Unusually High Thermal Stability and Peroxidase Activity of Cytochrome c in Ionic Liquids Colloidal Formulation

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Supporting Information

1. Materials, Methods and Experimental Details

1.1. Materials

Ionic Liquid (IL), 1-Ethyl-3-methylimidazolium ethylesulfate ($[C_2mim][C_2OSO_3]$) with stated purity higher than >95% mass fraction was purchased from Sigma Aldrich. Choline dioctylsulfosuccinate [Cho][AOT] was synthesized in our laboratory. All the chemicals used for synthesis of ILs were of AR Grade. Choline Chloride (>98%) was purchased from Sigma Aldrich. Sodium dioctylsulfosuccinate (>99 %) was purchased SDFCL. Brief procedure of synthesis is as follows: Equimolar amounts of choline chloride and [Na][AOT] were dissolved in water and stirred for 24 h at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction the product was extracted into DCM layer, followed by washing of DCM layer with water for several times to completely remove the chloride ion present. The washing was performed until the aqueous layer gave a clear solution even with the addition of excess 1M $AgNO_3$. The DCM layer is then distilled to get pure [Cho][AOT]. Further, [Cho][AOT] is dried under vacuum for several hours to remove the moisture present and stored in desiccator. The synthesized [Cho][AOT] was characterized by using ^{1}HNMR (Fig. S1), LC-MS, CHNS and DSC (Fig. S2).

[1]H-NMR: 200MHz (DMSO-d6): δ_H (ppm) 0.86 (m, 11H), 1.24 (m, 15H), 1.49 (m 2H), 2.85 (m, 2H), 3.10 (m, 5H), 3.34 (s, 9H), 3.65 (m, 1H), 3.88 (m, 5H), 5.26 (t, 1H)

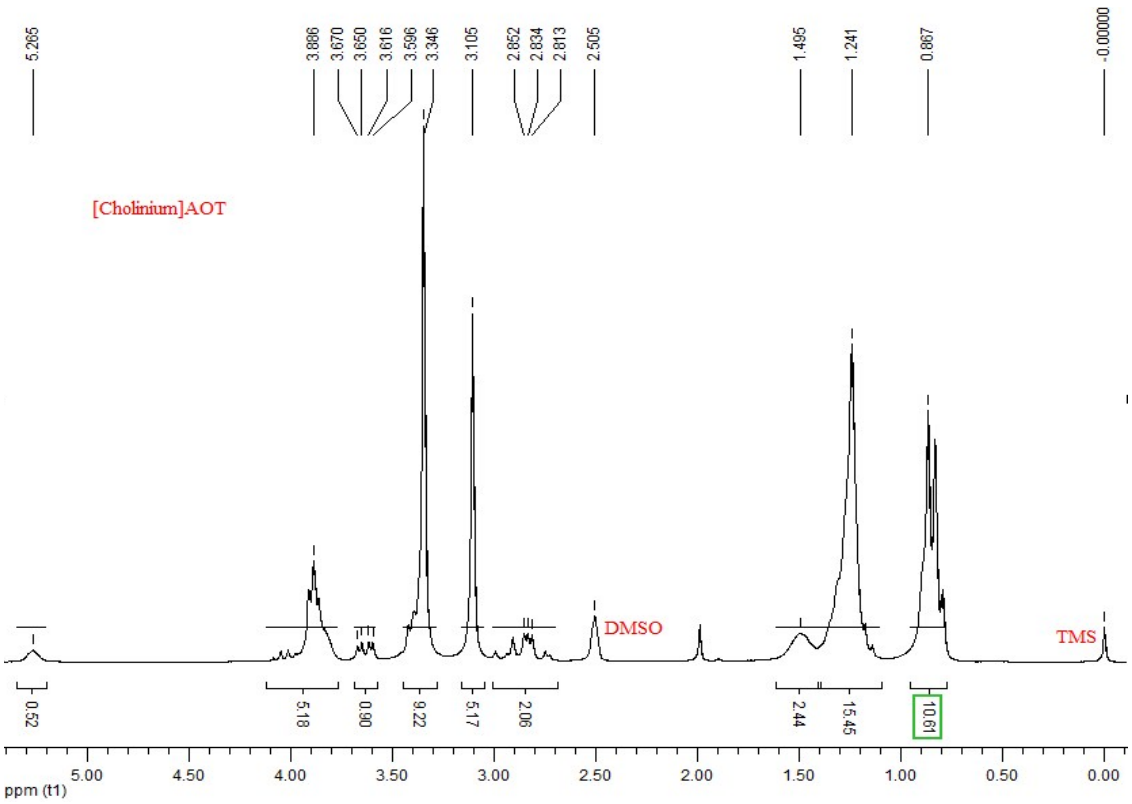


Fig. S1. [1]H-NMR spectra of [Cho][AOT]

ESI-MS: $[C_5H_{14}NO]^+$ m/z:104.14, $[C_{20}H_{37}O_7S]^-$ m/z = 421.2208.

CHNS Analysis:

Atom	Theoretical	Experimental
C	57.11%	56.20%
H	9.78%	9.043%
N	2.66%	2.59%
S	6.10%	5.77%

Melting point of [Cho][AOT] was checked using differential scanning calorimetry (DSC) and was found to be -47.4°C (Fig. S2).

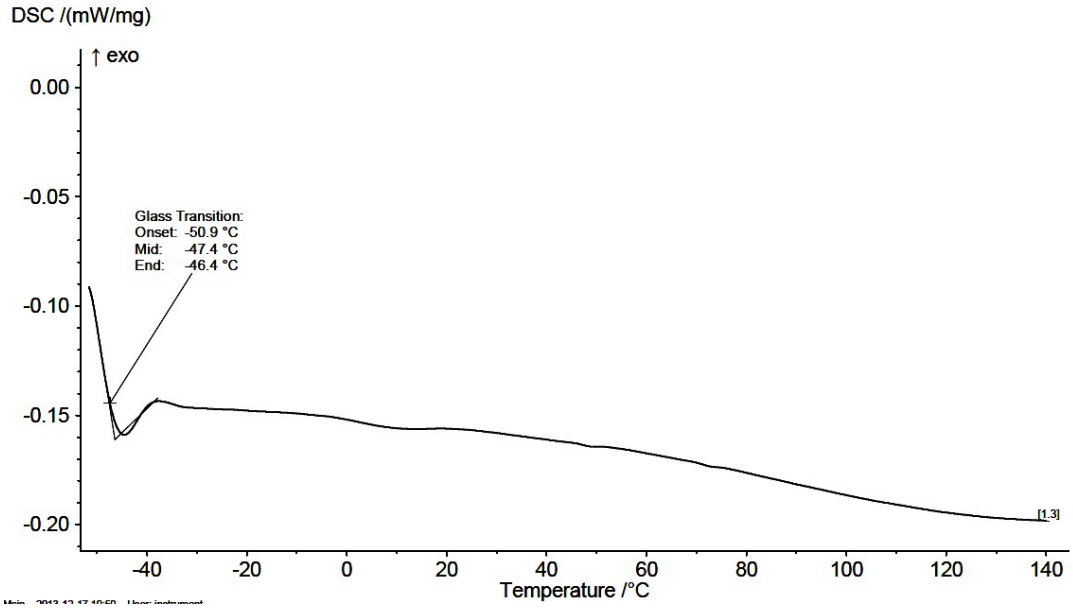


Fig. S2. Differential scanning calorimetric plot of [Cho][AOT].

