Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Deep Eutectic Solvents as Cryoprotective Agents for Mammalian Cells – Supplementary Information

Saffron J. Bryant,[1*] Aaron Elbourne,[1] Miyah Awad,[1] Andrew J. Christofferson,[1,2] Andrew V. Martin,[1] Nastaran Meftahi,[2] Calum J. Drummond, Tamar L. Greaves[1] and Gary Bryant,[1]

[1]School of Science, College of STEM, RMIT University, Melbourne, Australia.

[2]ARC Centre of Excellence in Exciton Science, School of Science, RMIT University, Melbourne, VIC 3001, Australia.

Email: saffron.bryant@rmit.edu.au

DES Molecular Weights

The molar concentrations of DESs are based on a molecular weight containing both components, at a ratio where the lowest integer is equal to one. For example, for ChCl-Gly, this is based on a 1:2 ratio, and thus the molecular weight is 323.8 g/mol, for ChCl-Gal, this is based on a ratio of 2.5:1, and thus a molecular weight of 529.1 g/mol.

This clarification is provided to avoid confusion that often arises in the DES literature regarding molar concentrations.

Differential Scanning Calorimetry

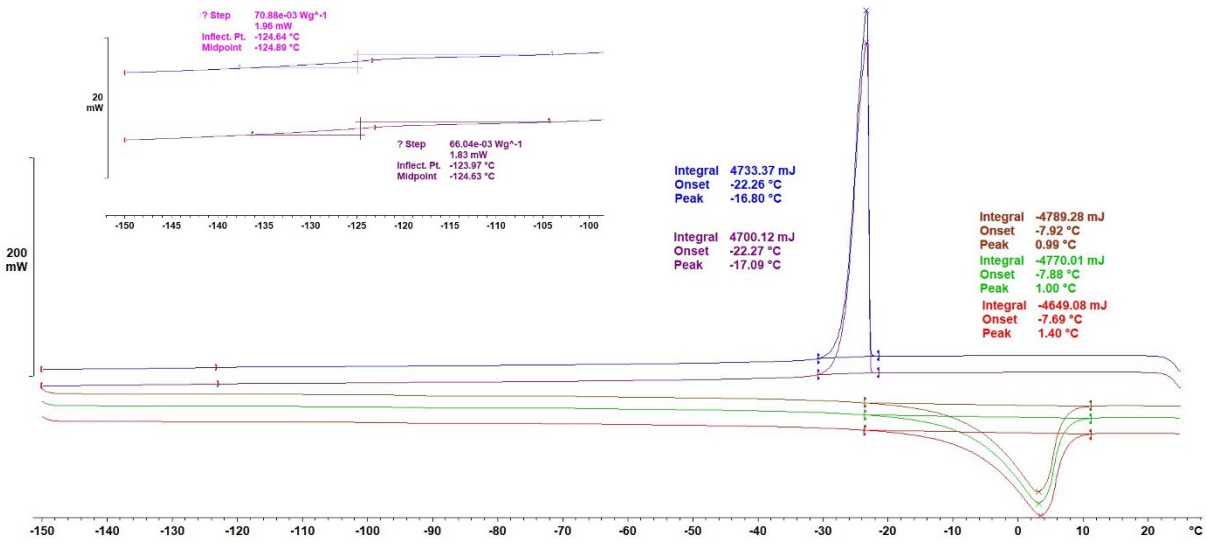

Figure S1. Thermogram of 10 wt% Prol-Gly in water. Inset shows zoomed-in portion demonstrating glass transition.

