Supporting Information for

A Synthetically Scalable Poly(ampholyte) which Dramatically Enhances Cellular Cryopreservation

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Additional Methods

Osmotic Fragility. 20 mL Sheep blood in Alsever's solution was added to a 50 mL centrifuge tube and centrifuged at 2000 rpm for 5 min to concentrate the solution, 14 mL of the supernatant was removed and replaced with 14 mL PBS solution. 10 mL of this solution was kept in the refrigerator as a control for unfrozen blood cells in later testing. Polymer solutions were made up at 200 $mg \cdot mL^{-1}$ concentration to ensure the correct final cryoprotectant concentration. Using the remaining 10 mL of blood cells in PBS, 0.5 mL of the blood solution was added to 0.5 mL of the polymer solution in 2 mL cryovials. These were then incubated in the fridge for 30 min before freezing in liquid nitrogen vapour. After 1 h, samples were thawed in a water bath at 45 °C for 10 min and recombined into one 15 mL centrifuge tube. This was centrifuged at 2000 rpm for 10 min, after which the supernatant was removed and the sample made up to its original volume with PBS. A serial dilution of salt solutions were prepared at 2 M, 1 M, 0.5 M, 0.25 M, 0.125 M, 0.0625 M, 0.03125 M, and finally pure DI water. 0.2 mL of each salt solution was pipetted into six Eppendorf tubes, and 0.2 mL of frozen blood was added to three of these, and unfrozen blood added to the other three. These samples were then left for 20 min, after which the samples were centrifuged at 2000 rpm for 5 min. 40 μL of the supernatant was removed and added to 750 μL of AHD solution. After vortexing, the samples were pipetted into a 96 well plate in triplicate (3 x 200 μL per sample) and the absorbance recorded at 580nm in a plate reader. Samples were compared against unfrozen PBS and lysis buffer as the 0 and 100% lysis samples respectively.

Nile Red Staining. A549 cells were seeded at $4 \cdot 10^4$ cells per well in 200 μL of cell culture medium with indicated concentrations of polyampholyte in 96-well plates. Cells were incubated with polymer for 10 min and exchanged against completed cell media or incubated with polymer for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Following the incubation period, 200 nM Nile Red (Sigma-Aldrich) in PBS was added to the wells. Plate was incubated for 10 min at room temperature. Nile Red solution was removed and wells were washed with PBS. Cells were then imaged using a CKX41 microscope with pE-300-W LED illumination (CoolLED, Andover, UK), the XC30 camera, and processed using the CellSens software.

Confocal Nile Red Staining. A549 cells were seeded at $5 \cdot 10^5$ cells per well in 1 mL of cell culture medium with indicated concentrations of polyampholyte in 12-well plates

(ThermoFisher) with 12 mm coverslips (Appleton Woods). Cells were incubated with polymer for 10 min and exchanged against completed cell media or incubated with polymer for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Following the incubation period, 200 nM Nile Red in PBS was added. Plate was incubated for 10 min at room temperature. Nile Red solution was removed and wells were washed with PBS. Cells were subsequently stained with NucBlue™ Live ReadyProbes™ nuclear stain reagent (ThermoFisher), fixed with 4% paraformaldehyde and imaged. Confocal imaging was completed using a Zeiss LSM 710 inverted microscope with 63x oil immersion objective lenses, equipped with three photomultiplier detectors (GaAsP, multialkali and BiG.2) and multichannel spectral imaging with an ultra-sensitive GASP detector. The UV and VIS Laser Modules allowed selection of six lasers with wavelengths of 633, 594, 561, 543, 514, 488, 458, 405 and 355 nm. Zeiss ZEN (blue edition) 2.3 lite was utilised for image collection and processing.

Confocal Polyampholyte Uptake. A549 cells were seeded at $5 \cdot 10^5$ cells per well in 1 mL of cell culture medium with indicated concentrations of rhodamine-6G tagged polyampholyte in 12-well plates with 12 mm coverslips. Cells were incubated with tagged polymer for 10 min and exchanged against completed cell media or incubated with polymer for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Following the incubation period, cells were stained with NucBlue™ Live ReadyProbes™ nuclear stain reagent, fixed with 4% paraformaldehyde and imaged as detailed previously.

Neutral Red Staining. A549 cells were seeded at $4 \cdot 10^4$ cells per well in 200 µL of cell culture medium with indicated concentrations of polyampholyte in 96-well plates. Cells were incubated with polymer for 10 min and exchanged against completed cell media or incubated with polymer for 24 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Following the incubation period, 100 µL neutral red was added and the plate was incubated for 2 h at 37 °C. Plate was washed with PBS and then 150 µL of destain solution, 50% ethanol (VWR International, Leicestershire, UK), 49% deionized water, 1% glacial acetic acid (Sigma-Aldrich), was added for 10 min with shaking. Cells were then imaged using a CKX41 microscope, the XC30 camera, and processed using the CellSens software.

