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Deep eutectic solvents for the preservation of concentrated proteins: the case of lysozyme in 1 : 2 choline chloride : glycerol

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Experimental section

Materials: Chicken egg white lysozyme (Lyz, >98%), choline chloride (>99%), glycerol (>99.5%), sodium phosphate monobasic dihydrate (>99.5%), sodium hydroxide (>99%), sodium azide (NaN_3, Sigma, >99.5%) and D_2O (Sigma, 99.9 %D) were supplied by Sigma and used without further purification. Choline-d_9 chloride (98 %D) and glycerol-d_8 (99 %D) were supplied by Cambridge Isotope Labs and used as received. Water used for sample preparation was of Milli-Q grade, with a resistivity of 18.2 MΩ cm at 25 ˚C.

The chemicals needed for the preparation of the deep eutectic solvent (DES) were vacuum dried at 50 °C for 24 hours before preparation of the DES. 1:2 choline chloride:glycerol (ChCl:Glyc) and its deuterated version were prepared by mixing the required amounts of each chemical under continuous stirring and heating at 60 °C until a clear liquid was formed. After cooling, the DESs were equilibrated at room temperature in a desiccator for a minimum of two days before use. Once ready, the DES were sealed and stored under a dry atmosphere to avoid water absorption. The residual water in the (neat) DES was measured using Karl-Fischer titration to an average water content of 0.47 wt% for ChCl:G.

Sample preparation: Protein stock solutions were prepared in water or D_2O by adding the required amounts of Lyz. The stock solutions were centrifuged at 13 000 rpm for 1 min, followed by pipetting of the supernatant into new tubes to remove any aggregates from the solution. Protein samples in the DES were prepared by mixing the required amount of DES and protein aqueous stock solution and, subsequently, samples were freeze-dried to remove the excess water using an optimised protocol as explained below. This approach has been used before to incorporate sensitive biomolecules to DES.[1, 2] Sample for the SANS experiments were prepared using deuterated DES and D_2O and following the protocol presented for the protiated analogues. Aqueous protein solutions were prepared in 10 mM, pH 7, phosphate buffer following the same dilution protocol presented above.

All samples contained 0.2 mg/ml NaN_3 to avoid bacterial proliferation.

Methods: Freeze-drying was performed on an Epsilon 2-6D LSCplus from Martin Christ. Samples in glass vials were loaded in the instrument tray, and quenched to -40 ˚C. Once the samples solidified, the pressure in the instrument was gradually reduced to 0.8 mbar to sublimate the water in the sample. The temperature of the tray was kept at -15 ˚C during the primary drying stage that lasted for 24 hours. Subsequently, the temperature of the system was gradually increased to room temperature over a period of 24 hours. Finally, the instrument automatically closed the vials under dry atmosphere and the vessel was vented to reach ambient conditions. No bubbling was observed during any of the freeze-drying stages. Control samples of the DES without protein were tested for water content upon drying using Karl-Fischer titration showing that minor variations in the water content (±0.1 wt%) occurred during this process.

UV-Vis absorption measurements were performed using the Varian Cary 50 UV-Vis Spectrometer. Quartz cells with a 1 mm light path, 10 mm width (Hellma Analytics) were used. The absorbance was measured in the range 190-500 nm at a scan speed of 600 nm/min, using the dual beam mode and baseline correction. The absorption from the solvents were subtracted to the data. The first-derivative UV spectra were calculated from the UV-vis spectra in the wavelength range between 240 and 310 nm. The first-derivative data were smoothed using a Gaussian function and the smoothed data were subsequently used to calculate the second-derivative UV spectra. First- and second-derivative data did not introduce any major differences in the trends or artifacts before and after smoothing (See Figure S1).

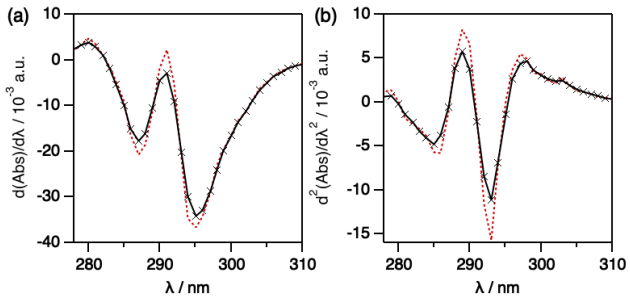

Figure S1 (a) First- and (b) second-derivative spectra of the UV-vis absorption of 100 μM Lys in ChCl:Glyc before (red dotted line) and after (black solid line) smoothing.

