

Electronic Supplementary Information

Superior performance of ionic liquids over deep eutectic solvents in stabilizing L-Asparaginase for sustainable biopharmaceutical applications

Diksha Dhiman^{a,†}, Ana S. C. Marques^{b,†}, Ana P. M. Tavares^b, Mara G. Freire^{b,} and Pannuru Venkatesu^{a,*}*

^a Department of Chemistry, University of Delhi, Delhi-110 007, India.

^b CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

[†] These authors contributed equally.

The Electronic Supplementary Information contains 9 Figures and 10 Tables in 16 pages

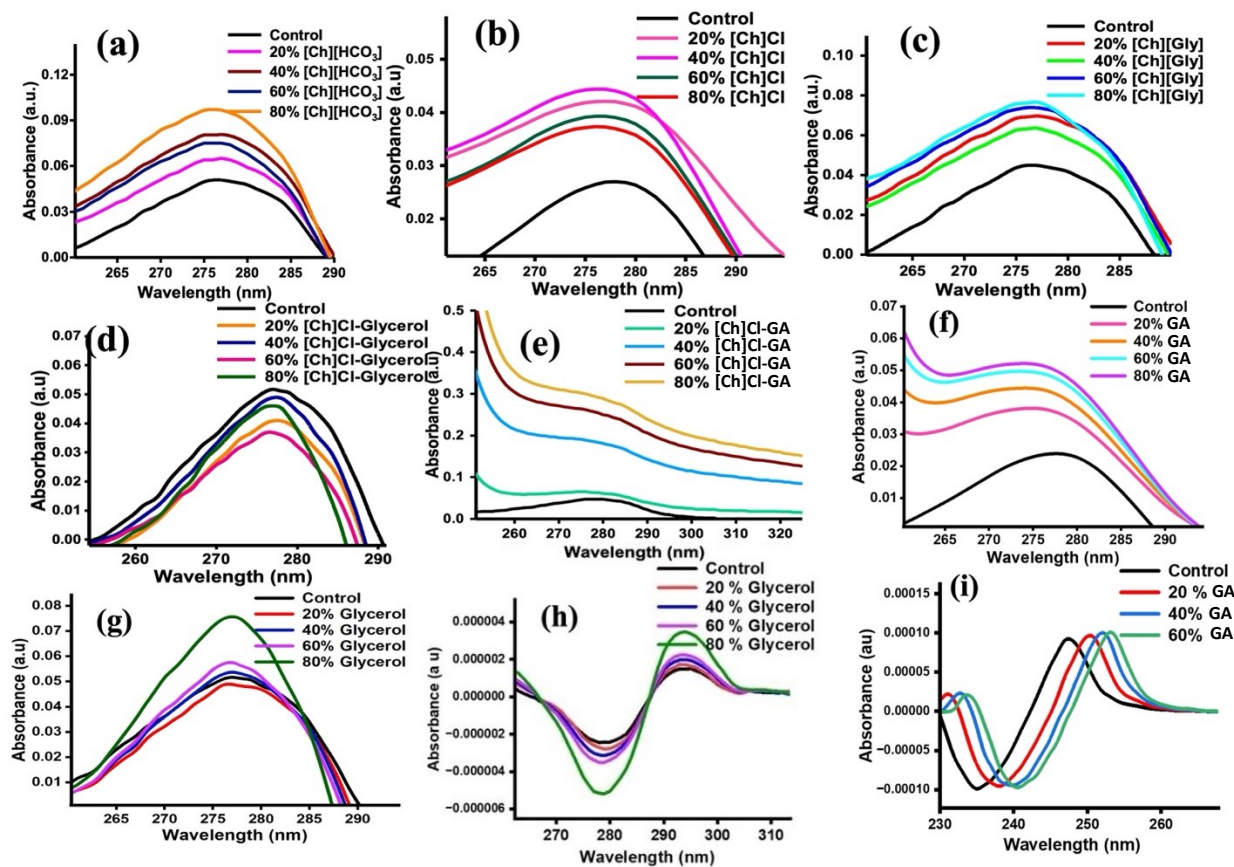


Figure S1. UV – absorbance spectra of L-ASNase in the presence of (a) [Ch][HCO₃] (b) [Ch]Cl (c) [Ch][Gly], (d) [Ch]Cl-Glycerol and (e) [Ch]Cl-GA at concentration range (0-80 wt%). Second-derivative UV-visible absorption spectra of L-ASNase in the presence of various concentrations of (h) Gly (i) GA at 40 wt %.

