Supporting information

Unravelling the Stabilization Mechanism of Mono-, Di and Tri-Cholinium Citrate-Ethylene Glycol DESs towards α-Chymotrypsin for Preservation and Activation of the Enzyme

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This supporting information contains materials and methods used in the manuscript, Synthesis and characterization of ILs and DESs, 7 Tables and 8 Figures.

Materials and Methods

Materials

α-Chymotrypsin (α-CT) (CAS No. 9004-07-3) from bovine pancreas, choline citrate [Chn][Cit] >99.0%, ethylene glycol (EG), cholinium bicarbonate (~80 wt% pure in H_2O) and Tris-HCl were purchased from Sigma-Aldrich, U.S.A. Casein (Hammarsten), trichloroacetic acid (TCA) and sodium acetate were purchased from Sisco Research Lab (SRL), India. All chemicals were used without further purification because they were of high purity and analytical grade. The concentration of enzyme was 0.5 mg/mL. All the samples were prepared in buffer. All chemicals were of high purity and of analytical grade. Prior to measurements all samples were incubated for 30 min at room temperature.

Methods

Spectroscopic measurements

UV- visible absorption spectroscopy

Absorption, fluorescence and UV-CD measurements were performed to analyze the behaviour of α-CT in presence and absence of varying concentration of DESs. Absorption spectra were obtained using Shimadzu UV-1800 (Japan) spectrophotometer. The spectrophotometer has the highest resolution (1 nm) using 1 cm path length quartz cuvette and having wavelength accuracy ±0.3 nm. The spectra were produced at room temperature in the range of 200- 600 nm.

Steady State fluorescence spectroscopy

Steady-state fluorescence experiments were performed using Cary Eclipse spectrofluorimeter from Varian optical spectroscopy instruments, Mulgrave, Victoria (Australia). The emission spectra were recorded at a constant room temperature (25 °C) employing a Peltier device. The excitation wavelength was set at 295 nm to examine the contribution of Trp residues. The slit width was kept 5 nm for both excitation and emission.

Time Resolved fluorescence spectroscopy

Time-resolved fluorescence spectroscopy was utilized to assess the fluorescence lifetimes. The fluorescence decay of α-CT was observed both in the absence and presence of DESs (at a concentration of 60 mg/mL). A single-photon counting spectrophotometer from Horiba Scientific (Delta Flex Jobin Yvon, technology, Glasgow, UK) was employed for recording, operating at 25°C and pH 7.4. Calibration was conducted using a ludox solution, with the maximum counts set to 10,000 and the bandpass to 8 nm. The acquired curve was subjected to tri-exponential fitting using dedicated software, allowing for determination of the average lifetime <τ> through a specific equation.

$$\tau_{avg} = \frac{\sum_{i=i}^{n} \alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i} \qquad \text{……. } \textit{(S1)}$$

Here, α_i, τ_i represent the relative contributions and associated lifetimes of various rotamers in relation to the total number of decay event.

Circular dichroism (CD) spectroscopy

The CD spectra were recorded on Jasco-185 spectrophotometer (USA) equipped with a Peltier system for controlling the temperature with an accuracy of ±0.1 °C. Calibration was performed using (1S) -(+)-10-camphor sul-fonic acid with and a molar ellipticity (Θ) of 2.36 M cm^{-1} at wavelength 295 nm and 34.5 Mcm^{-1} molar extinction coefficient (ε) at 285 nm Far and near-UV CD spectra were observed in the range 200-250 nm and 250-350 nm, respectively. The bandwidth, response time and scan speed were set as 1 nm, 1 s and 50 nm/min respectively.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of α-CT in the absence and presence of choline-based DESs were recorded in the range of 4000–500 cm^{-1} using Shimadzu FTIR spectrometer. For the sample preparation, buffer was prepared in D_2O and maintained at pD ~7.0. The concentration of α-CT was kept 0.5 mg/mL and allowed to pre-equilibrate at room temperature for 1-2 hour. Before recording the spectra for samples, spectra for background and buffer were recorded under the same condition. About 40 µL of sample was placed in between the ZnSe windows by using a spacer of 50 µm path length. Total of 256 interferometer scans was performed with 2 cm-1 resolution. Utilizing OPUS 6.0 (Brukekr) software, the data are processed and the spectrum is collected.

Thermal Fluorescence Spectroscopy

The thermal unfolding studies of α-CT in presence of DESs were analyzed using same Cary Eclipse spectrofluorimeter over the temperature range 20 to 85 ˚C. The heating rate was 1 °C min^{-1} and an excitation wavelength fixed at 295 nm. All thermal unfolding transitions were determined by assuming the two-state unfolding mechanisms as shown in equation S2.

$$\textit{Folded} \leftrightarrow \textit{Unfolded} \qquad \textit{.....(S2)}$$

The difference in free energy between unfolded and the native state (ΔG_u) is calculated according to equation S3.

$$\Delta G_u = -RT(lnK) \qquad \textit{.....(S3)}$$

Here R is the universal gas constant, T absolute temperature and K is equilibrium constant.