Toxicity of Components

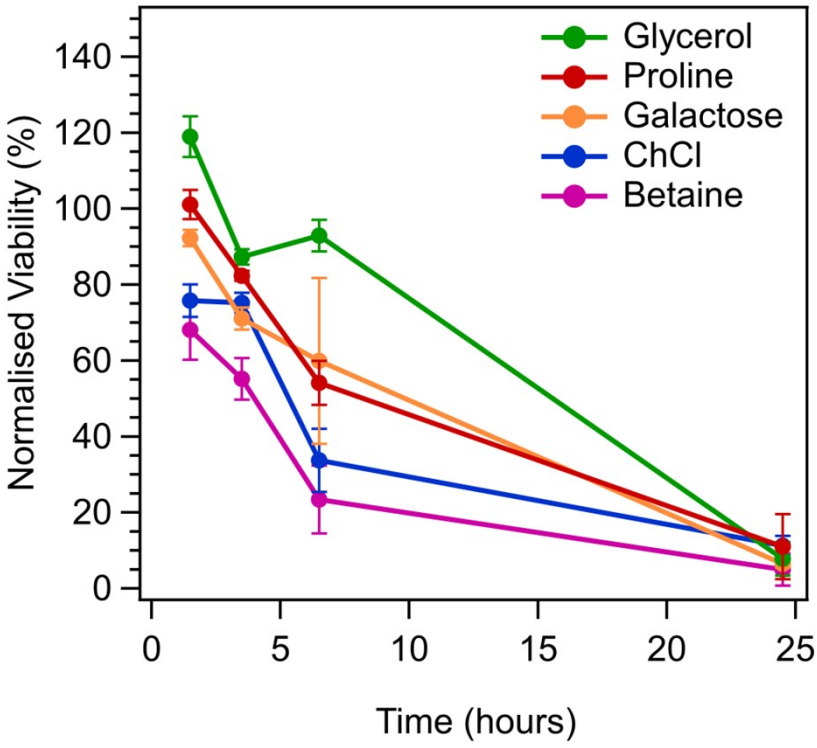

Figure S2. Viability of THP-1 cells over time with exposure to 10 wt% of the DES components.

Normalising Viability

In some cases where the CPA is extremely toxic or ineffective as a cryoprotective agent, cells will break down. This means that they are not included in the 'dead' count. This can result in artificially inflated viabilities (e.g. 50%), if based solely on live vs. dead counts. However, this is clearly inaccurate if the expected cell density is 10^6 and only 100 cells are observed. Therefore, total cell number for all cases was compared to the known frozen cell density and if there was significant variation, the true viability was taken as the number of live cells divided by the expected cell density.

In general, viabilities below 15% cannot be accurately determined and so comparisons between values below this are not made.

To avoid this complication, data for the four different cell types frozen with Prol-Gly, or glycerol or proline individually (Figure 8) was normalised to an 'ideal value'. This was the number of live cells obtained from a sample of cells frozen at the *same* density, at the *same* time using DMSO. The live cell number of this sample on thawing is considered the positive control, as it is what would 'normally' be achieved, following the standard cryopreservation procedure. Thus, values below 100% indicate that there were fewer live cells than in the 'ideal' sample, while values above 100% indicate that there were more live cells than the 'ideal' sample (and thus, that Prol-Gly is a more effective CPA than DMSO).

Two Parameter Formalism

The two parameter formalism is described in the literature[34, 36, 38] and is based on two primary equations, one for water flux (1):

Equation 1:

$$\frac{dV_w}{dt} = -L_p ART(M^e - M^i)$$

and the other for solute flux (2):

Equation 2:

$$\frac{dN_s}{dt} = P_s A(M_s^e - M_s^i)$$

Where L_p is the water permeability (hydraulic conductivity), *A* is the cell area, *R* is the universal gas constant, *T* is absolute temperature, P_s is the solute permeability, subscript w refers to water, subscript s refers to permeating solute, and M^i and M^e are respectively the intracellular and extracellular osmolality.

Equation 3:

$$V_c = V_w + V_s + V_b$$

Where V_c is the total cell volume, V_w is the partial molar volume of water, V_s is the solute volume determined by Equation 4 (below), and V_b is the osmotically inactive volume (including cell solids). The value of V_b was set at 0.279±0.008 for THP-1 cells and 0.4181±0.092 for HaCat cells as determined by the Boyle van't Hoff relationship[73] (see Figure S5).

Equation 4:

$$V_s = N_s \bar{V}_s$$

Where N_s is the osmoles of solute inside the cell and $\bar{V}_s$ is the partial molar fraction.

The validity of this method and of the fixed parameters was confirmed by using DMSO as a control. As shown in Figure S2 and S3, a good model fit was achieved not only for all of the PBS data, but also for 10 wt% DMSO which had a P_s = 1.9 µm/min with THP-1 and 7.0 µm/min for HaCat. These values are well within the expected range based on studies of similar cell types in the literature.[58-62]

Boyle van't Hoff Relationship

The osmotically inactive volume (V_b) can be determined using the Boyle van't Hoff Relationship[73]:

$$\frac{V}{V_o} = \frac{V_w^o \pi_o}{V_o \pi} + \frac{V_b}{V_o} = (1 - b)\frac{\pi_o}{\pi} + b$$

where V is the cell volume, V_o is the total cell isotonic volume, V_w^o is the isotonic volume of water in the cell, and π and π_o are the osmolality and isotonic osmolality respectively. Shrink-swell experiments were performed on THP-1 and HaCat cells using various concentrations of phosphate buffered saline (a non-penetrating solute), the results of which are shown in Figures S2 and S3. The final/minimum volume for each solute concentration was determined and plotted against the normalised inverse osmolality (Figure S5). The intercept of the trendline for this data is equal to the osmotically inactive volume, i.e. V_b and was determined to be 0.279±0.008 for THP-1 and 0.4023±0.101 for HaCat.