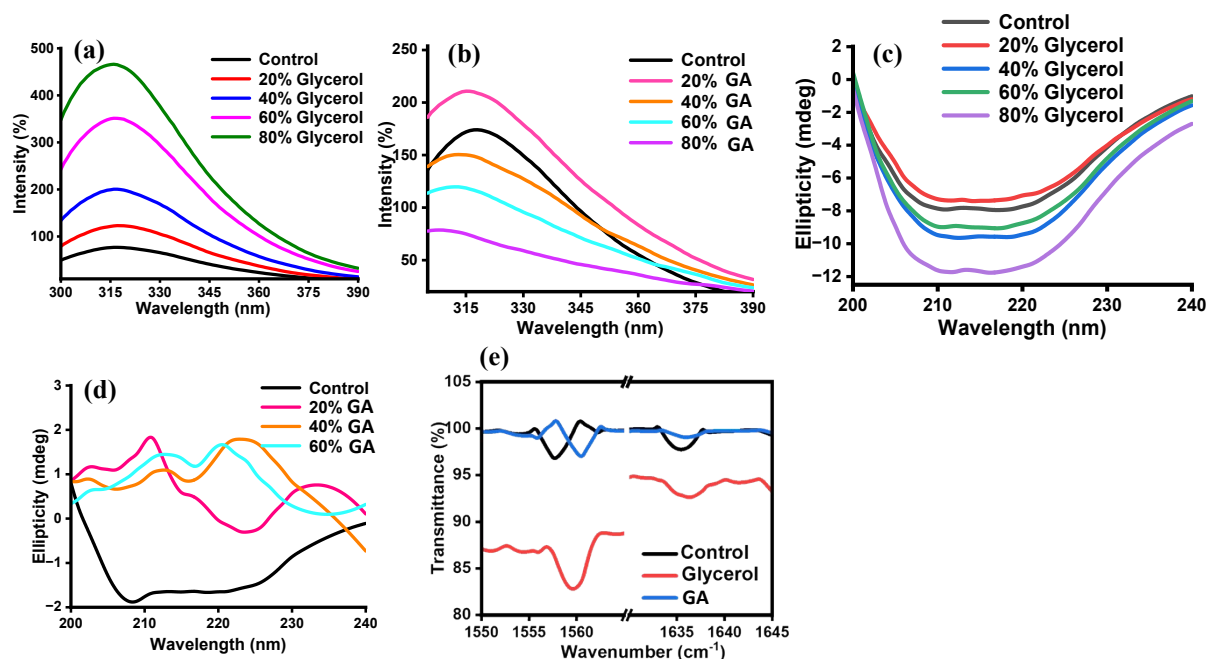


Figure S2. Fluorescence emission spectra of L-ASNase in the presence of (a) Glycerol (b) GA at a concentration range (0-80 wt%). Far-CD spectra of ASNase in the presence of (c)

Glycerol (d) GA at a concentration range (0-80 wt%). (e) FT-IR spectra of ASNase in the presence of PBS, Glycerol and GA.