At equilibrium, ΔG_u is always zero, thus, the temperature at which ΔG_u turns out to be zero is said to be the T_m value for the protein. The value enthalpy change of unfolding (ΔH_m) at T_m can be obtained by analysis of the plot of ΔG_u versus T. The slope of this plot at T_m illustrates the entropy change of unfolding (ΔS_m). The ΔH_m was calculated through equation S4:

$$\Delta H_m = T_m \Delta S_m \qquad \textit{.....(S4)}$$

The value of heat capacity change (ΔC_p) at 25 °C was calculated using Gibbs-Helmholtz equation given below-

$$\Delta G_u(T) = \Delta H_m[1 - \frac{T}{T_m}] - \Delta C_p[(T_m - T) + T\, ln(\frac{T}{T_m})] \quad \text{.....(S5)}$$

Dynamic light scattering (DLS) Size and Zeta-Potential measurements

To determine the hydrodynamic diameter (d_H) and zeta-potential of α-CT in buffer and various concentrations of DESs, dynamic light scattering (DLS) measurements are performed by operating Zetasizer Nano instrument (ZS90), U. K. The scattering angle is set as 90° with the fixed operating wavelength at 633 nm. The instrument is fitted with 4 mW He−Ne laser having laser attenuator (automatic). The detection range span is fixed from 0.1 nm to 10 μm and temperature was maintained at 25.0 °C. Each measurement was the average of three concordant reading. Stokes-Einstein equation is employed by instrument software for assessing the diffusion coefficient and subsequently calculate d_H.

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed for the examination of the morphology of α-CT in additives utilizing a cryo TEM operating at 200 kV (Talos, Thermo Scientific). The equipment features digital imaging and utilizes a 35 mm photographic technology. Samples were prepared using copper grids with a mesh size of 200 square mesh. A volume of 20 μL of the sample was deposited onto the carbon side of the TEM grid and allowed to settle undisturbed for 2 hours to ensure moisture elimination from the grid. Images were captured at various magnifications to obtain comprehensive information on size and surface characteristics. Image J software was utilized for the analysis of all acquired images.

Enzymatic activity of α-CT

The activity of α-CT is assayed using UV-vis spectrophotometer (UV-1800 Shimadzu) by taking 0.25 mL denatured casein solution (2% w/v) as a substrate. substrate is prepared by heating at 80-85°C for 15 min. The α-CT solution (0.25 mL) in absence and presence of DESs was incubated with 0.25 mL of denatured casein solution at pH 7.0 for 10 mins at 37 °C. Reaction time was 10 min and after that ice-cold 110 mM trichloroacetic acid (TCA) solution was added to stop the reaction. Afterwards, to remove the undigested casein, the samples were centrifuged for 7 mins at 10000 rpm. Finally, the absorption of the supernatant was recorded at 275 nm, the reaction product was corelated with the absorbance of a reagent blank. The activity (units per mL) of α-CT was calculated by using a standard curve of absorbance with the noted quantities of the Tyrosine (Tyr). The activity is described as the amount of the μmoles of Tyr equivalents liberated from casein per min and is calculated using the following equation S6:

$$\text{Activity} = \frac{(\text{mole tyrosine equivalents released})\ (\text{V})}{(\text{time of assay in minutes})\ (\text{v1})\ (\text{v2})} \quad \text{.....(S6)}$$

Here, V is the total volume of the assay in mL, v1 is the volume of enzyme used in mL and v2 is the volume of sample used in mL.

The percentage relative activity was determined by assuming that the pure α-CT in Tris-HCl buffer at pH 7.4 exhibited 100% activity. To investigate the effects of time on storage, the remaining samples were kept at room temperature for 15 days, after which the activity measurement was repeated. This process was repeated at regular intervals, and the results were

compared with the initial measurements. Further, Michaelis Menten constant (K_m) and maximum rate (V_{max}) were calculated using following Michaelis Menten equation S7 and prism software:

$$V = \frac{Vmax\ [S]}{Km + [S]} \qquad \text{.....(S7)}$$

Here, V is the rate of reaction, V_{max} is maximum rate, [S] is substrate concentration and K_m Michaelis Menten constant.