Table S1. Purity Specification of Ionic Liquids

Ionic Liquid	**Purity (mass fraction)**	**Chloride Content (ppm)** (Volhard Method)	**Moisture content (ppm)** (Karl Fischer Titration)
$[C_2mim][C_2OSO_3]$ (Sigma Aldrich)	≥ 95%*	< 25 ppm	< 75 ppm
[Cho][AOT] (Synthesized)	≥ 99%	< 10 ppm	< 50 ppm

* $[C_2mim][C_2OSO_3]$ was further purified by washing with toluene several times. The volatile impurities were removed by heating the sample at 80°C in a rotary evaporator at zero vacuum for 24 h which also ensured removal of moisture. The purity of ionic liquids was determined by using ^{1}H NMR.[1]

The water content of the ionic liquid was measured from Karl Fischer Titration. The halide contents of ionic liquids were measured using the standard Volhard titration method.[1]

Enzyme Cyt c from horse heart was purchased SRL Pvt. Ltd. and was characterized for purity using spectroscopic techniques. The prominent bands at 409, 528 and 549 nm in the UV-Vis spectra and 40.1% α-helical content in far-UV CD spectra in Millipore water and phosphate buffer pH 7.0 validated the purity of enzyme (Fig. S3).

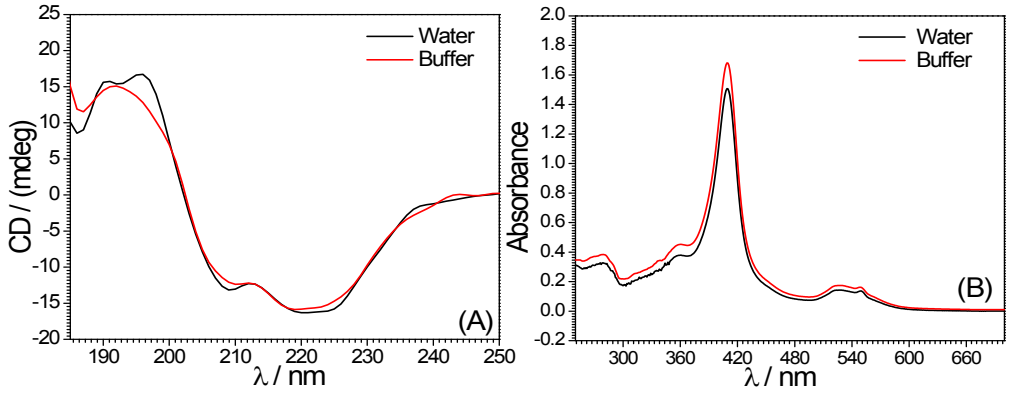
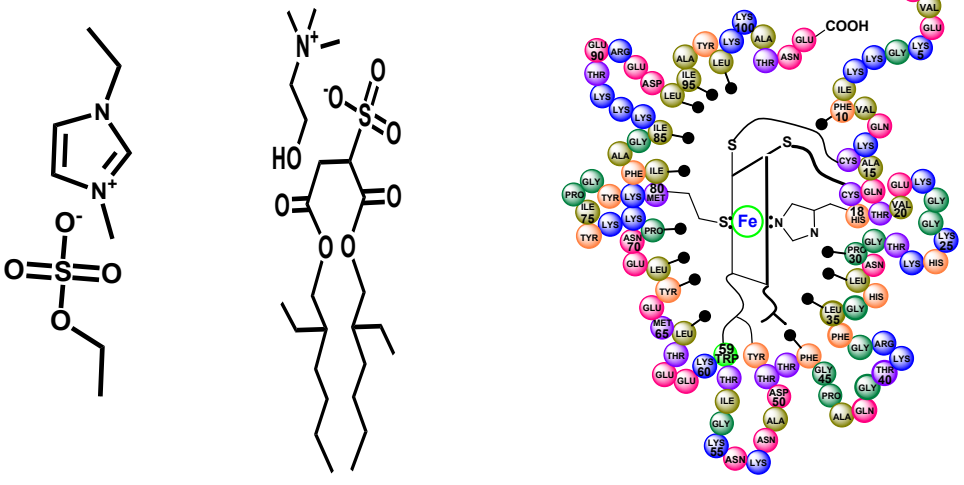


Fig. S3 (A) Far-UV CD spectra of Cyt c (B) UV-Vis-NIR spectra of Cyt c in water and phosphate buffer solution.

Guaiacol with stated purity of 98% was purchased from Sigma Aldrich. **Hydrogen peroxide** (H_2O_2) 30% (w/v) was purchased from Fischer Scientific. Buffer solution (50 mM, phosphate buffer) for activity analysis was prepared in degassed Millipore grade water using AR-grade potassium di-hydrogen phosphate (99%) and di-potassium hydrogen phosphate (99%) purchased from sd fine-chem Ltd.

Molecular Structure of the studied ionic liquids and structure of Cyt c with amino acid sequences are given in below. Structure of Cyt c is regenerated from reference 2 with permission.[2]



[C_2mim][C_2OSO_3] **[Cho][AOT]** **Cyt c**

1.2.Methods

^{1}H NMR: The ^{1}H NMR spectra for both the pure ILs and their mixtures were recorded using a Brüker 500MHz spectrophotometer. The proton chemical shifts were referenced with respect to an external standard TMS (δ = 0.000 ppm) in C6D6 (deuterated benzene).

ESI-MS: Electrospray ionization mass spectrometry (ESI-MS) was done with Q-Tof microTM 23 Micromass, U.K.