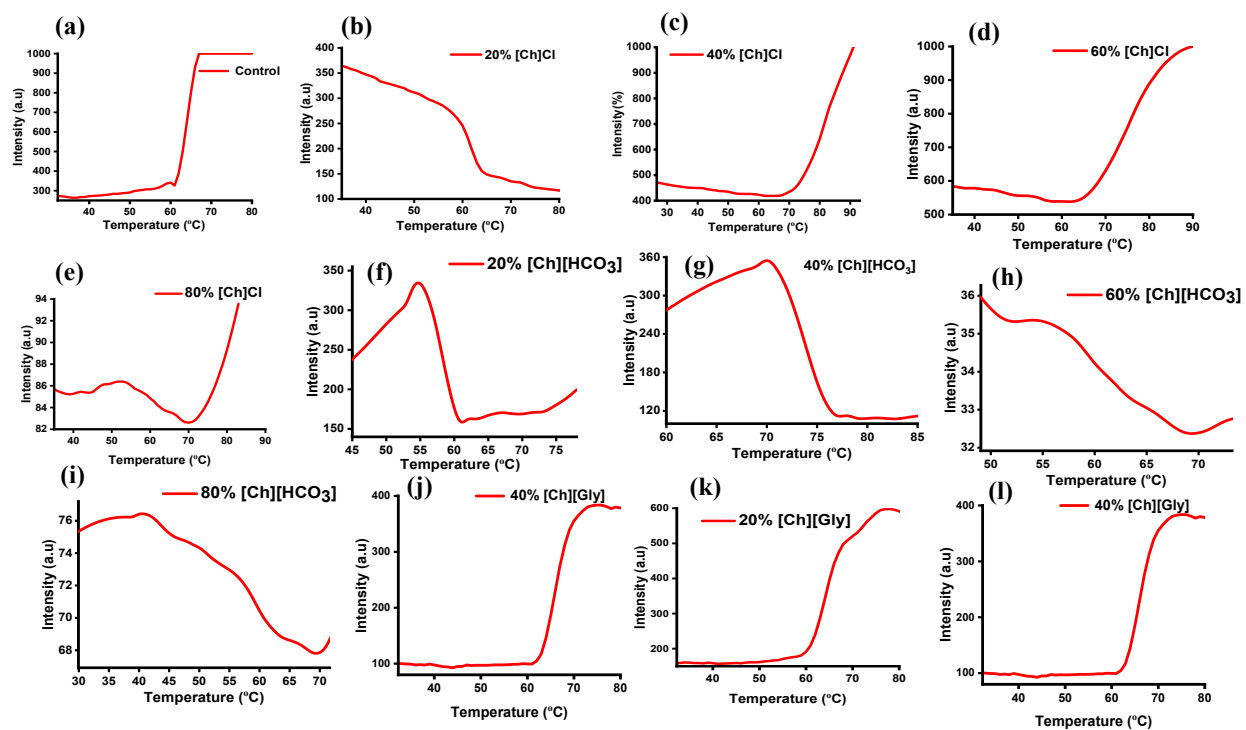


Figure S3. Transition Temperature (T_m) curves of L-ASNase in the presence of (a) PBS (pH=7), (b-e) [Ch]Cl, (f-i) [Ch][HCO₃] (j-l) [Ch][Gly].