Synthesis and Characterization of $[Chn]_2[Cit]$ and $[Chn]_3[Cit]$

Little modifications were made to the published approach in order to synthesized ILs based on di and tri cholinium.1 In a typical reaction aqueous cholinium bicarbonate (80 wt % in water) was added into a round-bottom flask containing cholinium dihydrogen citrate in the stoichiometric ratio (1:1 and 2:1), under continuous magnetic stirring at temperature 60°C for 12 h in oil bath in inert atmosphere a clear homogenous mixture was obtained. Ultimately, ethylacetate was used to wash the synthesized ILs. In order to remove moisture and drying, the obtained ILs were kept in nitrogen environment at 60°C for 24 hours in vacuum line. Fig. S1 and S2 displays the FTIR spectra and 1H NMR of $[Chn]_2[Cit]$ and $[Chn]_3[Cit]$ in D_2O respectively. Dicholinium citrate ($[Chn]_2[Cit]$); 1 H NMR (400 MHz, D2O): δ (ppm) 3.84 (m, J = 6.2 Hz, 4H, $-CH_2$); 3.42 (t, J = 5.1 Hz, 4H, $-CH_2$); 3.12 (s, 18H, $-CH_3$); 2.25 (m, 4H, $-CH_2$). Tricholinium citrate ($[Chn]_3[Cit]$); 1 H NMR (400 MHz, D2O): δ (ppm) 3.92 (t, J = 6.3 Hz, 6H, $-CH_2$); 3.37 (t, J = 5.2 Hz, 6H, $-CH_2$); 3.06 (s, 27H, $-CH_3$); 2.49 (m, 4H, $-CH_2$).

Characterization of ionic liquids:

(A) Di Cholinium Citrate

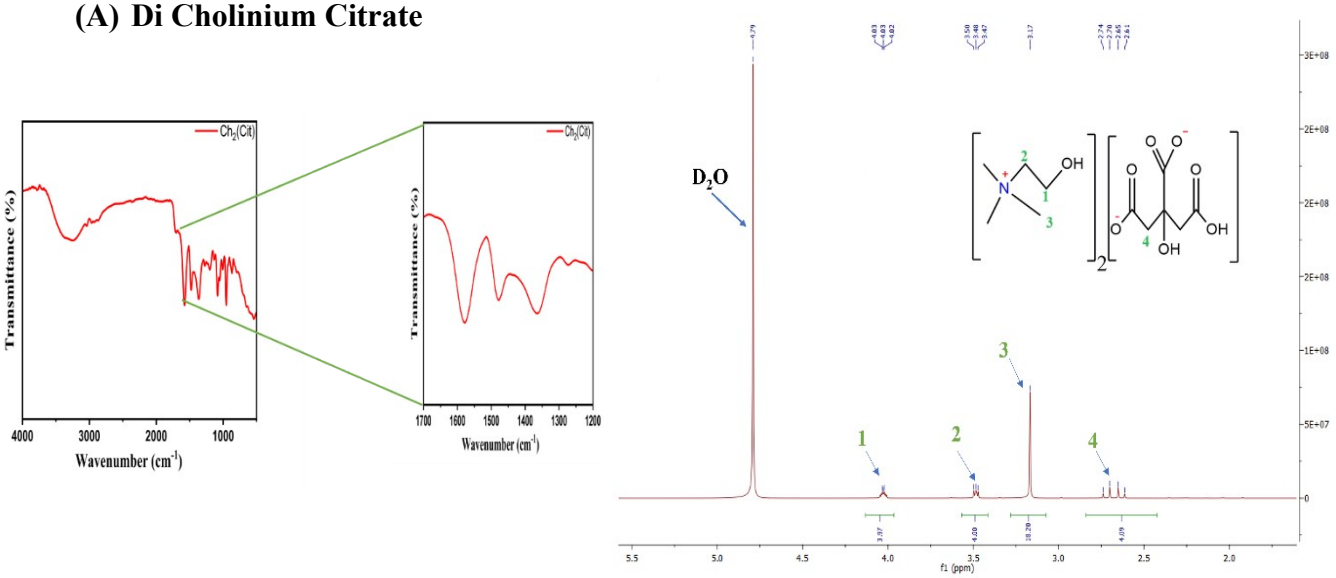


Fig. S1 FTIR and NMR spectra of $[Chn]_2[Cit]$ D_2O.