Additional Data

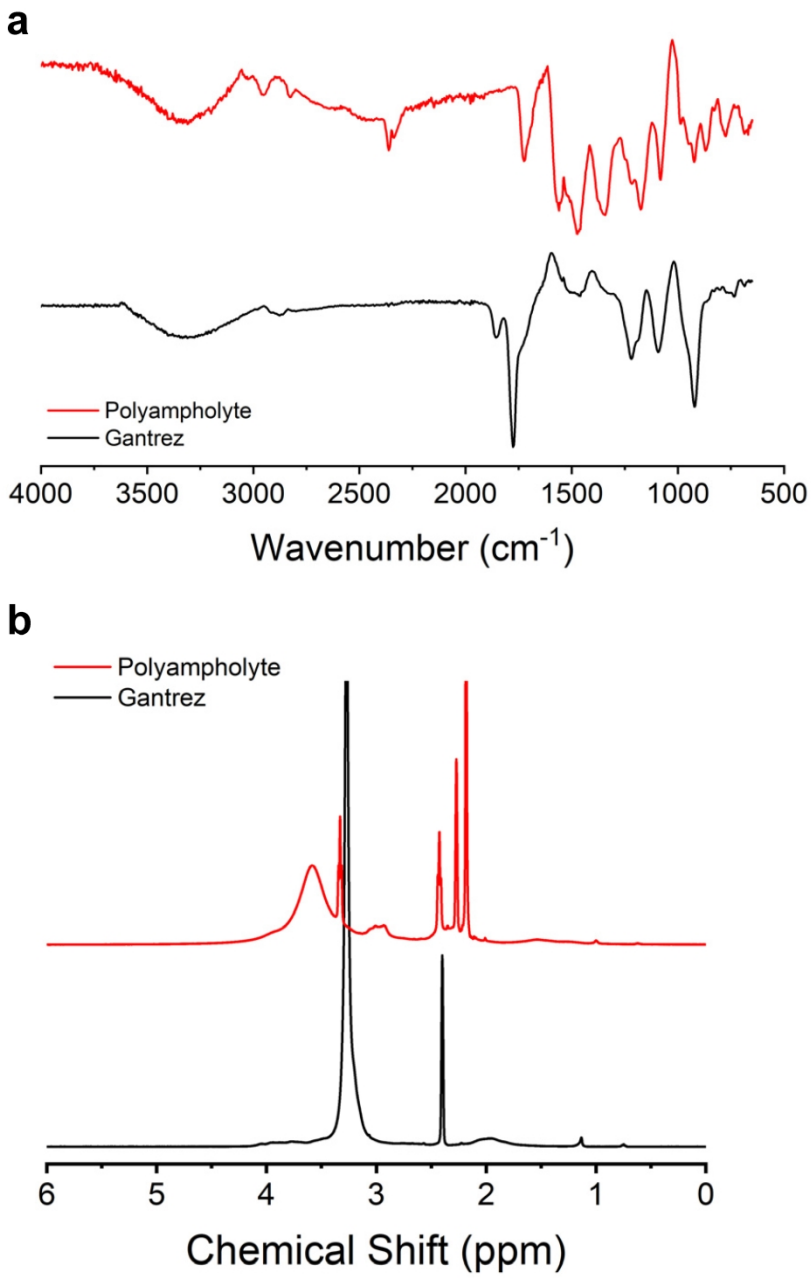

Figure S1. Polymer characterization. a) Infrared spectra of the poly(methyl vinyl ether-*alt*-maleic anhydride) precursor (black) and the aminoethanol functionalised polyampholyte (red), the anhydride peak at ca. 1750 cm^{-1} is removed, and the new carboxylate and ester stretches produced; **b)** ^{1}H NMR spectra of the poly(methyl vinyl ether-*alt*-maleic anhydride) precursor (black) and the aminoethanol functionalised polyampholyte (red).