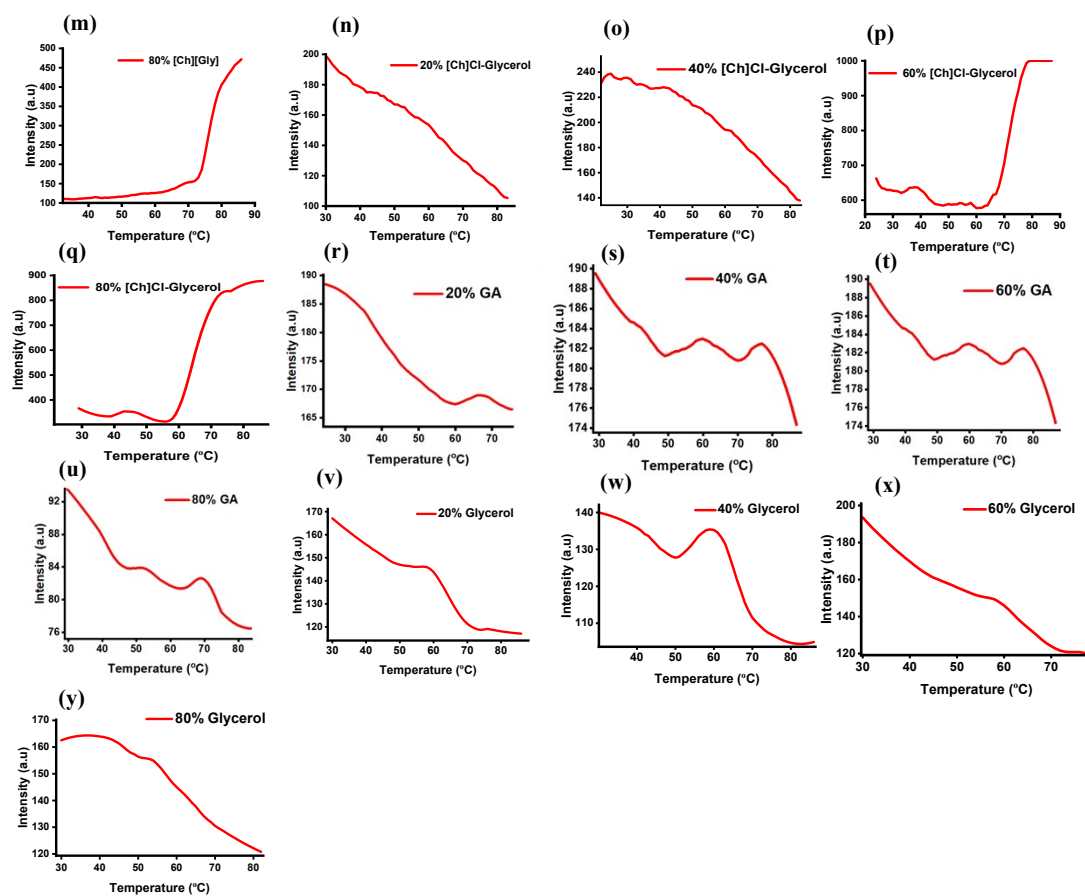


Figure S4. Transition Temperature (T_m) curves of L-ASNase in the presence of (m) [Ch][Gly], (n-q) [Ch]Cl-Glycerol, (r-t) GA, (v-y) Glycerol.

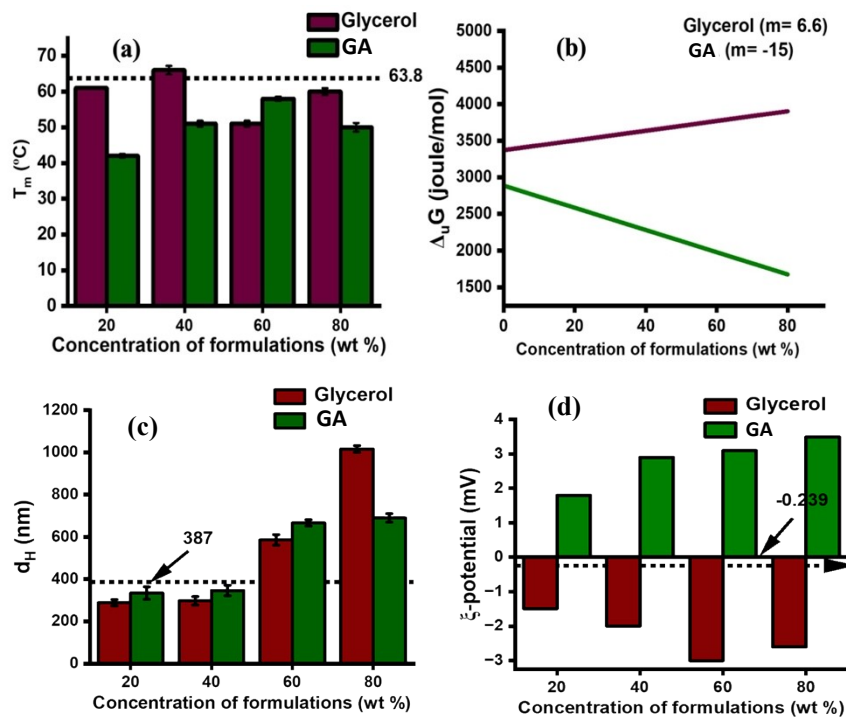


Figure S5. (a) T_m of ASNase as a function of the concentration of Glycerol and GA, (b) $\Delta_u G$ of ASNase as a function of concentration of Glycerol and GA, (c) d_H of ASNase as a function of the concentration of Glycerol and GA, (d) ζ -potential of ASNase as a function of the concentration of Glycerol and GA.