The peaks in the tyrosine/tryptophan region (280-300 nm) in the second-derivative UV spectra were fitted using two or three Gaussian functions and a flat baseline to accurately extract the positions and amplitudes of the peaks (See Figure S2).

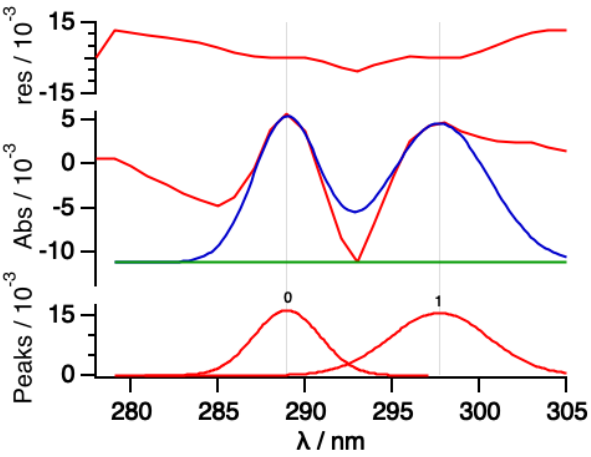

Figure S2 Analysis of the peak positions and amplitudes using two Gaussian functions and a flat baseline.

Circular dichroism (CD) measurements were performed on a Jasco J-715 with a temperature-controlled sample stage fixed at 25 ˚C. The instrument configuration covered a wavelength range between 250 and 190 nm, with a scan speed of 50 nm min^{-1}, a spectral bandwidth of 1 nm, and a response time of 1 s. Samples were loaded in a 0.1 mm quartz cuvette. The solvent contributions were measured and subtracted from the sample signal. Data are presented as mean residue ellipticity ($[\theta]_{MR}$) in deg cm^2 $dmol^{-1}$, calculated as:

$$[\theta]_{MR} = \frac{\theta}{nl[BSA]}$$

where θ is the measured ellipticity in mdeg, n is the number of amino acid residues in Lyz (129), l is the cuvette path length in cm, and $[BSA]$ is the concentration of protein in the sample in dmol cm^{-3}.

SANS measurements were performed on D22 at the Institut Laue-Langevin (Grenoble, France). Samples were loaded into 1 mm path length, 1 cm width, quartz Hellma cells and placed in a temperature-controlled sample changer at 25 ˚C for measurement. The front and rear detectors were placed at 1.7 m and 8 m from the sample position, respectively. Measurements were conducted using a neutron wavelength of 6 Å. The configurations used provided a combined q-range of 0.006-0.65 $Å^{-1}$. The raw data were reduced according to the D22 protocols using the GRASP software. The reduced data are presented as scattered intensity in absolute scale, I(q) in cm^{-1}, versus momentum transfer, q in $Å^{-1}$.

SANS data of Lyz in dilute conditions were analysed using the indirect Fourier transform (IFT) method, which is a real-space approach used to determine the pair-distance distribution function of the scatterer ($p(r)$).[3] The p(r) accounts for all distances within the scatterer and provides information on its shape and size. The function is parametrised using the maximum dimension of the scatterer (D_{max}) and a regularisation constant (α). From the p(r) the radius of gyration of the scatterer (R_g) and extrapolated zero angle (forward) intensity ($I(0)$) can be calculated using the following equations:

$$R_g{}^2 = \frac{\int_0^{D_{max}} p(r) r^2 dr}{2\int_0^{D_{max}} p(r) dr}$$

$$I(0) = 4\pi \int_0^{D_{max}} p(r) dr$$

The aggregation number (N_{agg}) is a descriptive parameter of the self-association equilibrium and was calculated from I(0) using the following equation:

$$N_{agg} = \frac{V}{V_m}$$

$$V = \sqrt{\frac{I(0)\, M_w}{c\, (\Delta SLD)^2\, N_A}}$$

where V is the experimental volume of the scatterer, V_m is the volume of the protein monomer, c is the concentration of protein in the sample (in g cm^{-3}), ΔSLD is the scattering excess (i.e. the difference between the scattering length density of the protein and that of the solvent, SLD_p-SLD_s), and N_A is Avogadro's number.