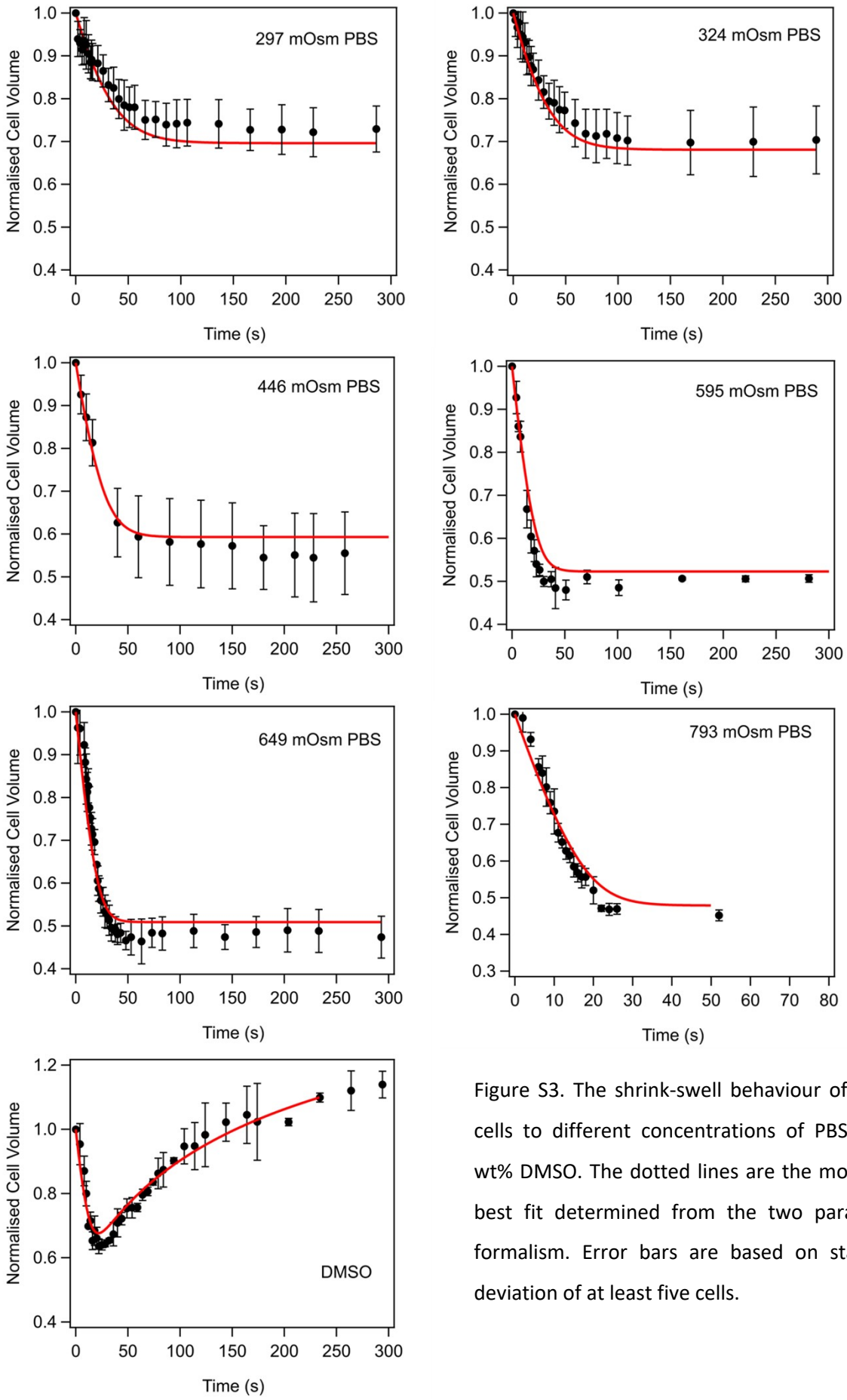

Figure S3. The shrink-swell behaviour of THP-1 cells to different concentrations of PBS or 10 wt% DMSO. The dotted lines are the models of best fit determined from the two parameter formalism. Error bars are based on standard deviation of at least five cells.