Differential Scanning Calorimetry (DSC): The melting point of [Cho][AOT] was determined by using differential scanning calorimetry measurements using a Mettler Toledo DSC822 thermal analyser at a scan rate of 5^{o}C/minute. Uncertainty in DSC measurements has been found to be ±0.06%.

CHNS Analysis: CHNS analysis of [Cho][AOT] was carried out using CHNS analyser (Elementer Vario Micro cube). The uncertainty in measurements is found to be ±0.6.

Karl-Fischer Analaysis: The Karl-Fischer analysis was done in Mettler Toledo DV705, T50 Autotitrator using dry methanol as solvent and Karl-Fischer reagent. The moisture content of [C_2mim][C_2OSO_3] and [Cho][AOT] were found to be, < 75 ppm and < 50 ppm.

Pyrene Fluorescence: Pyrene fluorescence spectra in [C_2mim][C_2OSO_3] as a function of [Cho][AOT] concentration were taken in Fluorolog (Horiba Jobin Yvon) spectrometer. In a typical experiment 20 µl of 10^{-4} M pyrene solution in DCM was taken in a quartz cuvette. The DCM was evaporated at room temperature for 30 minutes followed by addition of 2 ml of [C_2mim][C_2OSO_3]. The solution was then stirred for 12 hours for complete mixing. The emission spectra of pyrene were recorded at $\lambda_{ex} = 334nm$ with continuous addition of 75 mM [Cho][AOT]. At least 4 spectra were recorded at each concentration after solution stirring for 5 minutes. The final I_{373} / I_{383} value was taken as an average of four recorded spectra.

Isothermal Titration Calorimetry (ITC): Enthalpy changes (dH) due to aggregation of [Cho][AOT] in [C_2mim][C_2OSO_3] and Binding to Cytc was measured using MicroCal ITC200 microcalorimeter, with an instrument controlled Hamiltonian syringe having volume capacity of 40 µL. The titration was done by adding 2µL aliquots of [Cho][AOT] stock solution into sample cell containing 200 µL [C_2mim][C_2OSO_3] or 15 µM Cyt c solution in [C_2mim][C_2OSO_3] with continuous stirring (500 rpm). The parameters like time of addition and duration between each addition were controlled by software provided with the instrument. The enthalpy change at each injection was measured and plotted against concentration by using origin software provided with the instrument. The concentrations of

stock solutions taken were 75mM of [Cho][AOT] in [C_2mim][C_2OSO_3]. The Uncertainty in ITC measurements was found to be ±0. 5%.

Dynamic Light Scattering (DLS): DLS measurements were performed at 298.15 K, using a NaBiTec SpectroSize^{300} light scattering apparatus (NaBiTec, Germany) with a He−Ne laser (633 nm, 4 mW). The hydrodynamic measurements were carried out in quartz cuvette of 1 cm path length. For accurate measurement both viscosity and refractive index of the samples were considered. The analysis was carried out at multi-angle (30^o to 150^o) to validate the accuracy of the results. The error in the measurements was found to be ±10 nm.

Optical Microscopy: Bright field optical microscopic images of [Cho][AOT] colloidal particles in [C_2mim][C_2OSO_3] were taken from AXIO Imager M1 Carl Zeiss (Germany) Light Microscopy equipped with AxioCam HRc. Images were recorded at the magnification of 10 x 100X.

UV-Vis Spectroscopy: The changes around the heme cleft of Cyt c upon binding with [Cho][AOT] in different concentration regimes in [C_2mim][C_2OSO_3] were measured using a UV 3600 Shimadzu UV-vis-NIR spectrophotometer at 298.15 K. The concentrated solution of [Cho][AOT] was titrated against a 15 μM Cytochrome c solution in a quartz cuvette of 1 cm path length. The measurements were taken 3 minutes after addition of [Cho][AOT] at each concentration in order to match the time gap of the energy changes after each addition in the ITC analysis. The temperature dependent UV-Vis studies were carried out by heating the sample solutions {Cyt c (30 μM) dissolved in [C_2mim][C_2OSO_3] or [Cho][AOT] (100 mM)+[C_2mim][C_2OSO_3]} in silicon oil bath on a hot plate equipped with temperature sensor for a specific time period and subsequently measuring the spectra. Prior to the measurements the baseline was corrected using neat [C_2mim][C_2OSO_3] as blank.

Circular Dichroism Spectroscopy. Alterations in the tertiary structure and region around heme cleft of Cyt c in vesicles of [Cho][AOT] at different temperatures were monitored using a Jasco J-815 CD spectrometer from 30^oC to 95^oC. The temperature of the measurement cell was controlled with a Julabo water thermostat within ±0.1 K. Spectra were collected in a 1 mm path length quartz cuvette at a scan rate of 100 nm per minute and a sensitivity of 100 mdeg. The response time and the bandwidth were 2 s and 0.2 nm, respectively. For temperature greater than 95^oC the measurements were carried out by heating the sample solutions {Cyt c (30 μM) dissolved in [C_2mim][C_2OSO_3] or [Cho][AOT] (100 mM)+[C_2mim][C_2OSO_3]} in silicon oil bath on a hot plate equipped with temperature sensor for a specific time period and subsequently measuring the spectra.

1.3. Experimental Details: Cyt c was dissolved in $[C_2mim][C_2OSO_3]$ by stirring for 3-4 days at 60°C. The concentration of Cyt c used for this work was from 15µM to 30µM. The images of Cyt c dissolved in $[C_2mim][C_2OSO_3]$ and [Cho][AOT] vesicles in $[C_2mim][C_2OSO_3]$ are shown in Fig. S4. Cyt c did not precipitate out even at a concentration as high as 300 mM. The retention of the structure in $[C_2mim][C_2OSO_3]$ was confirmed from UV-Vis experiments (Fig. S5).

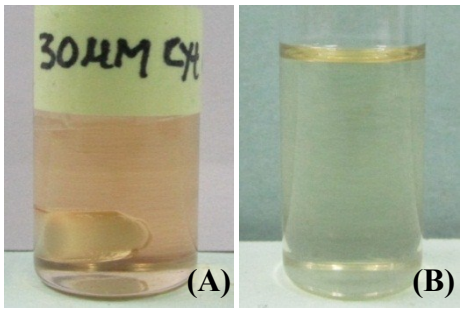


Fig S4. (A) Native Cyt c in $[C_2mim][C_2OSO_3]$. (B) 15µM Cyt c +300 mM [Cho][AOT] in $[C_2mim][C_2OSO_3]$

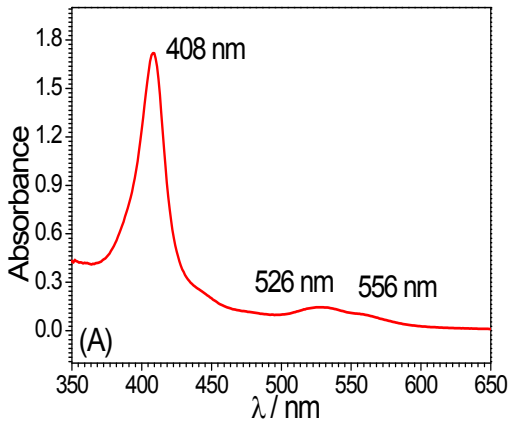


Fig S5. UV-Vis spectra of native Cyt c (15µM) in $[C_2mim][C_2OSO_3]$.