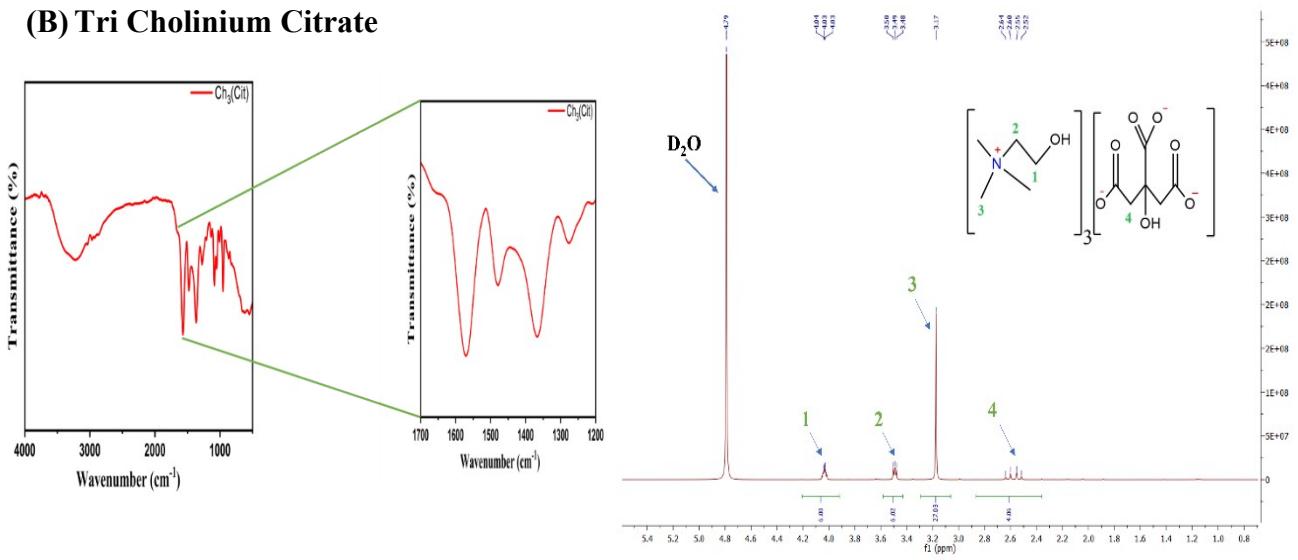


Fig. S2 FTIR and NMR spectra of $[Chn]_3[Cit]$ D_2O.

Synthesis and Characterization of Deep Eutectic Solvents

DES Preparation

For the current work, three DESs named as [Chn][Cit]-EG (DES-1), $[Chn]_2[Cit]$-EG (DES-2), and $[Chn]_3[Cit]$-EG(DES-3) were synthesized. Starting materials were mixed at a molar ratio of 1:2 in a glass vial containing one hydrogen bond acceptor (HBA) [Chn][Cit]/$[Chn]_2[Cit]$ / $[Chn]_3[Cit]$ and one hydrogen bond donor (HBD) EG for one to two hours, the entire mixture was magnetically swirled in an oil bath at 80 - 85 °C until a transparent, homogenous mixture appeared. After that, to reduce the percentage of moisture, the resulting solution was allowed to cool at room temperature and kept in a desiccator. The FTIR spectra of synthesized all three DESs are presented in Fig. S3.

Characterization of DESs

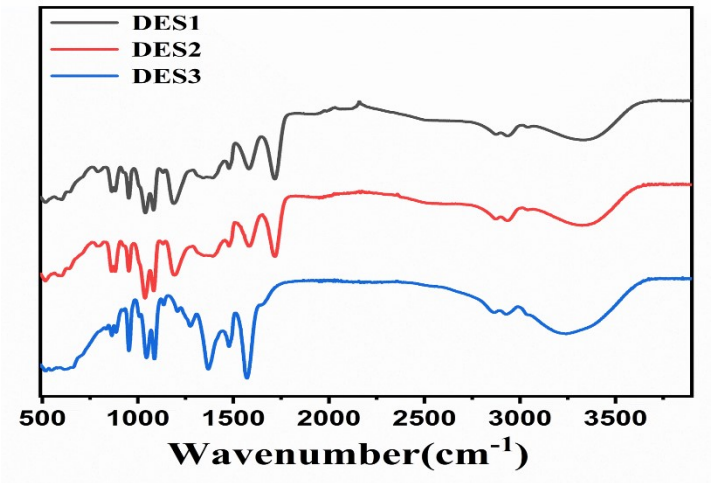


Fig. S3 FTIR spectra DES-1(black), DES-2 (Red) and DES-3 (blue) in D_2O.