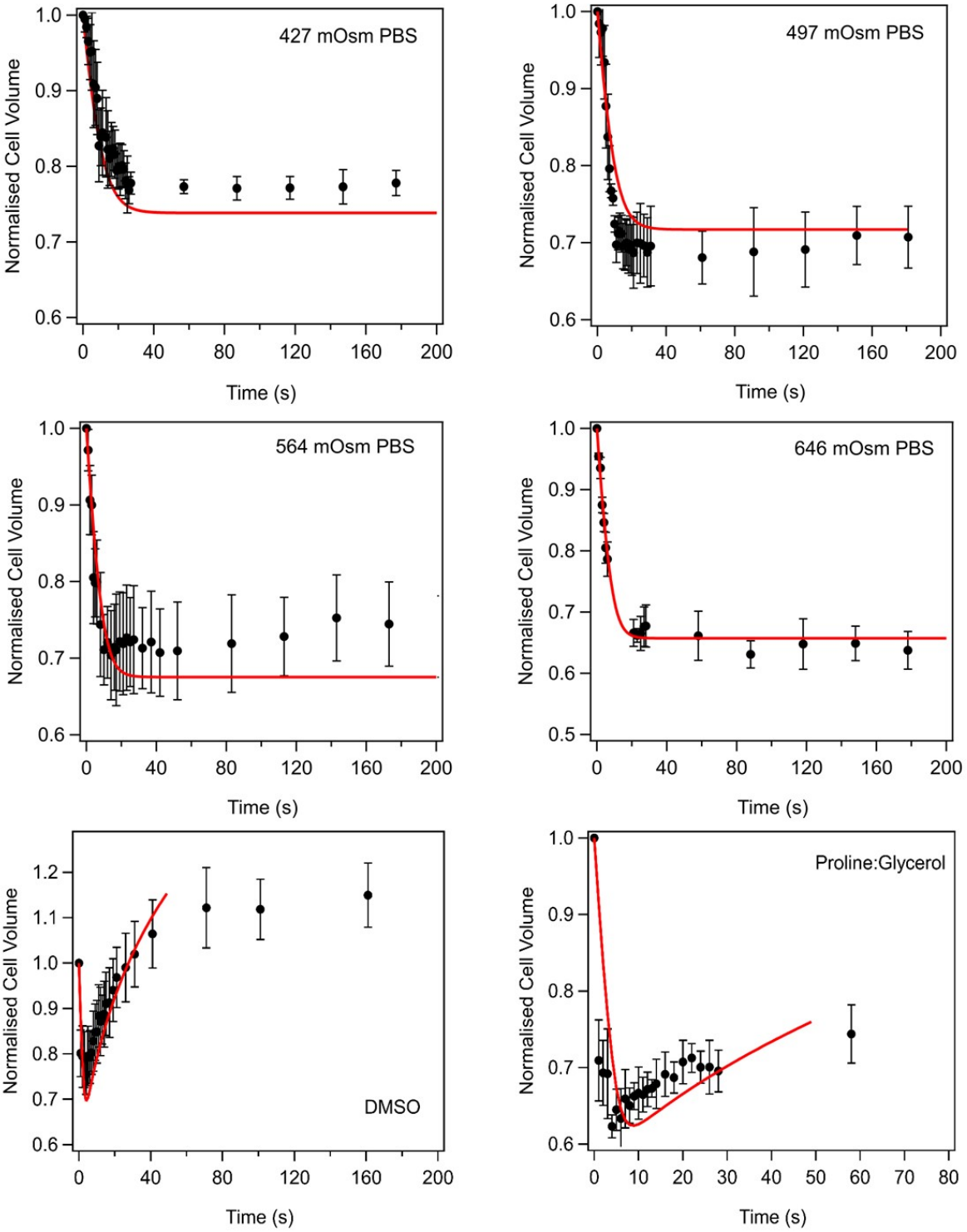

Figure S4. The shrink-swell behaviour of HaCat cells to different concentrations of PBS or 10 wt% of either DMSO or Prol-Gly. The red lines are the models of best fit determined from the two parameter formalism. Error bars are based on standard deviation of at least five cells. Because of the rapid swelling of DMSO and Prol-Gly, the model failed after ~50s, therefore, only the first 50s of data were used to determine the P_s.

