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Supplementary Information

Remarkable refolding effects of partially-immiscible ammonium–based ionic liquids on the urea-induced unfolded lysozyme structure

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Materials and Methods:

Salt-free lysozyme, urea, Nile red, butyltrimethylammonium bis(trifluoromethylsulfonyl)imide (IL-1) (≤0.2% water), ethyldimethylpropylammonium bis(trifluoromethylsulfonyl)imide (IL-2) (≤1.0% water) and diethylmethyl(2-methoxyethyl)ammonium bis(trifluoromethylsulfonyl)imide (IL-3) (≤500 ppm water) were purchased from Sigma–Aldrich Chemical Company (USA). All materials were used without further purification. Tris-HCl buffer solution (10 mM) of pH 7.0 was prepared using distilled de-ionized water at 18.3 MΩ and was used as a reference solvent for preparing the rest of the sample solutions containing 1% (v/v) of the ILs.

The samples containing protein and urea (at various concentrations) were shaken in water bath for 15 min so that the urea is well distributed throughout the solution and was left undisturbed for 30 min at 25 ^{0}C. After that the samples were directly used for the spectroscopic measurements. The protein concentration was fixed to 0.5 mg/mL for all the measurements.

Refolding experiments were performed after the addition of 1% (v/v) with the protein samples *pre-treated* with urea. After the addition of ILs, the samples were shaken in water bath for 15 min. Thereafter, the samples were left undisturbed for ~30 min at 25 ^{0}C before performing the spectroscopic measurements.

In order to investigate the polarity changes on the surface of the protein in the presence of urea and ILs, a solution of Nile red in methanol was added to each of the sample vial so that the total concentration of the extrinsic probe (Nile Red) is ~1.0 μM. The samples were agitated after the addition of the dye and the resulting mixtures were kept in the dark at room temperature for at least 30 min before performing the thermal fluorescence measurements.

Enzyme activity

Enzymatic activity of lysozyme was determined by using the *Micrococcus Lysodeikticus* as the substrate. A stock solution of 0.3 mg/mL *Micrococcus Lysodeikticus* cell suspension was prepared in 0.1 M phosphate buffer, pH 7.0. Lysozyme activity measurements were performed on suspended *Micrococcus Lysodeikticus* cells for each of the samples with urea, and 1% v/v of ILs added to lysozyme solutions *pre-treated* with urea. The decrease in absorbance of the solution at 450 nm was measured using a UV-Vis spectrophotometer for all samples. Absorption spectra for lysozyme in the absence and presence of various concentrations of ILs were recorded

on a Shimadzu UV-1800 (Japan) spectrophotometer with the highest resolution (1 nm) using matched 1 cm path length quartz cuvettes.

The scan of *Micrococcus Lysodeikticus* cells were taken separately in buffer, urea and ILs, in order to check whether any of these constituents contribute to the absorption spectrum. As presented in Fig. 1S, there is no effect of buffer, urea and ILs alone on the absorption spectra of *Micrococcus Lysodeikticus* cells suspension at 450 nm.