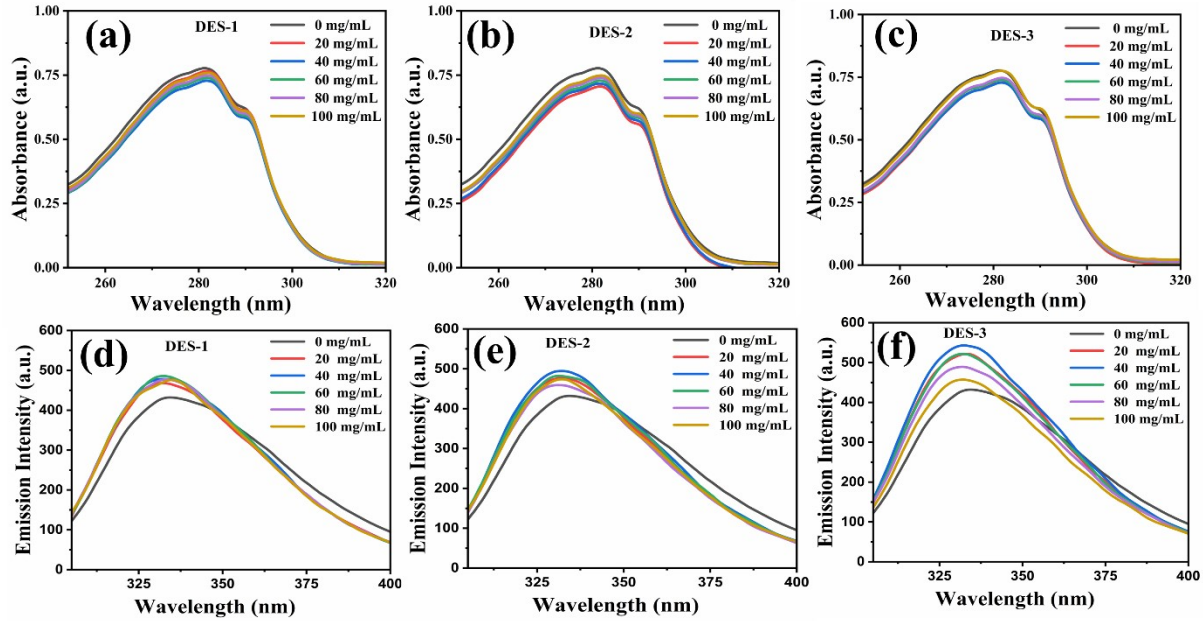


Fig. S4 UV-Visible spectra and fluorescence spectra of α-CT in the presence of varying concentrations of (a,d) DES-1, (b,e) DES-2, and (c,f) DES-3.

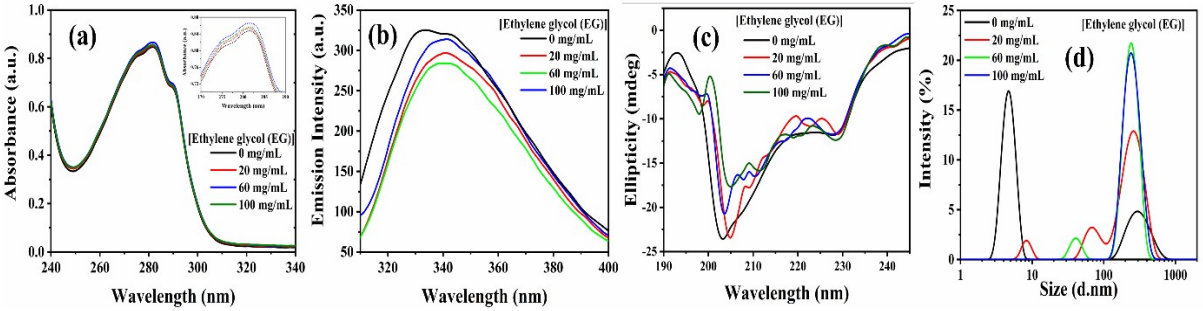


Fig. S5 (a) UV-visible spectra, (b) fluorescence spectra, (c) far UV-CD, and (d) hydrodynamic size of α-CT in the presence of varying concentrations of EG.

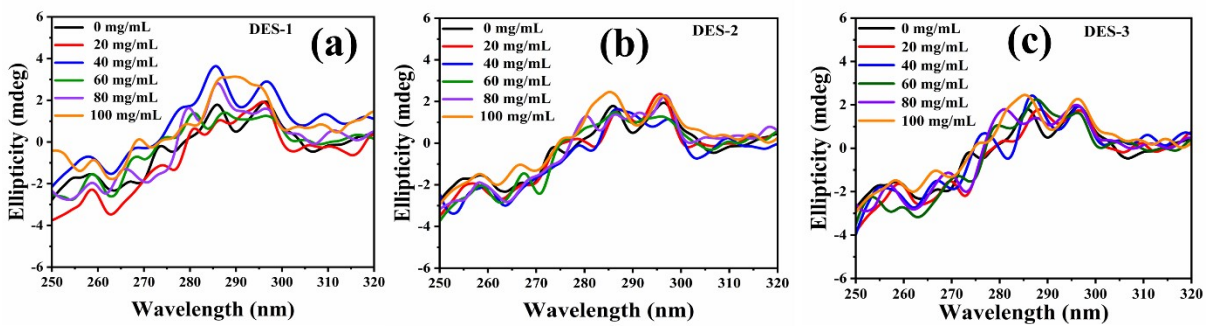


Fig. S6 Near UV-CD of α-CT in the presence of varying concentrations of (a) DES-1, (b) DES-2 and (c) DES-3.