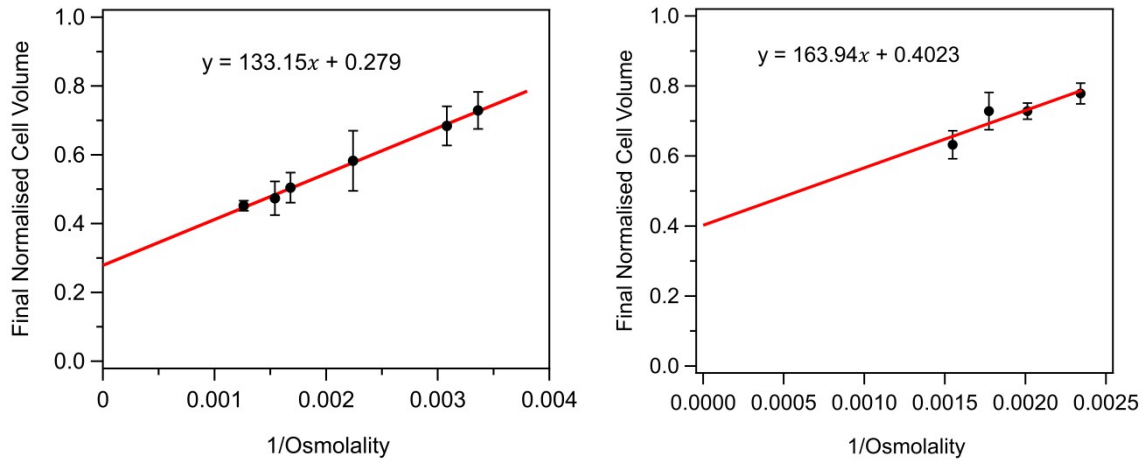

Figure S5. The final normalised cell volume of THP-1 (left) or HaCat (right) cells exposed to different concentrations of phosphate buffered saline. The intercept point (0.279±0.008 for THP-1 and 0.4023±0.101 for HaCat) is equal to the osmotically inactive volume (V_b),