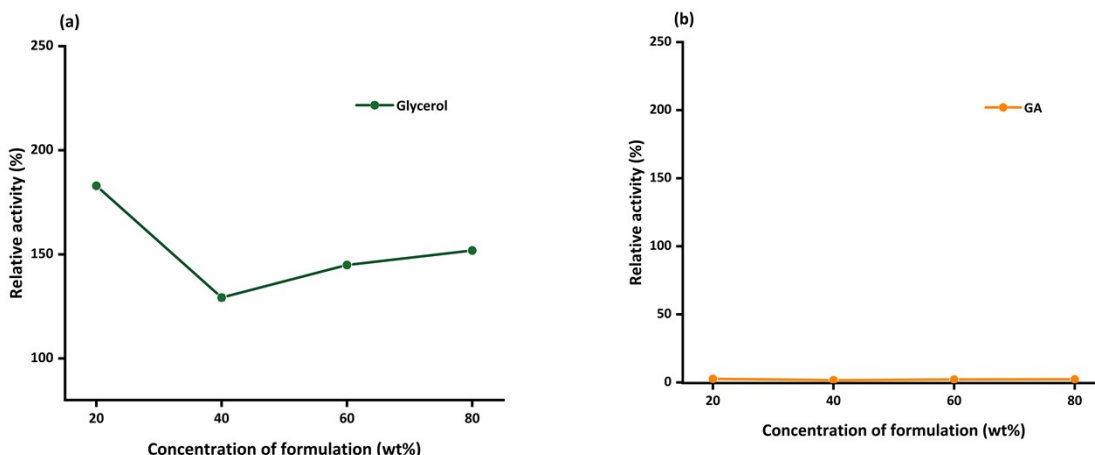


Figure S6. Effect of concentration (%wt.) of (a) Glycerol and (b) GA on the enzymatic activity of the commercial $0.4 \text{ mg}\cdot\text{mL}^{-1}$ of ASNase during 15min. The absolute value of 100% of relative activity corresponds to $149 \pm 2.44 \text{ U}$. The results represent the average of three independent experiments $\pm$ standard deviation errors. Solid lines are only a guide to the eye.

^1H -NMR of $[\text{Ch}][\text{Gly}]$ (400MHz, D_2O , ppm relative to TMS): δ (ppm) 2.95 (s,9H, NCH); 3.27 (m,2H, NCHCHOH); 3.66(s, H,OCHOO); 3.79 (m,2H, NCHCHOH), Figure S1(a). ^1H -NMR of $[\text{Ch}]\text{Cl-Gly}$ (1:1) (400MHz, D_2O , ppm relative to TMS) : δ (ppm) 3.02 (s,9H, NCH); 3.39 (m,2H, NCHCHOH); 3.66 (s, HOCHOO); 3.82 (m,2H, NCHCHOH) Figure S1(b). ^1H -NMR of $[\text{Ch}]\text{Cl-G.A}$ (1:1) (400MHz, D_2O , ppm relative to TMS): δ (ppm) 3.02 (s,9H, NCH); 3.39 (m,2H, NCHCHOH); 3.66(s, H, OCHOO); 3.82 (m,2H, NCHCHOH), Figure S7 (c).

Figure S7 (d) shows the spectrum of components of $[\text{Ch}][\text{Gly}]$ (red); $[\text{Ch}][\text{HCO}_3]$ (yellow) and GA (orange). The $\text{C}=\text{O}$ vibrational stretch of G.A appears around 1550 cm^{-1} , however, the $\text{C}=\text{O}$ vibrational stretch of IL $[\text{Ch}][\text{Gly}]$ was not observed. The characteristic vibrational peak of the quaternary ammonium groups of the cholinium cation appears around 1610 cm^{-1} , which gets shifted to 1570 cm^{-1} in $[\text{Ch}][\text{Gly}]$ IL, which further suggests a strong interaction between the COOH groups of the free fatty acid (GA) and the quaternary ammonium groups of the cations.

Figure S7 (e) provides the FTIR spectrum of [Ch]Cl-Gly (green) and their components [Ch]Cl (red) and Gly (blue). Figure S7 (e) shows that the peak around 3500 cm^{-1} becomes broader comparative to their component peaks which can be due to the presence of extended hydrogen bonding in the case of DES formation.

The FTIR and ^1H NMR of synthesized [Ch]Cl- GA are provided in Figure S7 (c and f). Figure S7 (f) Purple peaks of [Ch]Cl-GA shows the shift of quaternary ammonia of cholinium cation which highlights the complex formation between [Ch]Cl and GA.