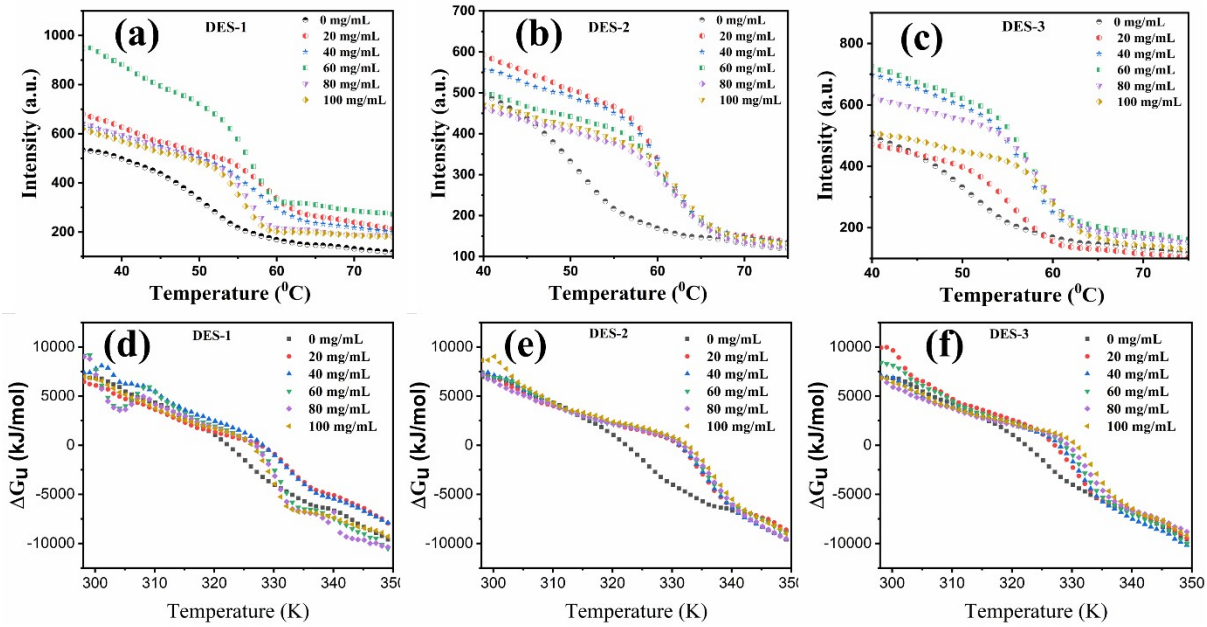


Fig. S7 Thermal transition curve and free energy plots of α-CT in presence of varying concentration of (a,d) DES-1, (b,e) DES-2 and (c,f) DES-3, respectively.

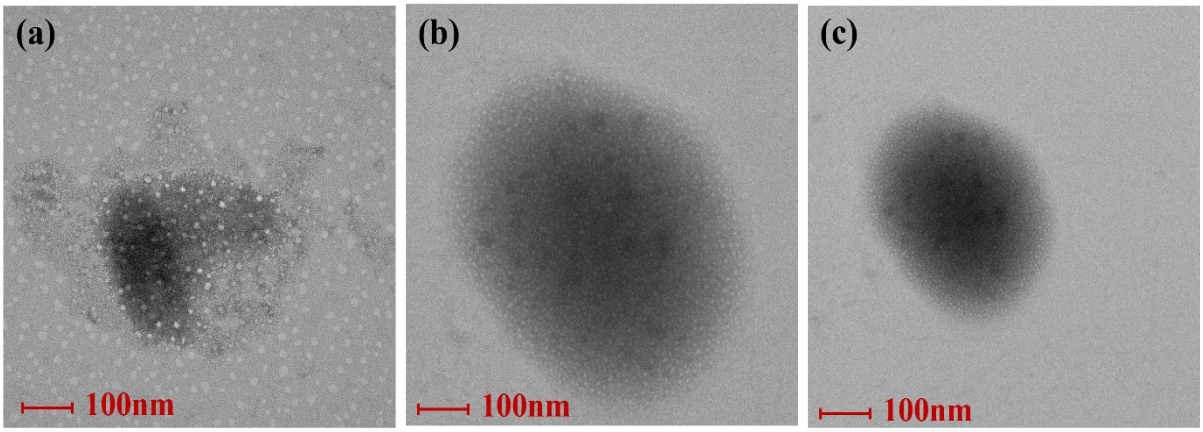
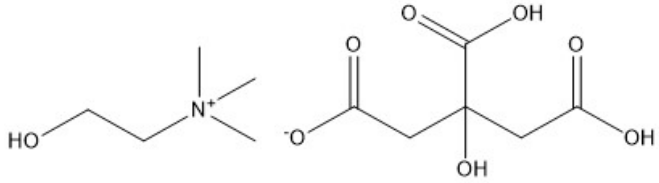


Fig. S8 Transmission electron microscopy of pure (a) DES-1, (b) DES-2, and (c) DES-3 at a concentration of 60 mg/mL.

Table S1. Composition, Chemical Structures, Nomenclature, and Molar Ratios of the DESs.

