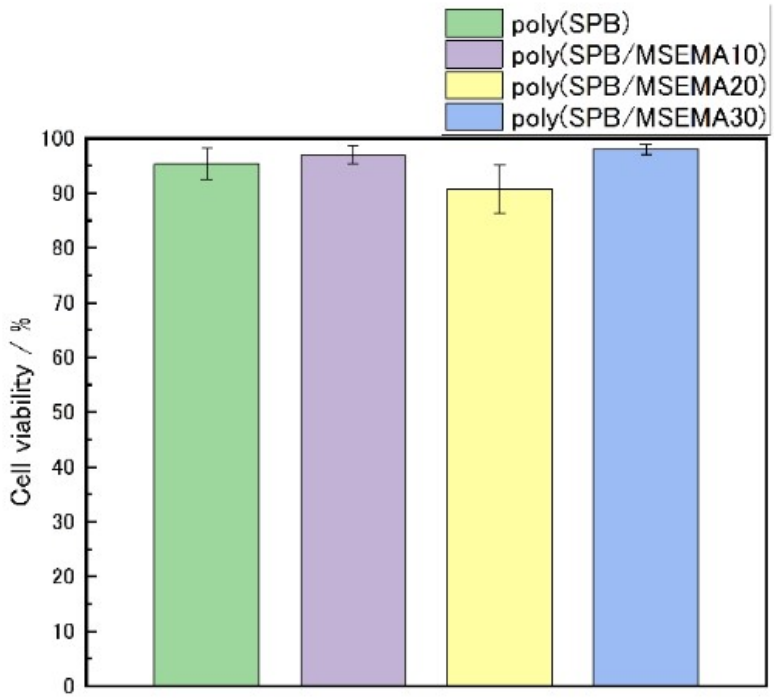


Fig. S8 Cytotoxicity of each polymer against L929 cells 1 hour after exposure.

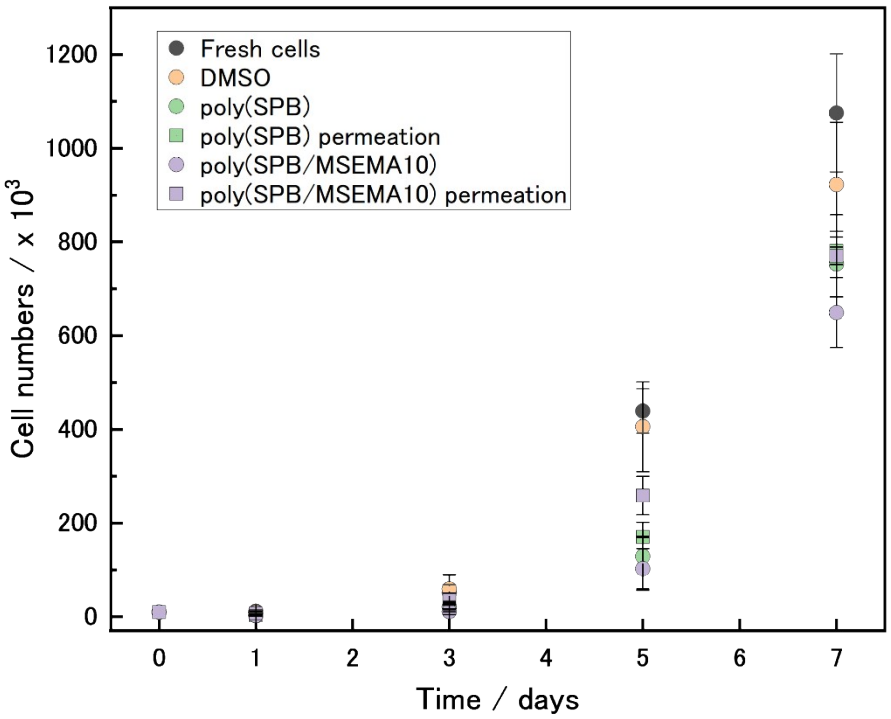


Fig. S9 Cell growth curve after freeze–thaw cycle.