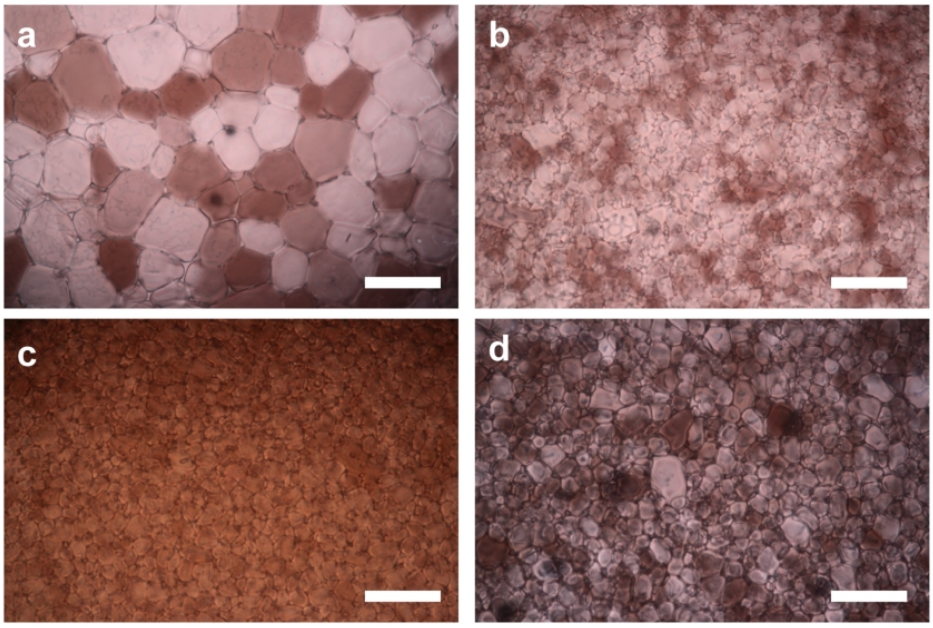

Figure S2. Example splat images. a) PBS, **b)** PVA 1 mg.mL^{-1}, **c)** AFP Type 1 0.5 mg.mL^{-1}, **d) P2** 20 mg.mL^{-1}. Scale bars represent 100 μm.

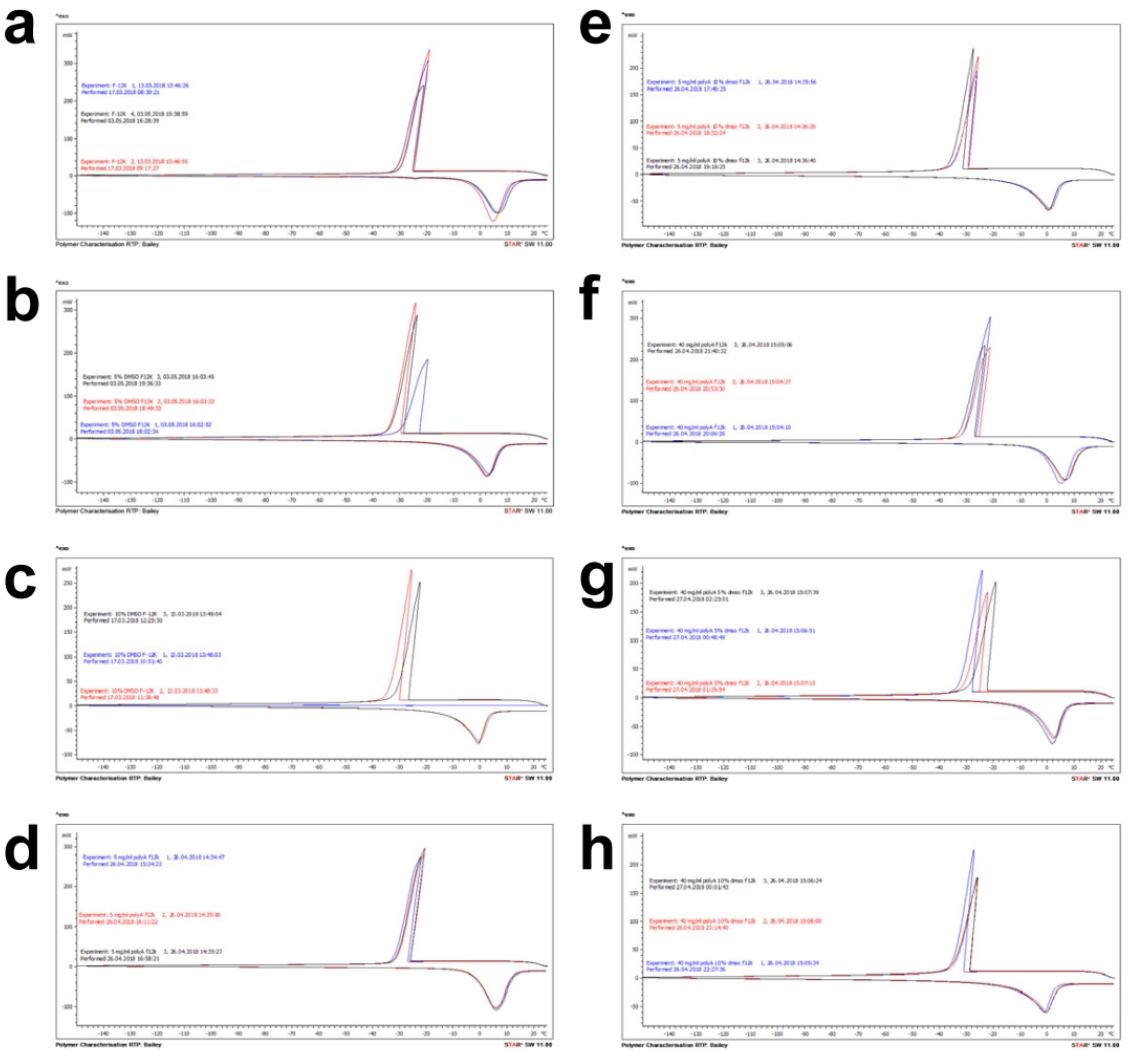

Figure S3. DSC traces of solutions tested. **a)** F-12K, **b)** 5% DMSO, **c)** 10% DMSO, **d)** 5 mg·mL^{-1} **P2**, **e)** 5 mg·mL^{-1} **P2** + 10% DMSO, **f)** 40 mg·mL^{-1} **P2**, **g)** 40 mg·mL^{-1} **P2** + 5% DMSO, **h)** 40 mg·mL^{-1} **P2** + 10% DMSO.