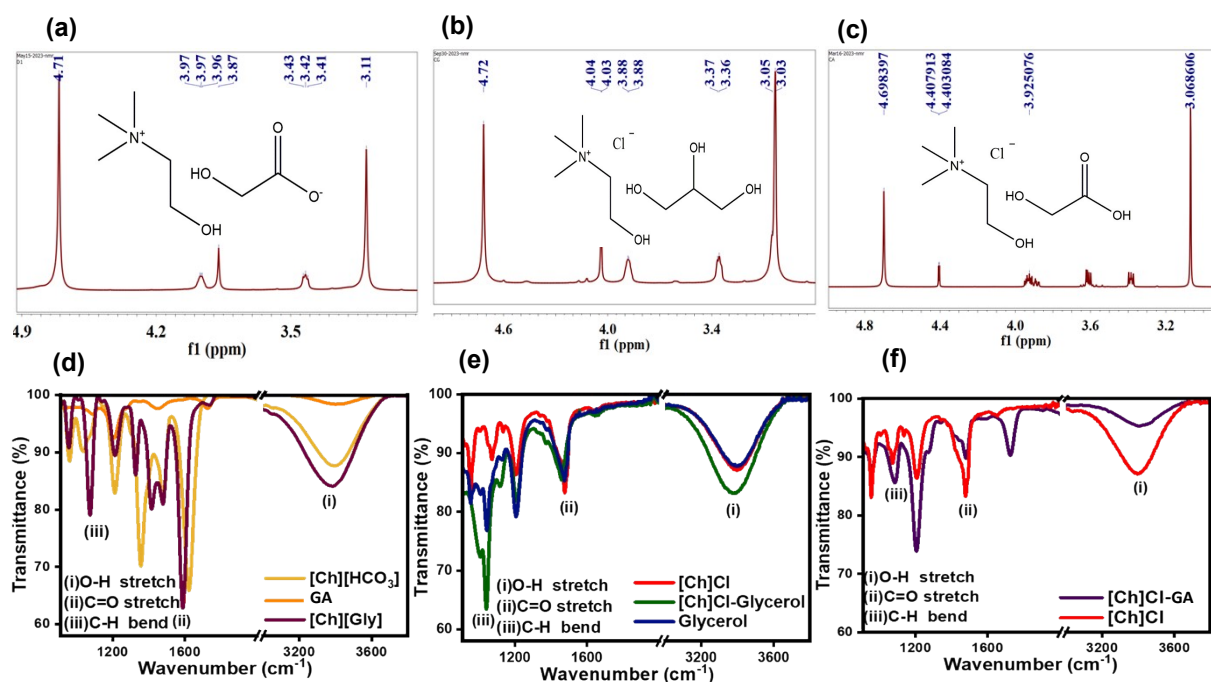


Figure S7. NMR spectra of synthesized formulations: (a) [Ch][Gly], (b) [Ch]Cl-Gly (c) [Ch]Cl-GA, at 400 MHz. FTIR spectra of synthesized formulations and their components (d) [Ch][HCO₃] (yellow), GA (orange) and synthesized IL [Ch][Gly] (maroon) (e) [Ch]Cl (red), synthesized DES; [Ch]Cl-Gly (green color) and glycerol (blue) (f) synthesized DES; [Ch]Cl-GA (purple) and [Ch]Cl (red).

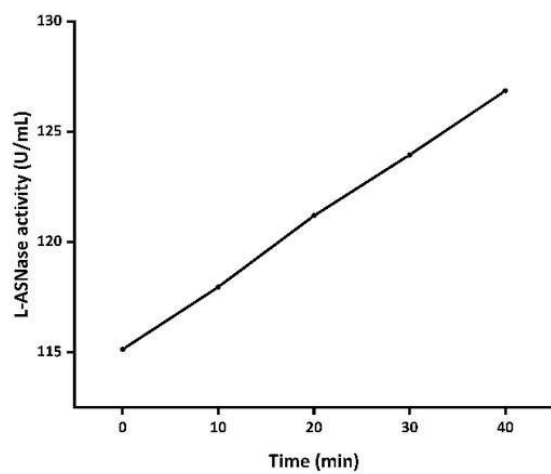


Figure S8. Linearity of the enzymatic reaction incubated at 37 °C over 30 min.

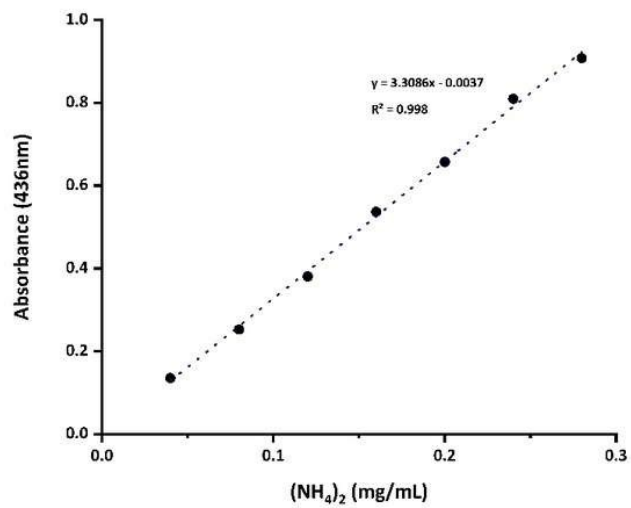


Figure S9. Calibration curve established by reacting Nessler's reagent with ammonium sulfate aqueous solution at various concentrations: 0.04; 0.08; 0.12; 0.16; 0.20; 0.24; 0.28 (mg/mL).