Hydrogen Bond Acceptor (HBA)	Hydrogen Bond Donor (HBD)	Molar ratio
 choline dihydrogen citrate	 Ethylene Glycol	1:2

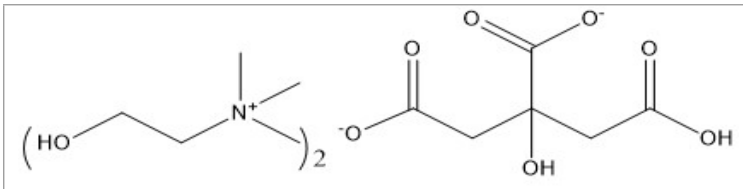
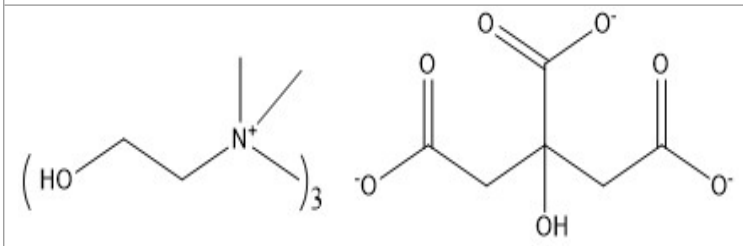
 Dicholine monohydrogen citrate	[redacted]	1:2
 Tri cholinium citrate	[redacted]	1:2

Table S2. Lifetime decay parameters of α-CT in DESs in 10 mM Tris-HCl buffer at pH 7.4

Systems	α_1	τ_1	α_2	τ_2	α_3	τ_3	χ^2	τ_{avg} (ns)
α-CT in buffer	0.42	1.39	0.48	4.01	0.09	0.27	1.02	3.37
DES-1(60 mg/mL)	0.37	1.62	0.39	4.03	0.24	0.76	0.99	3.16
DES-2(60 mg/mL)	0.31	2.01	0.35	3.79	0.33	0.87	0.99	3.00
DES-3(60 mg/mL)	0.33	1.76	0.45	4.02	0.21	0.80	1.33	3.30

Table S3 – The analysis of % secondary structure of α-CT in the presence of DES-1, DES-2, and DES-3 using curve-fitted FTIR spectra of the amide-I band.

Band (cm^{-1})	**Band assignment**	**Content (%)**			
		α-CT	**α-CT + DES-1**	**α-CT + DES-2**	**α-CT + DES-3**
1622	β - sheet	-	-	-	-
1623	β - sheet	-	-	-	9

1624	β - sheet	4	4	5	-
1635	β - sheet	-	4	-	21
1637	β - sheet	-	-	7	-
1641	Random coil	31	-	-	-
1645	Random coil	-	-	-	27
1646	Random coil	-	-	25	-
1647	Random coil	-	28	-	-
1653	α - helix	27	-	29	-
1655	α - helix	-	-	-	22
1658	α - helix	-	31	-	-
1663	β - turn	27	-	25	-
1664	β - turn	-	-	-	15
1670	β - turn	-	19	-	-
1672	β - turn	-	-	-	6
1674	β - turn	11	-	9	-
1680	β - turn	-	10	-	-
Total	α - helix	27	31	29	22
	β - (turn + sheet)	42	41	46	51
	Random coil	31	28	25	27

Table S4. Transition temperature (Tm), Gibbs free energy change of unfolding (ΔG_u), Enthalpy change (ΔH_m), Entropy change (ΔS_m), and Heat capacity change of unfolding (ΔC_p) at 25 °C determined by fluorescence analysis of thermal denaturation of α-CT in absence and presence of varying concentrations of DES-1, DES-2and DES-3.

DESs	**[Conc.] (mg/mL)**	**T_m (°C)**	**ΔG_u (kJ.mol^{-1})**	**ΔH_m (kJ.mol^{-1})**	**ΔS_m (kJ.mol^{-1}K^{-1})**	**ΔC_p (kJ.mol^{-1}K^{-1})**
DES-1	00	49.5±0.4	7.0±0.2	215.4±8.2	0.6±0.02	9.7±0.8
	20	58.6±0.3	6.4±0.2	378.5±7.4	1.1±0.03	18.1±0.8
	40	56.5±0.3	7.3±0.2	286.3±8.0	0.8±0.01	12.9±0.7
	60	55.8±0.4	9.1±0.2	453.1±8.1	1.3±0.04	22.3±0.8
	80	56.0±0.3	9.0±0.2	657.4±7.8	2.0±0.01	35.1±0.7
	100	54.9±0.3	7.0±0.3	540.5±7.9	1.6±0.05	30.0±0.8

DES-2	20	60.6±0.2	7.4±0.2	443.3±8.0	1.3±0.02	20.2±0.8
	40	60.7±0.3	7.3±0.2	383.5±7.9	1.4±0.04	17.0±0.7
	60	60.8±0.4	7.3±0.2	419.6±8.1	1.2±0.03	18.8±0.8
	80	61.6±0.2	7.1±0.3	394.4±7.8	1.1±0.04	17.3±0.7
	100	62.2±0.3	8.6±0.2	417.7±8.2	1.2±0.03	17.6±0.8
DES-3	20	54.9±0.3	9.9±0.1	340.6±8.2	1.0±0.01	15.0±0.8
	40	56.7±0.3	7.0±0.2	428.1±8.2	1.3±0.02	21.6±0.8
	60	57.1±0.4	8.4±0.2	414.0±7.9	1.2±0.04	19.7±0.7
	80	58.0±0.3	6.8±0.2	404.4±8.2	1.2±0.04	19.4±0.8
	100	59.6±0.4	6.8±0.2	454.4±8.2	1.3±0.01	21.6±0.8

Table S5. Hydrodynamic Diameter (d_H) values of α-CT in the absence and presence of varying concentrations of DESs.