Melting point by DSC.

DSC does not enable accurate nucleation temperatures to be obtained (as multipoint low volume assays are needed[4]) but onset melting temperature can be obtained (Fig. S2). Full DSC cooling/heating curves for the cryoprotectant formulations are also shown in Figure S2. In each case, there is clear crystallisation, showing none of the formulations enable a vitreous phase to be formed, crucial to the understanding of any post-thaw cell viability results. The melting

points were in line with colligative effects with the highest concentrations of the polyampholyte giving the lowest melting points.

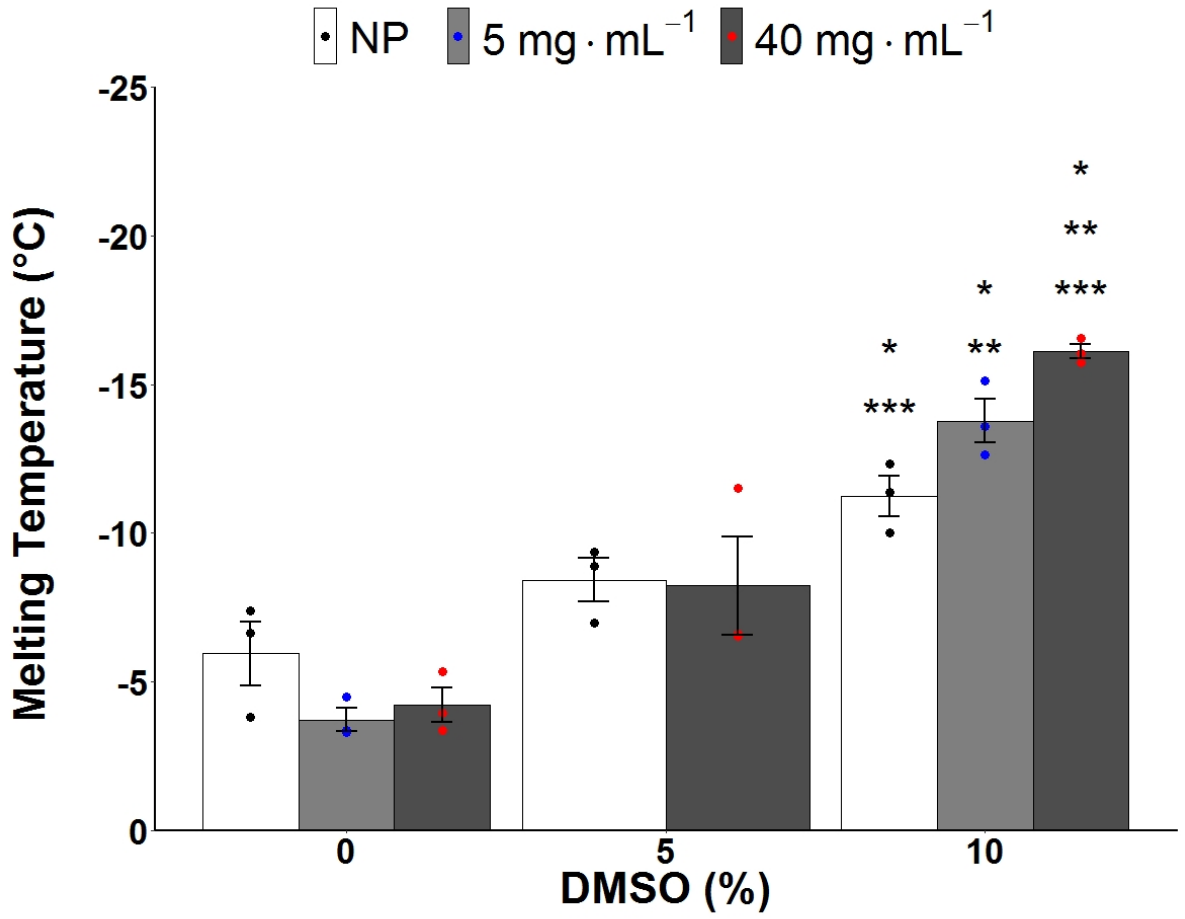

Figure S4. Melting Temperature. Melting temperature assessed by DSC for indicated concentrations of DMSO and polymer dissolved in F-12K with 10% FBS (N = 24, P = 0.0000002, * P < 0.001 from 0% DMSO w/NP, ** P < 0.001 from 0% DMSO w/5 mg·mL^{-1} **P2**, *** P < 0.001 from 0% DMSO w/40 mg·mL^{-1} **P2**). Error bars represent ± SEM of 3 independent experiments.