Activity Assay of Cyt c: The thermal activity of Cyt c in $[C_2mim][C_2OSO_3]$ or [Cho][AOT] (100mM)-$[C_2mim][C_2OSO_3]$ was investigated by incubating the Cyt c solution for 10 minutes at different temperatures (60-180°C) and observing the catalytic activity using guaiacol as substrate and H_2O_2 as an oxygen donor at room temperature. In a typical experiment the 100 µL solution of 30 µM Cyt c in buffer, $[C_2mim][C_2OSO_3]$ or $[C_2mim][C_2OSO_3]$[Cho][AOT] (100 mM) was taken in glass vial and heated at different

temperatures for 10 minutes. It was followed by the addition of 900 µL buffer solution + 100 µL of guaiacol (20 mM) + 100 µL of H_2O_2 (50 mM). H_2O_2 was added in the end as it can also oxidize the enzyme. It was followed by the UV-Vis measurement which could be started after minimum of 30 seconds due to the instrumental formalities to start the experiment like baseline correction and sample code etc. Cyt c catalyzes the oxidation of guaiacol to tetraguaiacol which gives orange colour and show absorption band at 470 nm. The activity at 100°C for 6 hrs. and at 130°C for 1 hrs. was measured by heating the samples for a specific period followed by mixing of 100 µL sample with 900 µL buffer solution + 100 µL of guaiacol (20 mM) + 100 µL of H_2O_2 (50 mM). The activity was observed qualitatively from the appearance of orange colour.

Thermal stability of Cyt c

The thermal stability of the Cyt c in both $[C_2mim][C_2OSO_3]$ and [Cho][AOT] (100mM)+ $[C_2mim][C_2OSO_3]$ was measured from CD and UV-Vis spectra. Since the in situ CD spectra can be measured only up to 95°C, we have carried out measurements above 95°C by heating the sample solutions {Cyt c (30 µM) dissolved in $[C_2mim][C_2OSO_3]$ or [Cho][AOT] (100 mM)+$[C_2mim][C_2OSO_3]$} immersed in silicon oil bath on a hot plate, equipped with temperature sensor for a specific time period and subsequently measuring the spectra. Similar procedure was followed to measure the UV-Vis spectra.

Annexure 1

1. Standard Gibbs free energy of calculation using mass action model is given by equation 1.[3]

$$\Delta G^o_{agg} = (2-\alpha)RT \ln x_{CAC} \quad \text{.....(1)}$$

In the equation 1 ΔG^o_{agg} is standard free energy of aggregation, α is free energy of counterion dissociation, R is gas constant, T is temperature and *x* is the mole fraction of the [Cho][AOT] at critical aggregation concentration.

2. The entropic contribution to the free energy of aggregation was calculated from standard Gibbs free energy equation.

$$-T\Delta S^o_{agg} = \Delta G^o_{agg} - \Delta H^o_{agg} \quad \text{.....(2)}$$

Where ΔS^o_{agg} is standard entropy of aggregation ΔH^o_{agg} is standard enthalpy of aggregation calculated from ITC experiments.

3. $q = \frac{4\pi n_r}{\lambda} \sin\frac{\theta}{2}$ .....(3)

Where q is scattering vector of light, λ is wavelength of laser light, n_r is refractive index of solution, θ is scattering angle.

2. Results

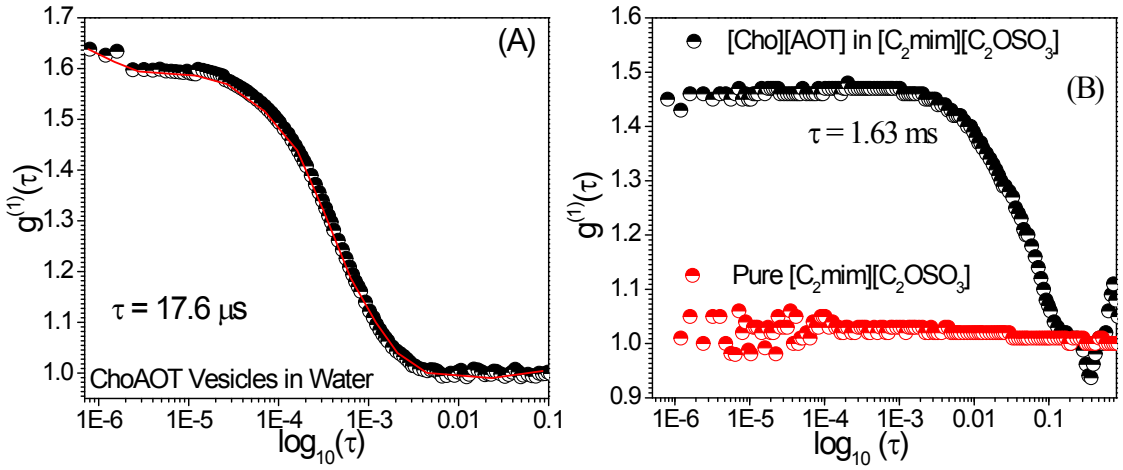


Fig. S6 Comparative autocorrelation functions of [Cho][AOT] vesicles in (A) Water and (B) $[C_2mim][C_2OSO_3]$. Red symbols in (B) showing intensity autocorrelation function of native $[C_2mim][C_2OSO_3]$ which is indicating that $[C_2mim][C_2OSO_3]$ do not contribute to the scattered light by vesicles