[DESs] mg/mL	**d_H of α-CT in DES-1 (nm)**	**d_H of α-CT in DES-2 (nm)**	**d_H of α-CT in DES-3 (nm)**
00	4.2±0.2	4.2±0.2	4.2±0.2
20	5.5±0.2	3.6±0.2	2.3±0.2
40	4.7±0.2	4.8±0.2	3.0±0.2
60	5.8±0.2	5.5±0.2	3.3±0.2
80	7.2±0.2	7.6±0.2	6.3±02
100	7.6±0.3	7.2±0.2	8.2±0.2

Table S6. Zeta- Potential values of α-CT in the absence and presence of varying concentrations of DESs and blank DESs

[DESs] (mg/mL)	**Zeta - Potential (mV) of**					
	DES-1	**α-CT in DES-1**	**DES-2**	**α-CT in DES-2**	**DES-3**	**α-CT in DES-3**

00	-	5.4±0.08	-	5.4±0.08	-	5.4±0.08
20	0.3±0.05	3.8±0.07	-1.2±0.06	-1.1±0.06	-9.2±0.2	-9.5±0.2
40	2.7±0.08	3.1±0.06	-1.3±0.09	-1.7±0.05	-10.1±0.2	-8.5±0.2
60	1.0±0.07	2.8±0.09	-1.4±0.08	-1.3±0.09	-9.9±0.2	-9.0±0.2
80	0.4±0.06	2.0±0.05	-1.5±0.06	-1.6±0.07	-8.7±0.2	-7.0±0.2
100	0.5±0.09	2.5±0.07	-1.7±0.06	-1.5±0.09	-8.2±0.2	-6.2±0.2

Table S7 - Enzyme activity of α-CT in presence of DES-1, DES-2 and DES-3 at 25°C and 55°C.

Day	**Sample**	**Enzyme Activity of α-CT in presence of various concentration of DESs (mM/min.)**				
		20 mg/mL	**40 mg/mL**	**60 mg/mL**	**80 mg/mL**	**100 mg/mL**
1st Day	α-CT	7.3 ± 0.02				
	α-CT + DES-1	1.5 ± 0.02	0.9 ± 0.01	0.7 ± 0.01	0.7 ± 0.02	0.6 ± 0.01
	α-CT + DES-2	6.7 ± 0.01	6.0 ± 0.03	5.4 ± 0.02	5.1± 0.03	4.7 ± 0.02
	α-CT + DES-3	7.4 ± 0.03	7.3 ± 0.02	7.4 ± 0.03	7.4 ± 0.01	7.3 ± 0.03
15th Day	α-CT	5.2 ± 0.01				
	α-CT + DES-1	0.8 ± 0.01	0.7 ± 0.02	0.5 ± 0.01	0.5 ± 0.01	0.6 ± 0.01
	α-CT + DES-2	6.7 ± 0.02	5.9 ± 0.03	5.4 ± 0.02	5.1 ± 0.02	4.7 ± 0.03
	α-CT + DES-3	7.2 ± 0.03	7.3 ± 0.01	7.4 ± 0.03	7.3 ± 0.04	7.5 ± 0.01
30th Day	α-CT	3.8 ± 0.02				
	α-CT + DES-1	0.8 ± 0.02	0.9 ± 0.01	0.8 ± 0.03	0.9 ± 0.01	0.7 ± 0.03
	α-CT + DES-2	6.7 ± 0.03	6.0 ± 0.04	5.4 ± 0.03	4.4 ± 0.01	3.4 ± 0.01
	α-CT + DES-3	7.1 ± 0.04	7.2 ± 0.01	7.2 ± 0.02	7.1 ± 0.03	7.2 ± 0.01
1st Day (at 50°C)	α-CT	1.1 ± 0.01				
	α-CT + DES-1	0.7 ± 0.01	0.6 ± 0.03	0.6 ± 0.01	0.5± 0.02	0.4 ± 0.01
	α-CT + DES-2	2.3 ± 0.02	1.7 ± 0.01	2.0 ± 0.02	2.2 ± 0.01	2.1 ± 0.02
	α-CT + DES-3	1.0 ± 0.01	1.0 ± 0.04	1.0 ± 0.01	1.8± 0.03	1.6 ± 0.01