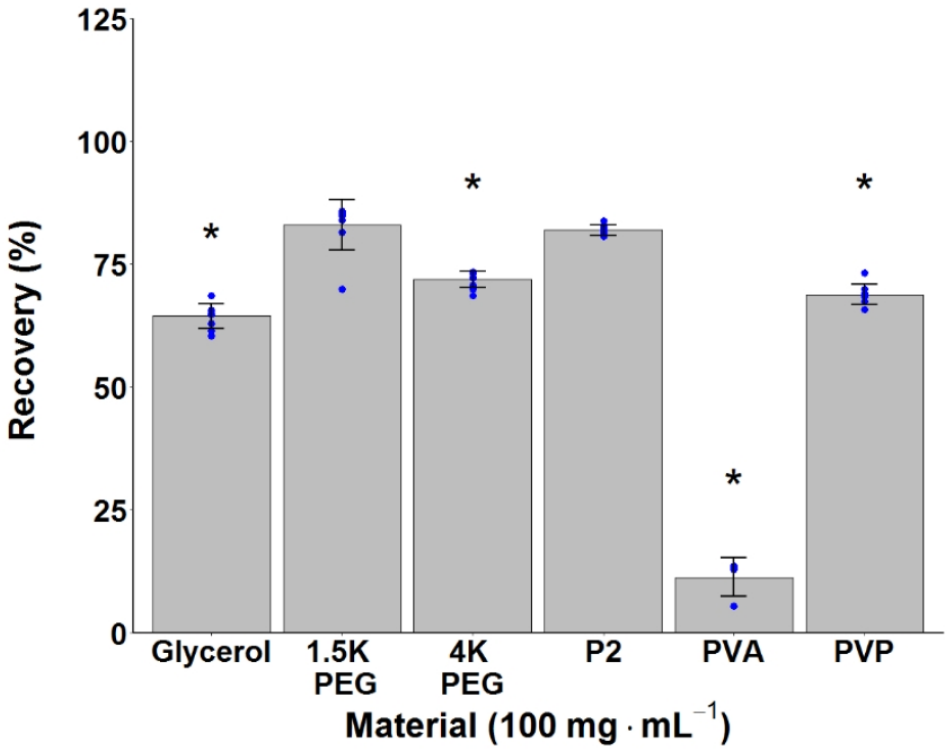

Figure S5. Blood freezing with other cryoprotective compounds. Comparison of recovery between **P2** and other commonly used cryoprotectants ($N = 49$, $P = 0.00000001$, * $P < 0.001$ from **P2**). Error bars represent ± SEM of 9 independent experiments.