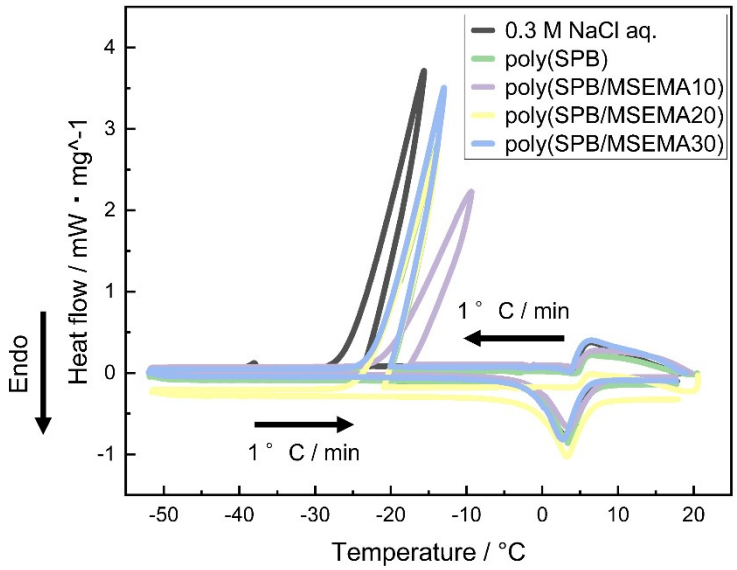


Fig. S10 DSC trace of polymers

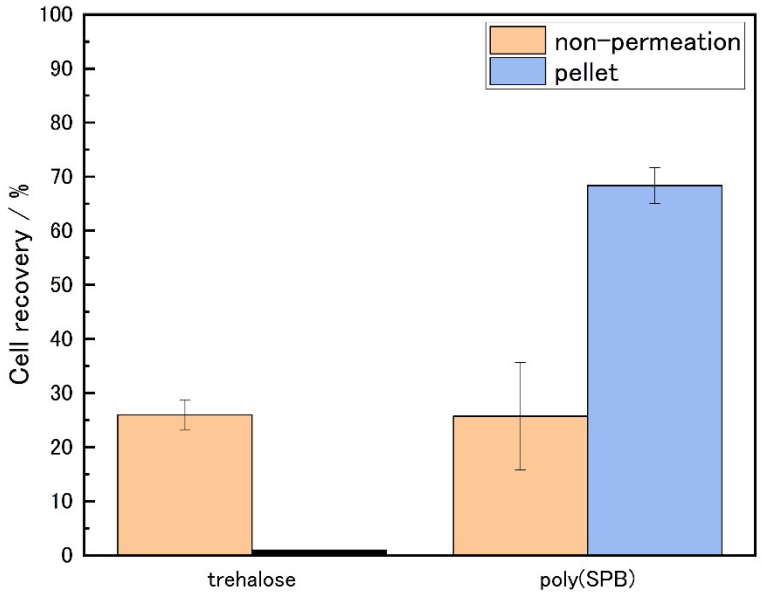


Fig. S11 Cryoprotective capacity of trehalose- and poly(SPB)-permeabilized cell pellets.

Table S1 Characterization of zwitterionic polymer.

	Composition (in feed)		Composition (NMR)[a)]		Molar ratio[b)]	Conversion / %[c)]	M_n $\times 10^4$ [d)]	M_w/M_n[d)]
	SPB	MSEMA	SPB	MSEMA				
poly(SPB)	100	0	100	0	100 : 1 : 0.2	96.7	1.47	1.25
poly(SPB/MSEMA10)	90	10	89.9	10.1	100 : 1 : 0.2	98.6	1.48	1.29
poly(SPB/MSEMA20)	80	20	81.8	18.2	100 : 1 : 0.2	98.5	1.25	1.24
poly(SPB/MSEMA30)	70	30	75.7	24.3	100 : 1 : 0.2	97.5	1.29	1.27

a) Using the integral value of the NMR peak in Fig. S3-6, the amount of MSEMEA was calculated by Eq.2.
b) [monomer]:[CTA]:[initiator]. c) Determined using vinyl protons near 5.8 ppm and 6.2 ppm based on the methylene peak of the polymer near 2.9 ppm by ^{1}H NMR. d) Determined by GPC (Fig. S7).

$$\text{MSEMA (\%)} = \frac{\frac{I(2.8\ \text{ppm})}{3}}{\frac{I(3.0\ -\ 3.6\ \text{ppm})}{12} + \frac{I(2.8\ \text{ppm})}{3}} \times 100$$

where *I* refers to the area under the peak corresponding to the chemical shift.