Shrink-Swell Behaviour of DES Components with THP-1 Cells

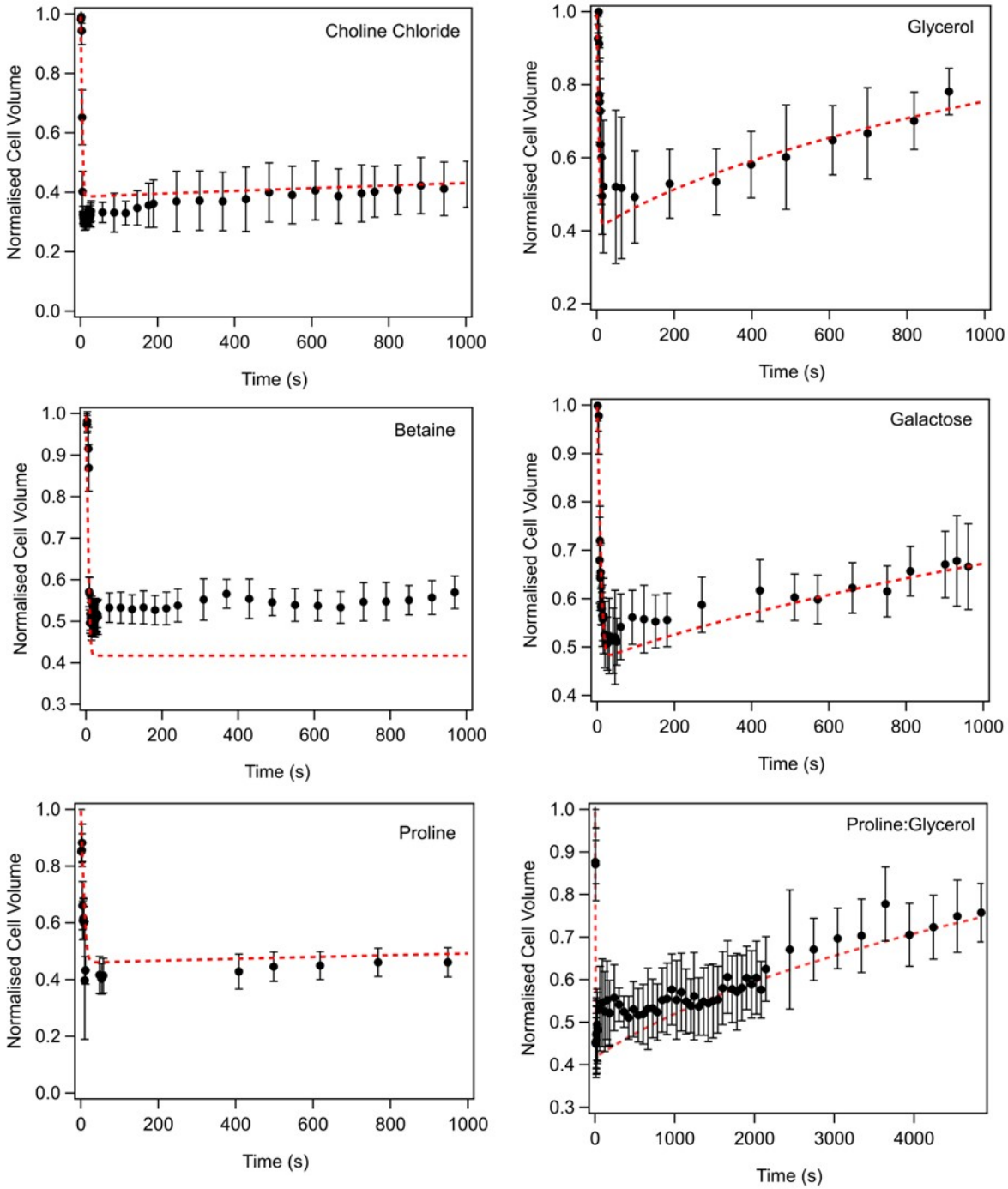

Figure S6. Shrink-swell behaviour of THP-1 cells exposed to individual DES components and Prol-Gly over an extended time period. Model fits to the data from the two parameter formalism are shown with red dotted lines and the solute permeabilities are given in Table S1. Error bars based on the standard deviation of measurements from ≥5 cells.

Table S1. Response of THP-1 cells to exposure of each DES component (10 wt%) with the P_s determined by data fitting to the two parameter transport formalism.

DES Component	**Solute Permeability (P_s) (μm/min)**
Glycerol	0.075
Betaine	No swell
Proline	0.005
Choline Chloride	0.005
Galactose	0.045

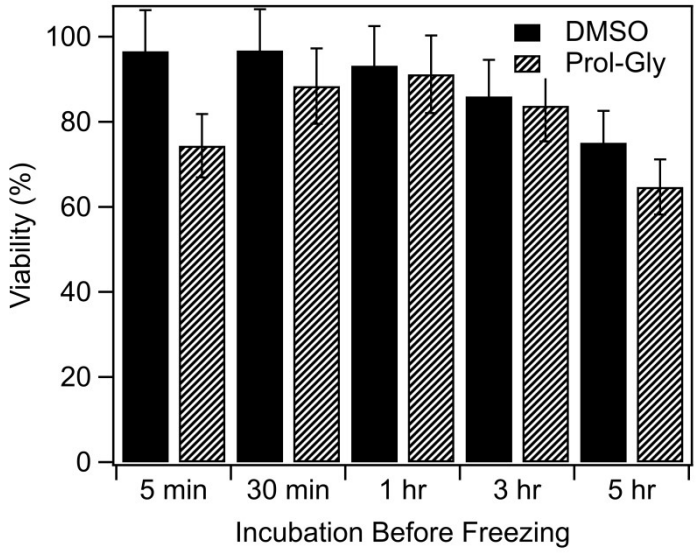

Figure S7. Viability of HaCat cells stored with either 10 wt% of either DMSO or Prol-Gly with incubation for different times prior to freezing. Error bars are based on 10 % observed variation in machine-reported viabilities.