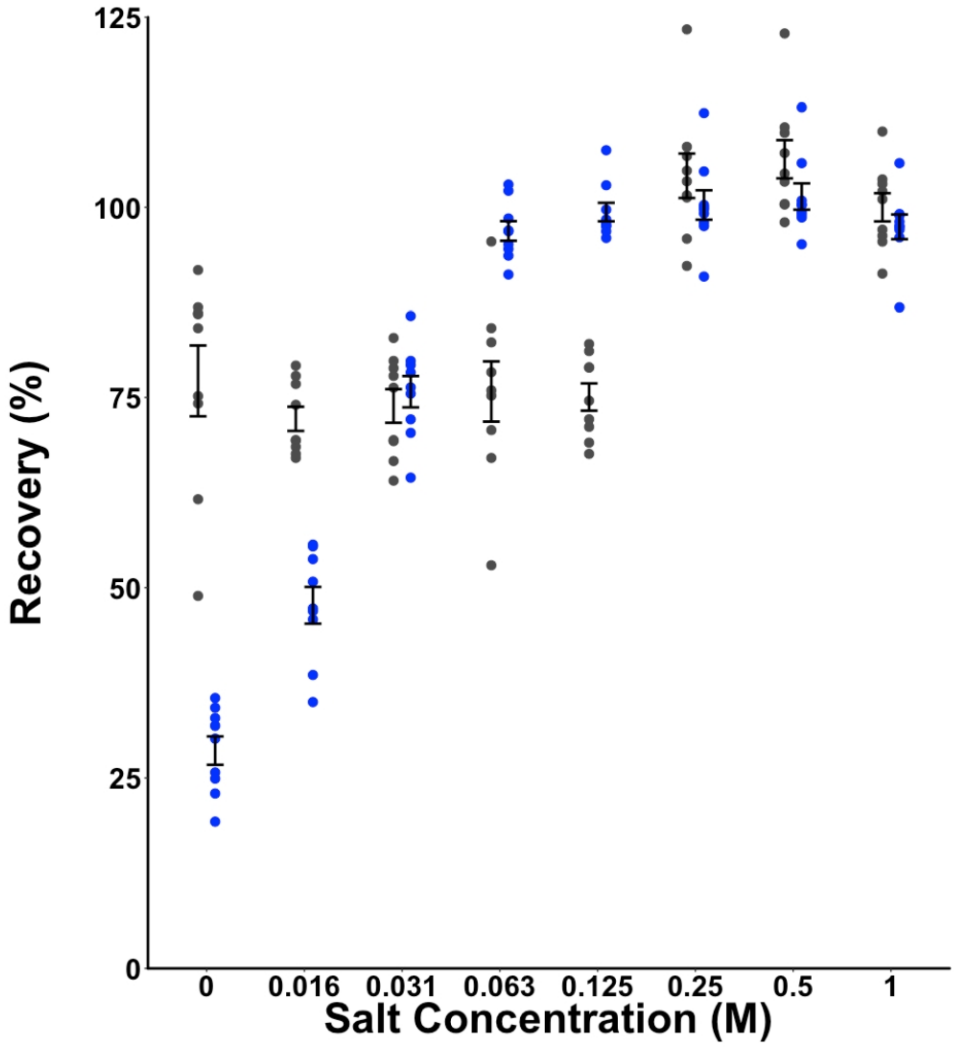

Figure S6. Osmotic Fragility. The osmotic stability of frozen (gray) and unfrozen (blue) blood was determined at a variety of salt concentrations. While the frozen blood appears to initially lyse at higher concentrations than the unfrozen sample (0.125M), the unfrozen blood reaches a much greater level of lysis at lower concentrations.

Mammalian Cell Phenotype

A circular phenotype was observed during the toxicity screening of **P2** (Fig. S7a,b). Concentrations above 10 $mg{\cdot}mL^{-1}$ showed some evidence of this phenotype at the 10 min incubation time point (Fig. S7a), while concentrations above 1 $mg{\cdot}mL^{-1}$ displayed the phenotype at the 24 hr time point (Fig. S7b). The origin of this phenotype was investigated. We first investigated if the circles were due to lipid droplets using Nile Red staining (Fig. S7c).

Had the phenotype been due to lipid droplets, the circles would have stained bright red/yellow, as the circles remained black, we ruled out the origin as lipid droplets. We next evaluated the circles as phagocytes/vacuoles within the cells utilising neutral red staining (Fig. S7d). The circles remained completely unstained, ruling out the presence of phagocytes/vacuoles. We next investigated the phenotype to be membrane disruption utilizing the LIVE/DEAD stain to test for membrane permeability (main text, Fig. 6c). We saw no staining of the circles, but we also did not see an increase in permeability for cells displaying the phenotype. Finally, we investigated the presence of encapsulated polymer rafts using a fluorescently tagged rhodamine-6G **P2** polymer (main text, Fig. 5c). We saw no retention of polymer, ruling out the origin as polymer rafts. Additionally, we did not see a decreased metabolic rate in all concentrations that resulted in the phenotype (main text, Fig. 3f). We can conclude that the phenotype present is not lipid droplets, vacuoles, or polymer rafts, but the circles do not appear to permeate the membrane or affect the metabolic rate of the cells and the normal phenotype is recovered post-thaw.