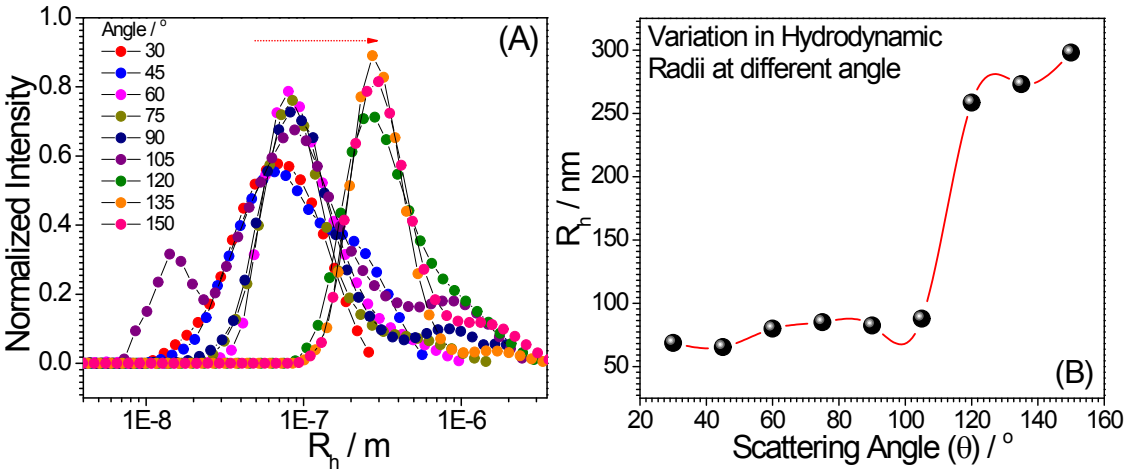


Fig. S7 (A) Hydrodynamic radii profile of [Cho][AOT] aggregates at different angle. (B) Hydrodynamic radii vs angle plot.

Increase in aggregate size with an increase in scattering angle in ILs is an unusual observation since the scattering vector (equation 3 annexure 1 ESI†) has the units of $rad.m^{-1}$ so the large size must be observed at small angle which is true for [Cho][AOT] aggregation in water.[4] Such an unusual angular dependence of aggregate size in IL medium needed to be explored by some expert photophysicist.

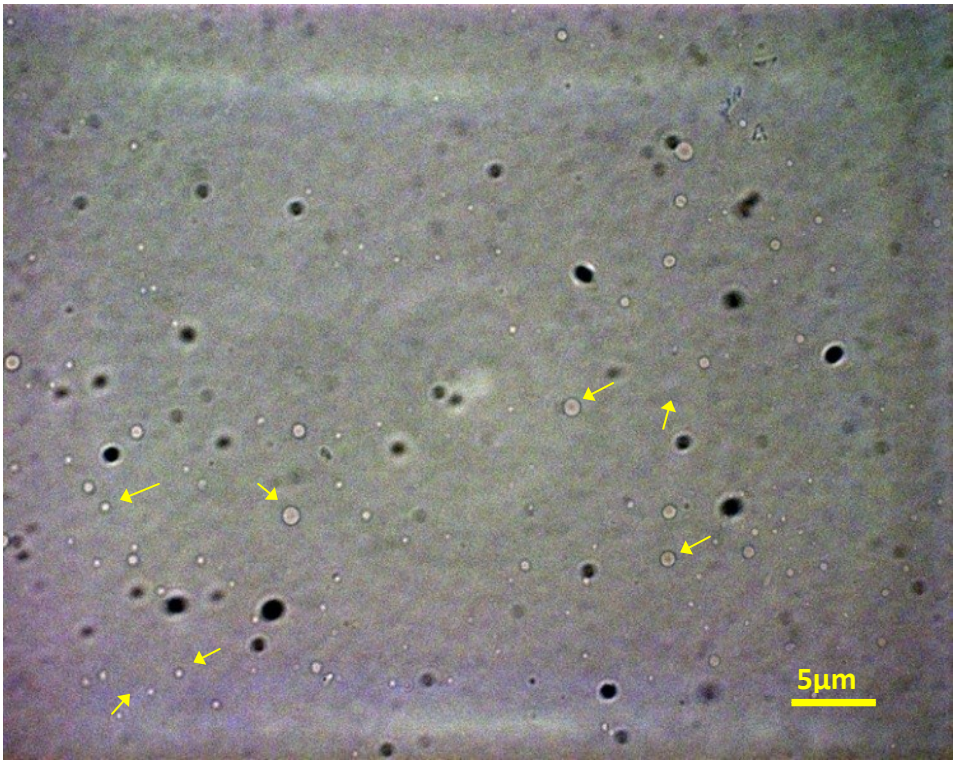


Fig. S8 (A) Optical microscopic images of polydispersed [Cho][AOT] vesicles in $[C_2mim][C_2OSO_3]$.

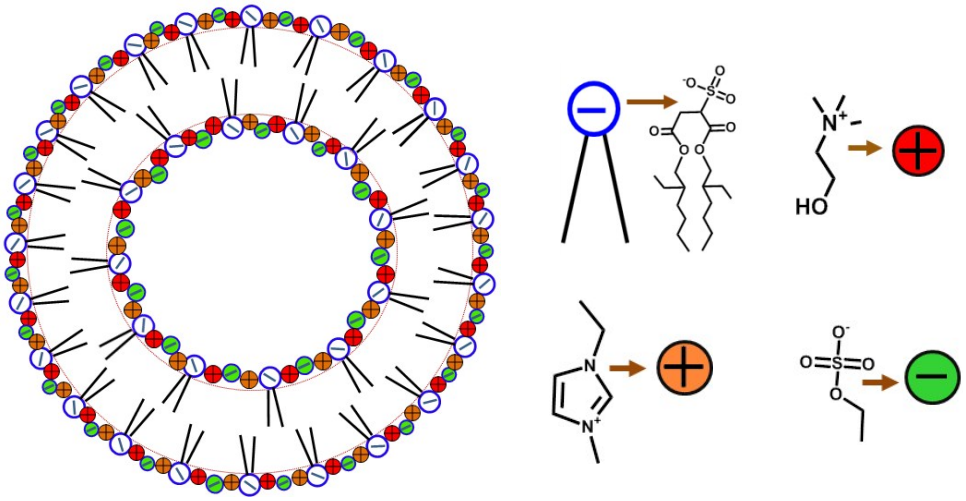


Fig. S9 Proposed structure of [Cho][AOT] vesicle in $[C_2mim][C_2OSO_3]$ medium.