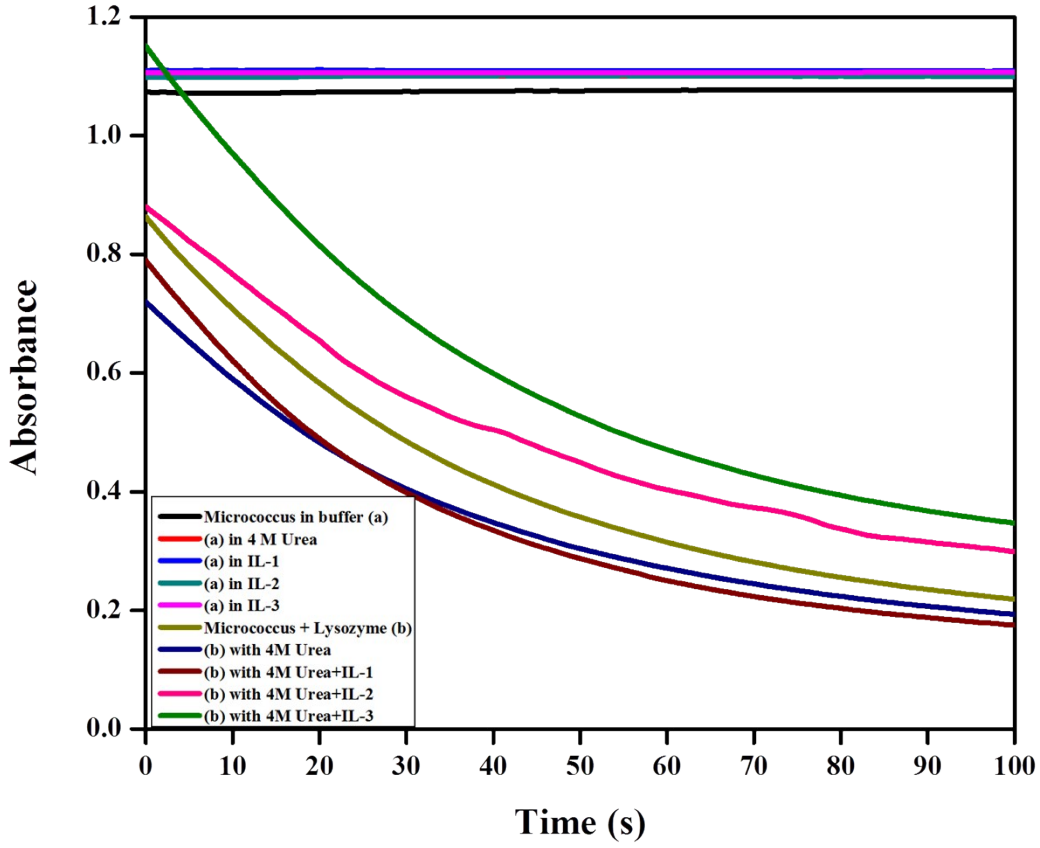

Fig. 1S. Absorbance vs time curve of a typical lysozyme activity for *Micrococcus Lysodeikticus* in buffer, urea and 1% (v/v) of ILs. The straight lines represent the absorption of *Micrococcus Lysodeikticus* alone in buffer, urea and ILs.

Fluorescence spectroscopy

Cary eclipse spectro fluorimeter (equipped with thermostat cell holders) from Varian optical spectroscopy instruments, Mulgrave, Victoria, Australia, was used to monitor the fluorescence emission spectra of lysozyme in urea as well as in the presence of ILs. The steady-state fluorescence measurements were conducted at a constant temperature of 25 ^{0}C using a circulating water bath controlled by a Peltier device attached to the sample holder of the

fluorimeter. The excitation wavelength was set at 290 nm in order to calculate the contribution of the tryptophan (Trp) residues to the overall fluorescence emission and 450 nm in order to monitor the Nile red fluorescence emission. The experiments were carried out at 25 ^{0}C by using a 1.0 cm sealed cell; both excitation and emission slit width were set at 5 nm, and corrected for background signal.

The fluorescence intensity at the emission maximum (λ_{max}) for the native enzyme in the presence of Nile red was continuously recorded at the excitation wavelength of 450 nm with temperature from 20 to 85 ^{0}C at an approximate rate of 2 ^{0}C min^{-1}. In brief, the fluorescence was monitored as a function of the temperature. As presented in Fig 2S, non-linear fits were applied to the thermal unfolding curves to obtain the unfolding temperature (T_m) for the lysozyme using Origin 8.0 software.

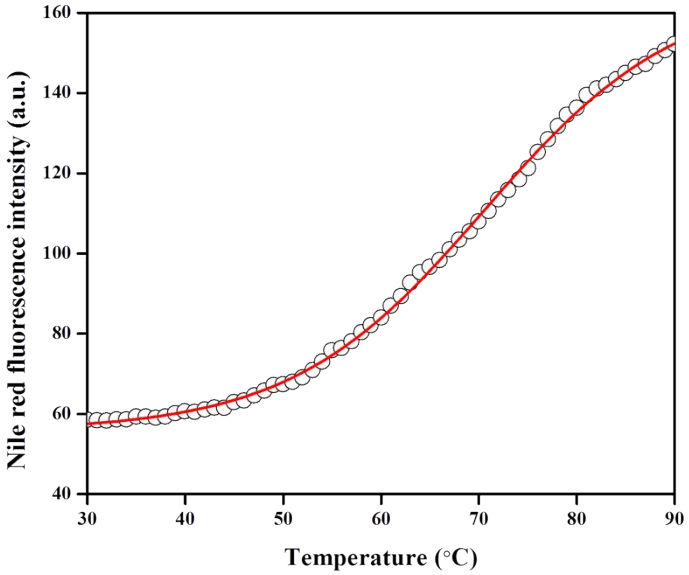

Fig. 2S. Applying non-linear curve fitting to the fluorescence thermal curve for the lysozyme in buffer using Origin 8.0. The red line indicates the fitted values.

Circular Dichroism

CD spectroscopic studies were performed using a Jasco-815 spectrophotometer equipped with a Peltier system for temperature control. CD calibration was performed using (1S)-(+)-10-camphorsulphonic acid (Aldrich, Milwaukee, WI), which exhibits a 34.5 M/cm molar extinction

coefficient at 285 nm and 2.36 M/cm molar ellipticity (Θ) at 295 nm. The sample was pre-equilibrated at the desired temperature for 15 min and the scan speed was fixed for adaptive sampling (accuracy of $\pm$ 0.01) with a response time of 1s and 1 nm bandwidth. Each sample spectrum was obtained by subtracting the appropriate blank media (without protein) from the experimental spectrum and was collected by averaging six spectra. The near-UV CD spectra for the samples were obtained in the range of 250-350 nm wavelengths.