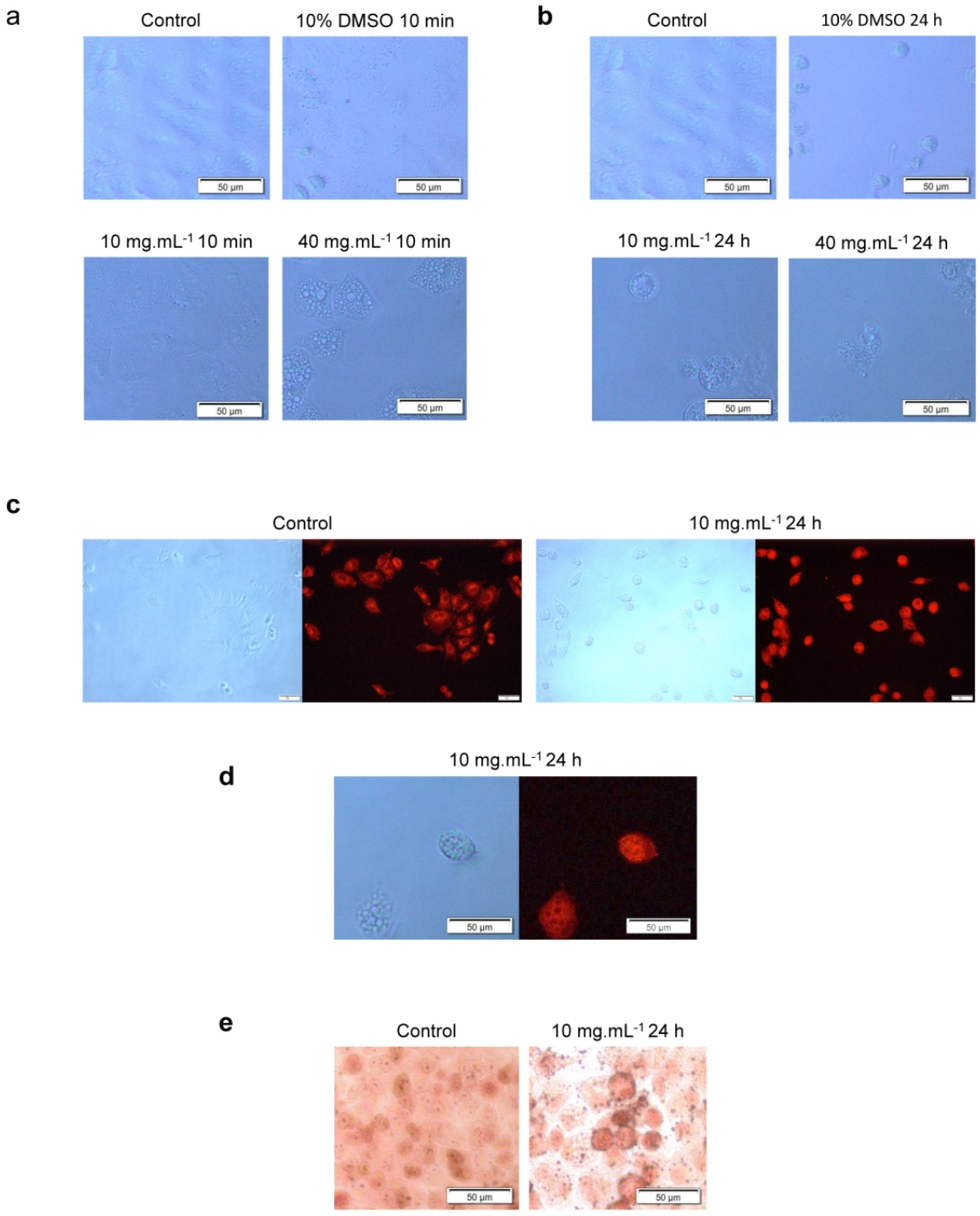

Figure S7. Cell phenotype. **a)** Images with 10 min **P2** incubation; scale bars represent 50 µm, **b)** Images with 24 h **P2** incubation; scale bars represent 50 µm, **c)** Nile red staining at 10X; scale bars represent 100 µm, **d)** Nile red staining at 20X; scale bars represent 50 µm, **e)** Neutral red staining; scale bars represent 50 µm.

DMSO Toxicity

A549 cells were incubated with varying concentrations of DMSO for 30 min and then evaluated for metabolic reduction of alamar blue (Fig S8). There was a significantly lower alamar blue reduction for cells incubated in 5 and 10% DMSO.

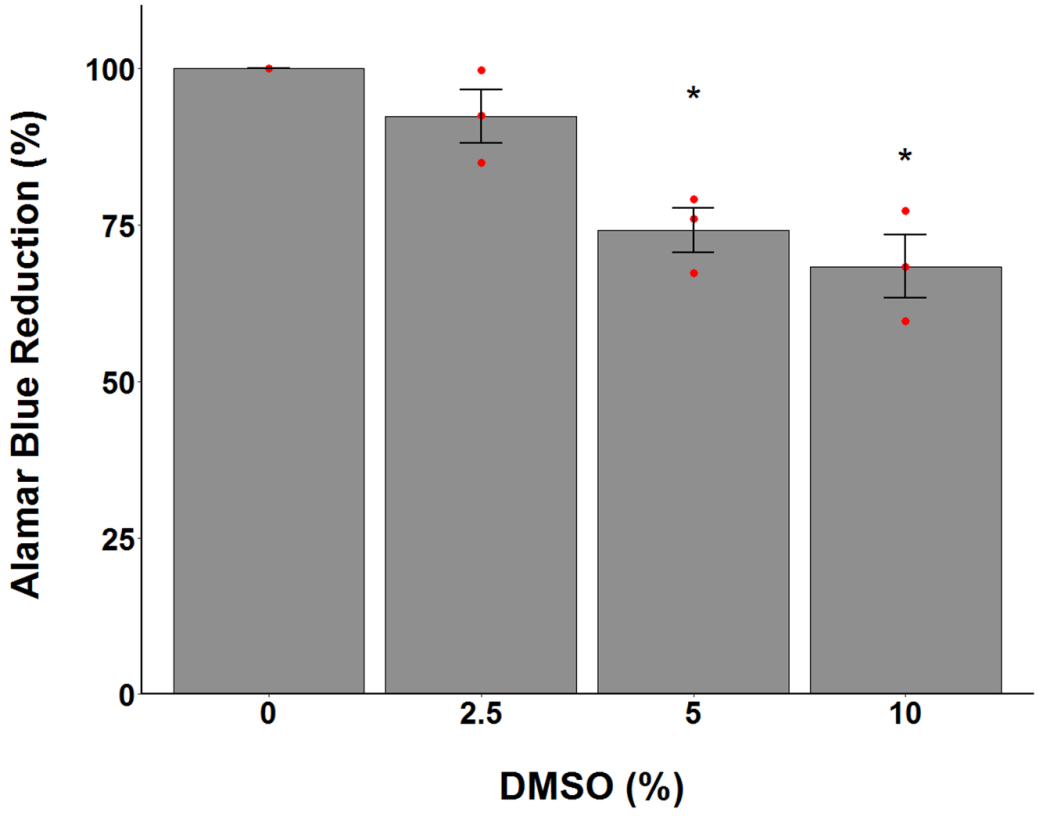

Figure S8. DMSO Toxicity. A549 cell alamar blue reduction following incubation in DMSO for 30 min ($N = 12$, $P = 0.001$, * $P < 0.01$ from 0% DMSO). Error bars represent ± SEM of 3 independent experiments.