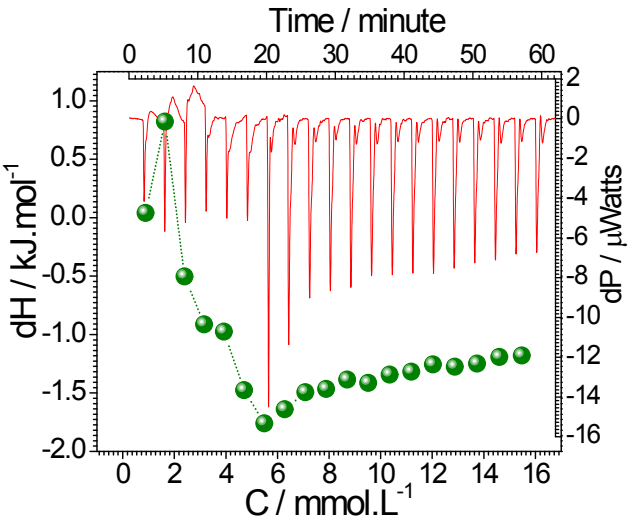


Fig. S10 Enthalpograms of [Cho][AOT] aggregation in Cyt c solution along with dP plot.

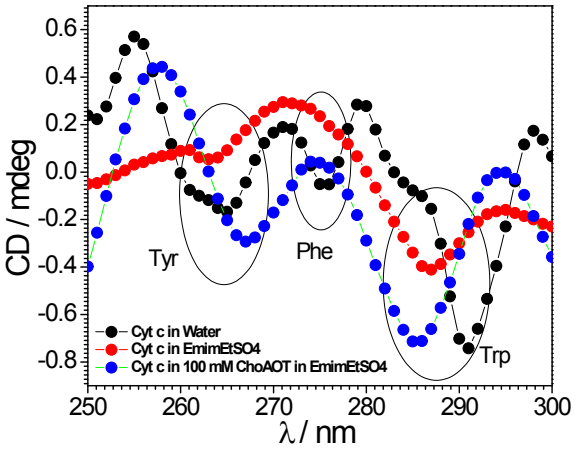


Fig. S11 Comparative mid-UV CD spectra of Cyt c in water, $[C_2mim][C_2OSO_3]$ and vesicles.

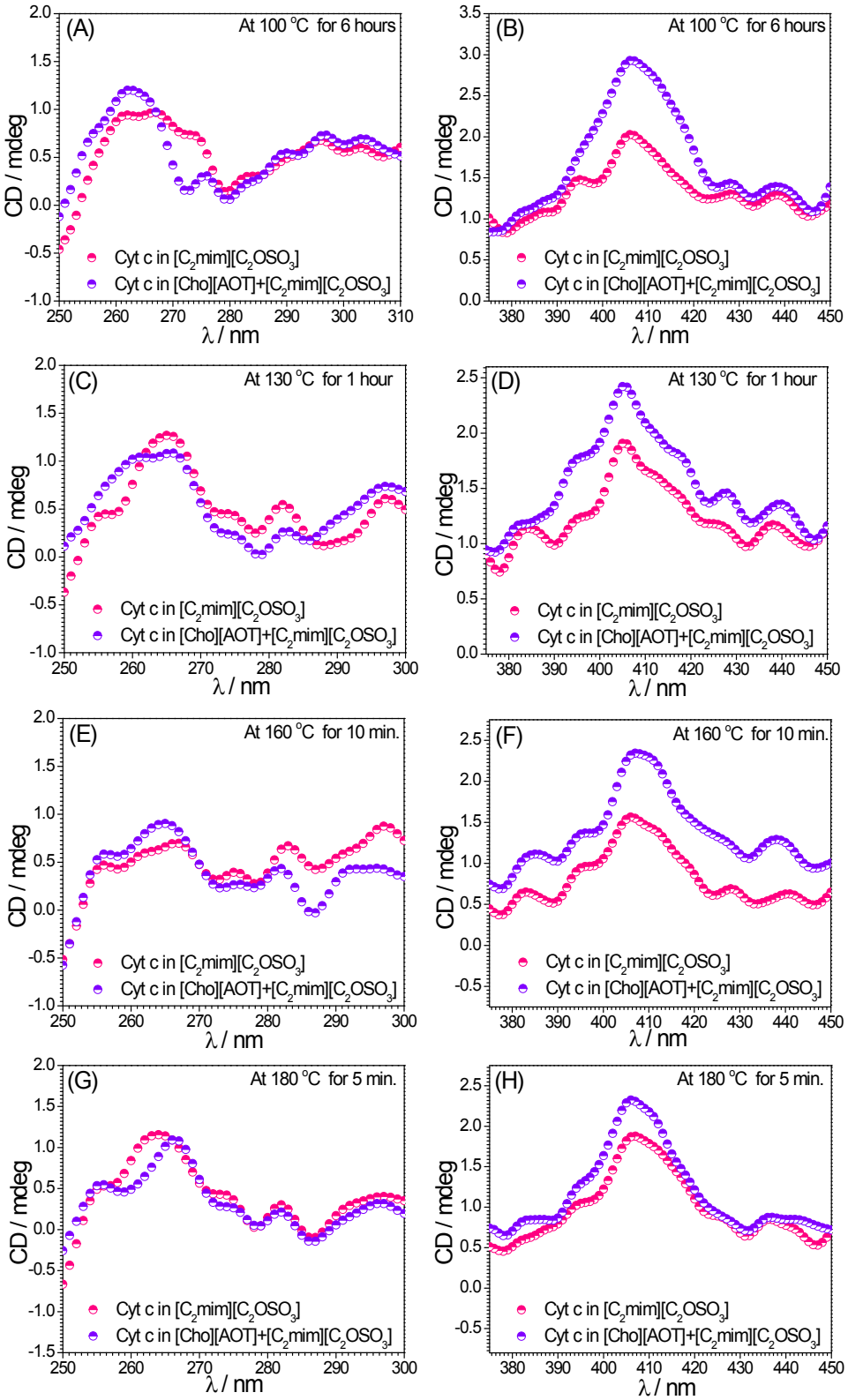


Fig. S12. CD spectrum of Cyt c in $[C_2mim][C_2OSO_3]$; and in [Cho][AOT] (100mM) in $[C_2mim][C_2OSO_3]$ in the near UV and Far-VISIBLE regions after heating at different temperatures and time periods (A) 100°C for 6 h (B) 130 °C for 1 h (C) 160 °C for 10 min and (D) 180°C for 5 min.