Data from Lyz at higher concentrations in DES were analysed using a model-based approach. The scattering in the small angle can be described as the product of intra-particle scattering and inter-particle scattering for the present case of near centro-symmetrical particles and interaction potential. The form factor (P(q)) accounts for the

morphology of the scatterer, while the structure factor (S(q)) depends on the interaction between the dispersed particles; the parameter K accounts for the composition and concentration of the sample.

$$I(q) = KP(q)S(q)$$

The effective structure factor was obtained from the SANS data using the following equation.

$$S(q) = \frac{I(q)}{KP(q)}$$

The scattering from the protein, i.e., P(q), was approximated as that from a prolate ellipsoid with a radius parallel to the rotational axis r_{eq} and a radius perpendicular to the rotational axis r_{po}. To account for structural deviations from the ellipsoid, each of the radius used a polydispersity of 0.15 described using a Schulz distribution.[4] This approach showed a good agreement when compared to the results from the IFT analysis. A Percus-Yevick excluded volume effect was employed to account for the interparticle scattering.[5] It has been previously shown that the colloidal interactions between charged particles in DES cannot be simply described using excluded volume interactions between particles due to the presence of some weak, long-range interactions.[6, 7] Thus, an effective excluded volume effect has been used. This method uses an effective particle volume fraction to parametrise the S(q), $\phi_{S(q)}$, that is not constrained to the actual volume fraction of particles, $\phi_{P(q)}$. This method has been shown to provide a good approximation for concentrated colloidal systems in DES.[7, 8] This approach allowed to determine the intra- and inter-particle scattering to fit the model to the experimental scattering.

The scattering length density and monomer volume of the protein were calculated using the sequence of each protein (PDB files: Lys – 1HEW) and the Biomolecular Scattering Length Density Calculator from the Science and Technology Facilities Council, UK.[9] The SLD of Lyz is 3.451×10^{-6} Å^{-2} and that of D_2O is 6.37×10^{-6} Å^{-2}. The IFT method implemented in the GNOM program in the ATSAS 2.8.3 suite was used.[10, 11] The analysis of data using the model-based approach was performed on SasView 5.0.3.[12]

The activity of Lyz was determined by measuring the turbidimetric rate of lysis of a *Micrococcus lysodeikticus* suspension (Sigma-Aldrich) as catalysed by Lyz in aqueous buffer.[13] A stock solution of the cell suspension of 157 µg/ml was prepared. Samples were diluted using reaction buffer (50 mM potassium phosphate buffer, pH 7.2) to a final cell concentration of 1.27 µg/ml, giving an absorbance at 450 nm of *ca.* 0.6. 900 µl ml of this cell suspension were added to a 4 mm path length quartz cuvette and equilibrated at room temperature. Subsequently, 100 µl of Lyz solution ([Lyz]=24 µM in 50 mM potassium phosphate buffer, pH 7.2) from each sample were added to the cell. Samples were quickly inverted and placed in the sample holder for measurement. The absorbance of the reacting sample was recorded at 450 nm for 180 s (5 s step, 25 ˚C, Biochrom Libra S60). Control solutions of the suspended bacteria in buffer (no Lyz present) showed no absorption change during the experiments. The specific activities (U/mg of protein) were obtained by normalizing the measured activity (U/ml) to the protein concentration. The relative specific activities were normalised to the specific activity of freshly prepared Lyz in aqueous buffer. Three repeats were performed and averaged. The error bars represent the standard deviation of the three measurements. Note that the reduction of protein concentration in the reconstituted samples is attributed to the dilution required prior to the dialysis, i.e., a 3-fold dilution of the protein-in-DES sample was done using buffer to increase the dialysis rate, or DES hydration.

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