Graphical Representation of Live/Dead Post-Freezing Imaging

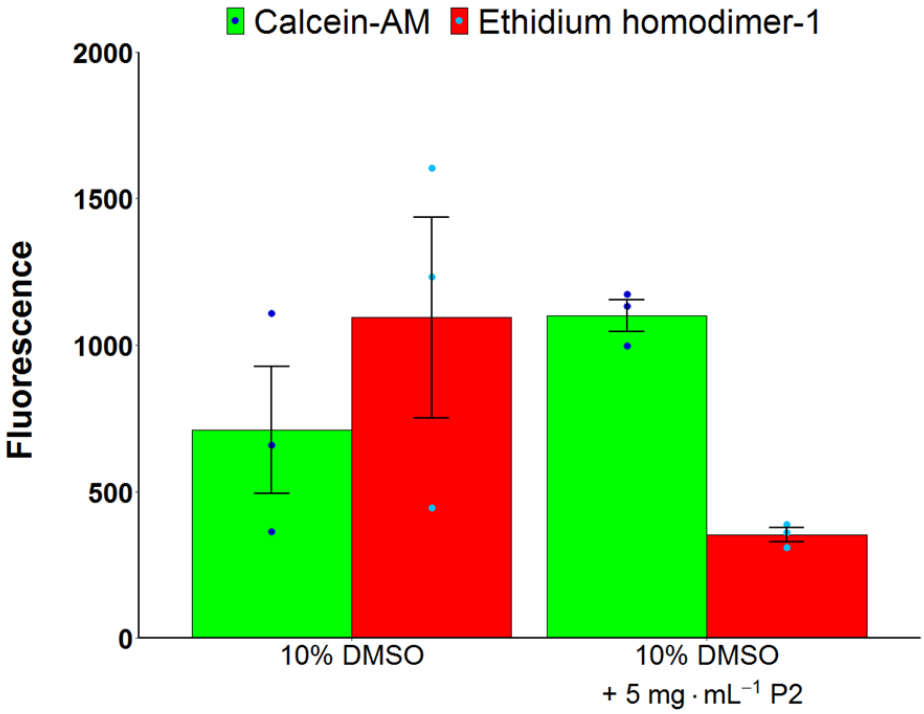

Figure S9. Graphical Representation of Live/Dead Staining Post-Freeze with A549 Cells. Post-freeze imaging of experimental solutions with control removed (N=3). Error bars represent ± SEM of 3 independent experiments.

Confirmation of Rhodamine-6G Tagged P2

A549 cells were incubated for 24 h in 100 mg·mL^{-1} of rhodamine-6G tagged **P2**. Cells were not washed and were imaged following the incubation period.

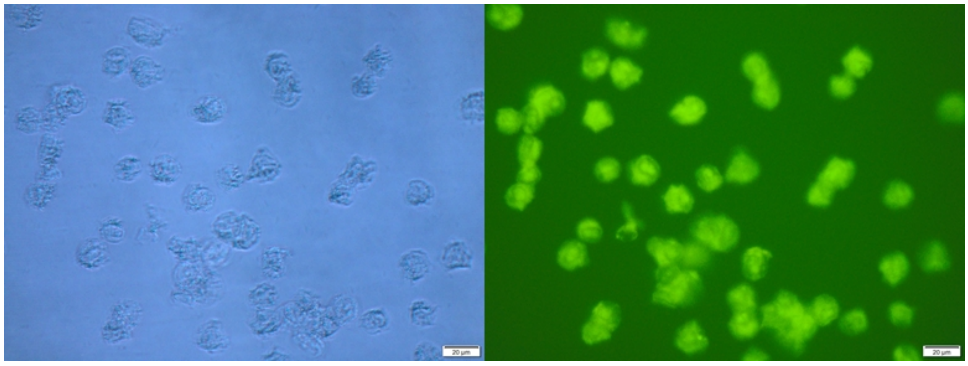

Figure S10. Rhodamine-6G Tagged Confirmation. A549 cells incubated with 100 mg·mL^{-1} rhodamine-6G tagged **P2**. Scale bar = 20 µm.

References

1. Stubbs, C., Lipecki, J. & Gibson, M. I. Regioregular Alternating Polyampholytes Have Enhanced Biomimetic Ice Recrystallization Activity Compared to Random Copolymers and the Role of Side Chain versus Main Chain Hydrophobicity. *Biomacromolecules* **18,** 295–302 (2017).
2. Su, M., He, C., West, C. A. & Mentzer, S. J. Cytolytic peptides induce biphasic permeability changes in mammalian cell membranes. *J. Immunol. Methods* **252,** 63–71 (2001).
3. Bailey, T. L. *et al.* Protective effects of osmolytes in cryopreserving adherent neuroblastoma (Neuro-2a) cells. *Cryobiology* **71,** 472–80 (2015).
4. Whale, T. F. *et al.* A technique for quantifying heterogeneous ice nucleation in microlitre supercooled water droplets. *Atmos. Meas. Tech.* **8,** 2437–2447 (2015).