Table S1. Maximum fluorescence emission intensity (I_{max}) and wavelength (λ_{max}) of ASNase in the presence of all cholinium-based formulations.

Concentration of formulation (wt%)	[Ch]Cl		[Ch] [HCO ₃]		[Ch][Gly]		[Ch]Cl-Glycerol		[Ch]Cl-GA		Glycerol		GA	
	λ_{max}	I_{max}	λ_{max}	I_{max}	λ_{max}	I_{max}	λ_{max}	I_{max}	λ_{max}	I_{max}	λ_{max}	I_{max}	λ_{max}	I_{max}
0	319	251	318	245	318	245	317	78	317	246	317	78	318	174
20	319	390	318	196	317	196	317	81	321	176	318	123	315	211
40	319	390	318	186	317	186	315	90	320	191	316	201	318	147
60	318	408	319	160	318	161	316	45	316	187	316	351	314	118
80	318	438	319	116	320	117	318	45	319	213	315	467	313	78

Table S2. Percentage Secondary Character of ASNase in the presence of various cholinium -based formulations

(a) ASNase % character in the presence of [Ch]Cl

[Ch]Cl (wt%)	α -helix (%)	β -pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	38.6	12.2	27.4	21.7
40	31.9	5.5	34.5	28.1
60	47.7	4.2	25.4	22.7
80	30.9	17.5	24.9	26.8

(b) ASNase % character in the presence of [Ch][HCO₃]

[Ch][HCO ₃] (wt%)	α-helix (%)	β- pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	20.0	33.0	13.0	28.2
40	31.6	17.4	20.3	29.4
60	19.3	30.1	21.2	34.4
80	26.6	16.4	17.7	40.1

(c) ASNase % character in the presence of [Ch][Gly]

[Ch][Gly] (wt%)	α-helix (%)	β- pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	42.2	5.0	23.6	40.5
40	26.2	19.3	25.8	31.4
60	23.7	6.7	10.5	67.1
80	16.6	5.4	10.3	65.8

(d) ASNase % character in the presence of [Ch]Cl-Gly

[Ch]Cl-Gly (wt%)	α-helix (%)	β- pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	44	2.5	22.2	31.2
40	44.5	4.1	20.4	31.1
60	42.9	3.6	21.3	32.3
80	56.5	1.3	16.9	25.3

(e) ASNase % character in the presence of [Ch]Cl-GA

[Ch]Cl-GA (wt%)	α-helix (%)	β- pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	26.2	41.4	10.6	10.0
40	4.7	14.8	10.3	62.8
60	6.4	10.7	20.1	60.8
80	17.7	6.5	20.7	55.1

(f) ASNase % character in the presence of Gly

Glycerol (wt%)	α -helix (%)	β -pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	42.9	5.6	23.8	26.7
40	50.1	1.1	23.6	25.0
60	55.9	3.7	24.1	16.2
80	62.3	1.9	18.0	17.9

(g) ASNase % character in the presence of GA

GA (wt%)	α -helix (%)	β -pleated (%)	Random coil (%)	Unordered coil (%)
0	47.1	7.3	22.9	22.7
20	22.6	35.6	22.8	35.3
40	22.7	36.2	22.7	34.2
60	22.5	35.0	22.5	21.0
80				

Table S3. Transition Temperature (T_m) of ASNase in the presence of all the formulations.

Concentration of cholinium - based formulations (wt%)	Transition Temperature (T_m) ($^{\circ}$ C) of ASNase						
	[Ch]Cl	[Ch][HCO ₃]	[Ch][Gly]	[Ch]Cl-Gly	[Ch]Cl-Glycerol	Glycerol	GA
Control	63.8						
20	61.2	58	64.0	58.5	55	61	42
40	82.0	74	67.0	65.0	57	66	51
60	74.0	61	70.1	72.0	60	51	58
80	78.0	55	75.0	66.0	54	60	50

Table S4. $\Delta_u G$ of ASNase in the presence of all the formulations.