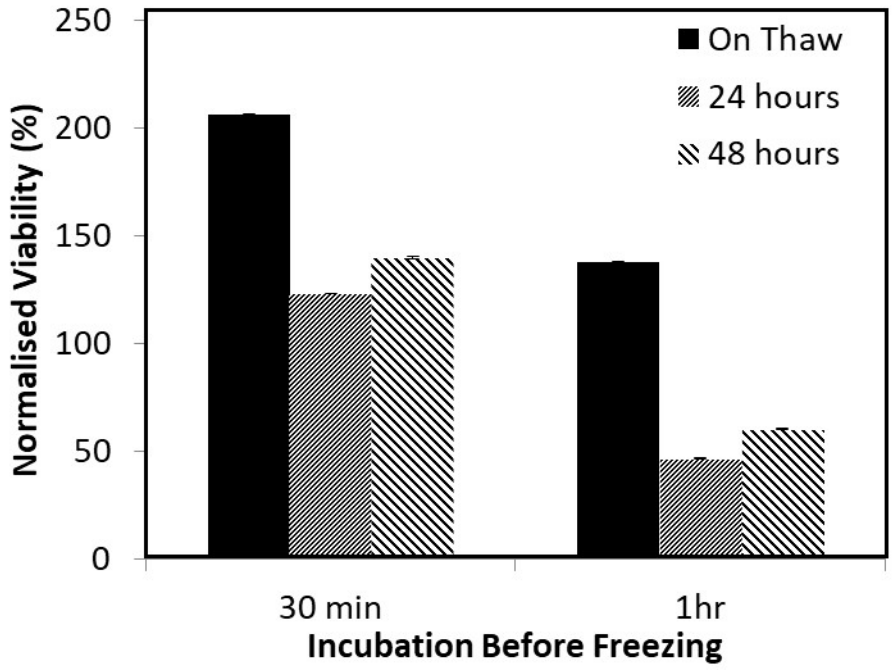

Figure S8. Viability of HaCat cells stored with 10 wt% Prol-Gly after thawing (immediately, 24 hours and 48 hours), based on the resazurin viability assay. Values are normalised to emission from cells stored with DMSO. Error bars are based on standard deviation of triplicate measurements.

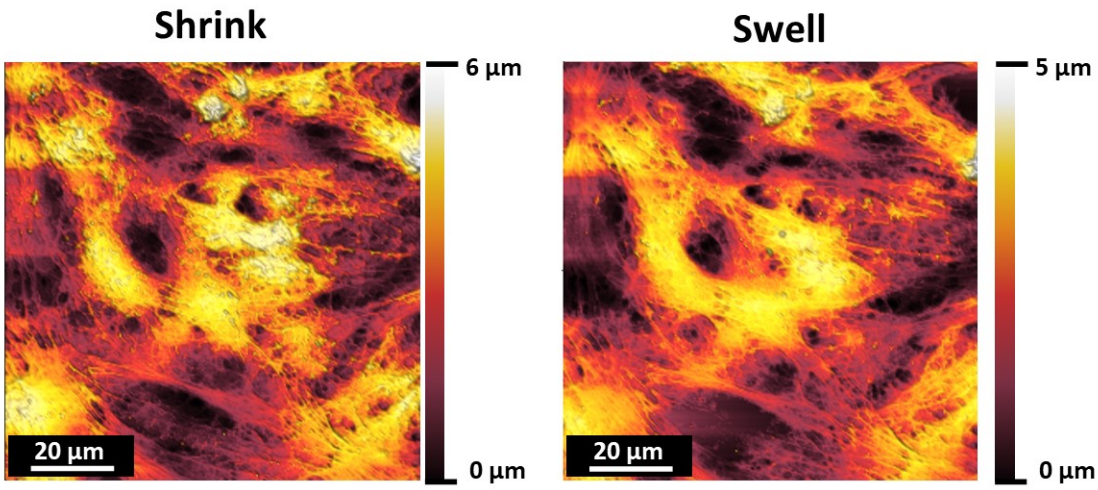

Figure S9. AFM images of HaCat cells immediately following exposure to ~10 wt% Prol-Gly (left), and ~40 minutes after exposure (right).

As shown in Figure S9, 'shrink-swell' behaviour can be observed via AFM for HaCat cells in their native adhered state. Due to the non-spherical nature of these cells, the degree of swelling cannot be quantified, furthermore, it was not possible to get an image before addition of Prol-Gly as addition of the DES itself causes the AFM tip to move. However, these images qualitatively demonstrate penetration of Prol-Gly into the HaCat cells, even when they are adhered to a surface.

Molecular Dynamics

Table S2. Composition of model membranes (per leaflet)

Lipid	**THP-1**	**HaCat**
Cholesterol	70	54
POPC	56	64
POPS	6	10
POPI	12	12
DOPG	16	
DOPE	30	40
SM	20	20
Total	210	200