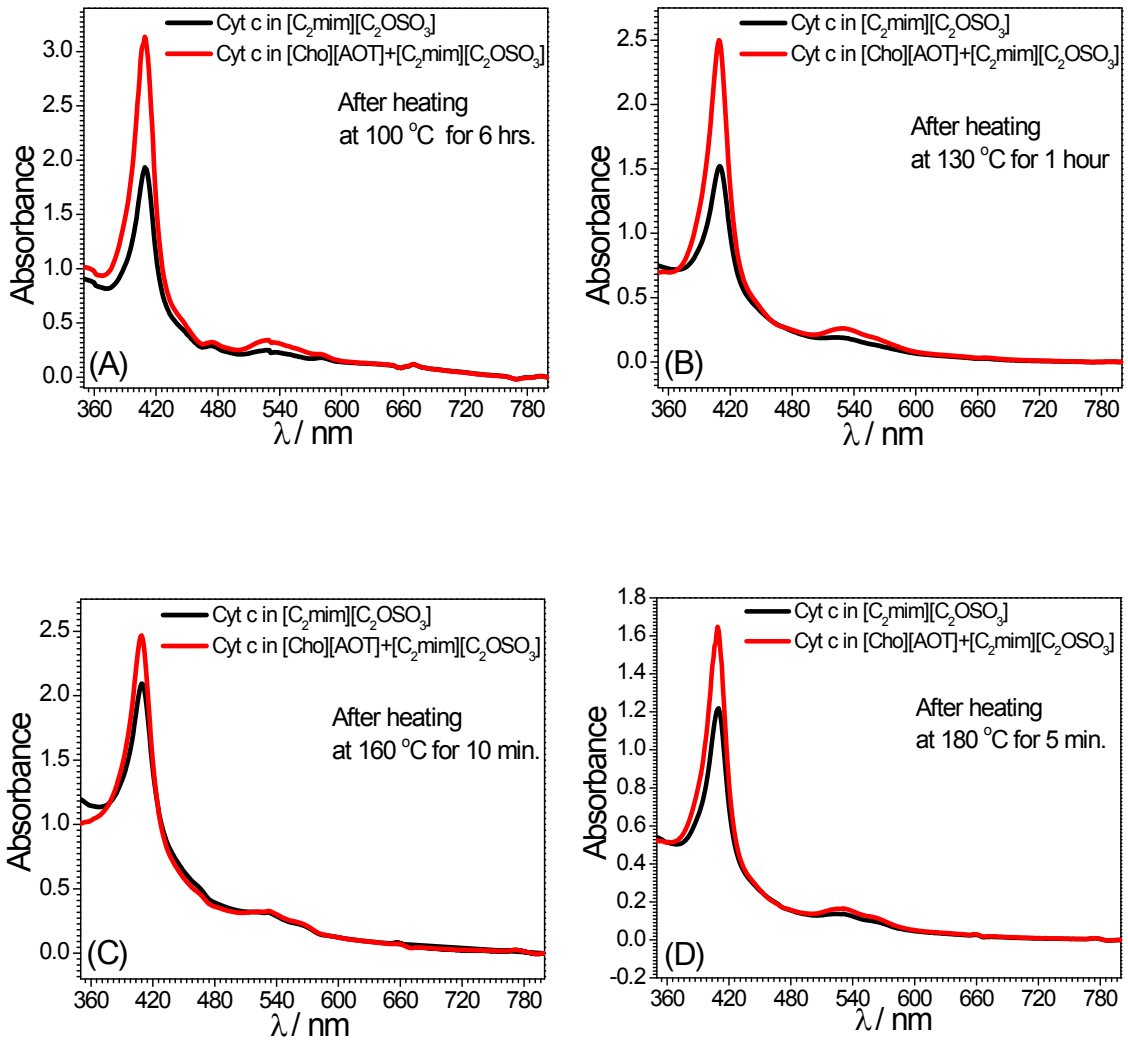


Fig. S13. UV-Vis spectra of Cyt c in $[C_2mim][C_2OSO_3]$; and in [Cho][AOT] (100mM) + $[C_2mim][C_2OSO_3]$ after heating at different temperatures and time periods (A) 100 °C for 6 hrs. (B) 130 °C for 1 hrs. (C) 160 °C for 10 min and (D) 180 °C for 5 min

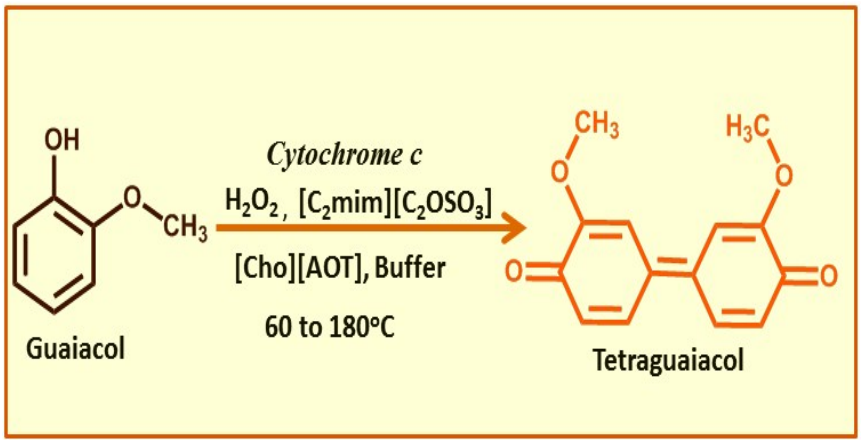


Fig. S14. Peroxidase reaction of tetraguaiacol formation from guaiacol

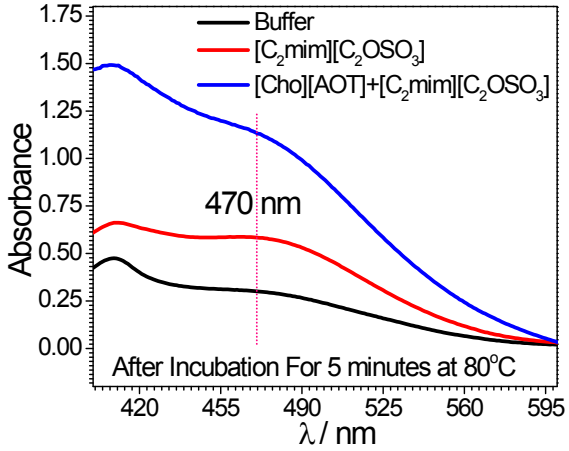


Fig. S15. Comparative UV-Vis spectra of tetraguaiacol after enzyme incubation at 80°C for 10 min and 5 min after the start of reaction in various solvent media.