Concentration of cholinium - based formulations (wt%)	Change in Gibbs free energy ($\Delta_u G$) (Joule/mol) of ASNase						
	[Ch]Cl	[Ch][HCO ₃]	[Ch][Gly]	[Ch]Cl-Gly	[Ch]Cl-Glycerol	Glycerol	GA
Control	896						
20	900	1508	900	1810	1530	1000	1000
40	1021	2143	1560	2275	2772	1500	700
60	1260	2028	2100	1722	1053	2028	1500
80	2694	2135	1100	2000	504	2500	500

Table S5. d_H of ASNase in the presence of all the formulations.

Concentration of cholinium-based formulations (wt%)	Hydrodynamic size (d_H (nm)) of ASNase						
	[Ch]Cl	[Ch][HCO ₃]	[Ch][Gly]	[Ch]Cl-Glycerol	[Ch]Cl-GA	Glycerol	GA
Control	387.2 ± 20						
20	291.0 ± 10	249.7 ± 10	299.9 ± 15	395 ± 15	460.0 ± 15	288.5 ± 15	334.0 ± 30
40	300 ± 25	330.5 ± 25	473.2 ± 25	247 ± 17	540.0 ± 25	297.5 ± 20	346.8 ± 25
60	857.0 ± 30	692.8 ± 30	666.6 ± 30	594 ± 15	1751.0 ± 20	585.6 ± 25	666.0 ± 15
80	986.0 ± 25	692.1 ± 25	669.0 ± 25	780 ± 18	2184.0 ± 25	1016.1 ± 16	690.0 ± 20

Table S6. x-potential of ASNase in the presence of all the formulations.

Concentration of cholinium-based formulations (wt%)	ξ -potential (mV)						
	[Ch]Cl	[Ch][HCO ₃]	[Ch][Gly]	[Ch]Cl-Glycerol	[Ch]Cl-GA	Glycerol	GA
Control	-0.239						
20	-4.95	-0.61	-6.9	-0.40	1.25	-1.5	1.8
40	-3.7	-6.8	-4.8	-0.11	2.61	-2.0	2.9
60	-0.68	-5.11	-3.6	-0.81	2.91	-3.0	3.1
80	-1.7	-4.3	-3.7	0.45	2.71	-2.6	3.5

Table S8. The surface potential (Vina Score) of ASNase in the presence of various formulation, calculated using CB-DOCK.

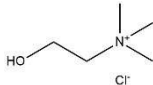
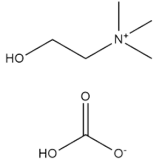
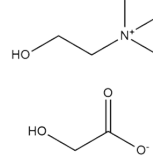
S. No.	Formulation	Vina Score (Kcal/mol)
1	[Ch]Cl	-3.7
2	[Ch][HCO ₃]	-3.7
3	[Ch][Gly]	-3.9
4	Gly	-4.7
5	GA	-4.4

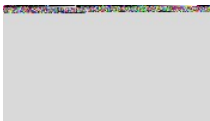
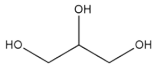
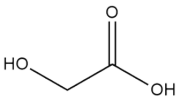
Table S9. Time-dependent studies: d_H of ASNase in the presence of several cholinium-based

	d_H (nm)		
Formulation	Day 1	Day 5	Day 10
Control	340	481	1293
[Ch]Cl	299	266	845
[Ch][HCO ₃]	332	359	385
[Ch][Gly]	415	592	810
[Ch]Cl-Glycerol	384	631	1083
[Ch]Cl-GA	489	602	615
Gly	352	770	1871
GA	575	854	995

formations across different days after storing at 4 °C.

Table S10. Chemical structure, acronym and pH of all cholinium-based formulations.

[Ch]-based IL (acronym)	Chemical structure	pH
Cholinium chloride ([Ch]Cl)		4.9
Cholinium bicarbonate ([Ch][HCO ₃])		8.7
Cholinium glycolate ([Ch][Gly])		5.7
[Ch]-based DES (acronym)	Chemical structure	pH

Cholinium chloride-glycerol ([Ch]Cl-Glycerol)	HBA 	HBD 	5.7
Cholinium chloride-glycolic acid ([Ch]Cl-GA)	HBA 	HBD 	1.6