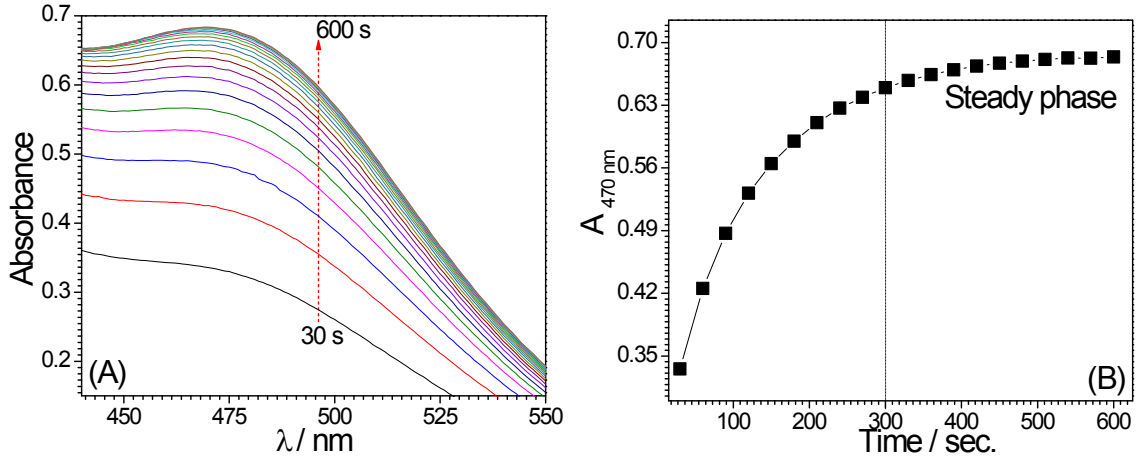


Fig. S16 (A) UV-Vis spectra of tetraguaiacol at room temperature for 10 min. (B) Changes in absorbance of tetraguaiacol at 470 nm at different time intervals for 10 min.

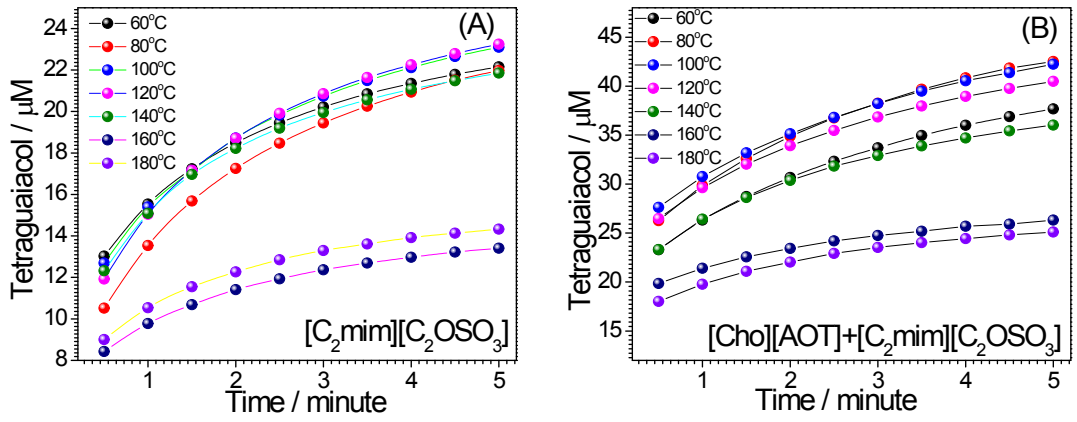


Fig. S17. Plots showing the amount of tetraguaiacol formed in (A) $[C_2mim][C_2OSO_3]$; (B) in $[Cho][AOT]$-$[C_2mim][C_2OSO_3]$ vesicular solution at various temperatures

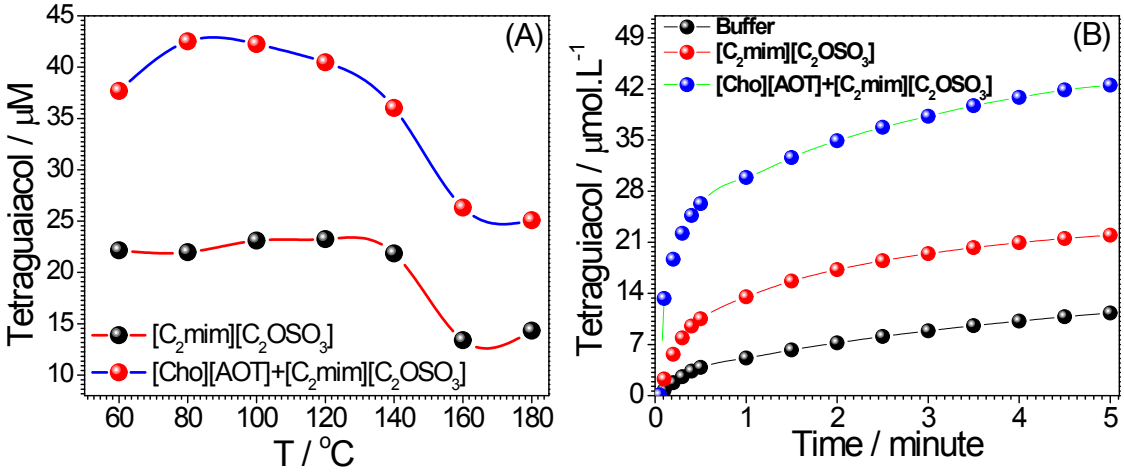


Fig. S18. (A) Temperature dependent activity of Cyt c (30 µM) in neat $[C_2mim][C_2OSO_3]$ and $[Cho][AOT]$ (100mM) + $[-[C_2mim][C_2OSO_3]$ solutions; (B) activity of Cyt c in buffer, neat $[C_2mim][C_2OSO_3]$ and $[Cho][AOT](100mM)+[C_2mim][C_2OSO_3]$ vesicular solutions after incubation at 80°C.

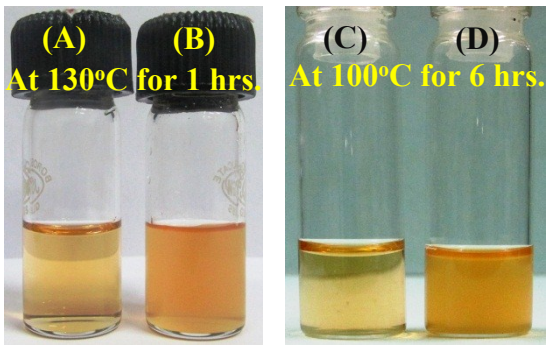


Fig. S18. Images showing functional activity of Cyt c in $[C_2mim][C_2OSO_3]$ (A), (C) and in [Cho][AOT]-$[C_2mim][C_2OSO_3]$ vesicles (B), (D) at incubation at 130°C for 1 h and 100°C for 6 h.

References:

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