Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

A novel amalgamation of deep eutectic solvent and crowder as a biocompatible solvent media for enhanced structural and thermal stability of bovine serum albumin

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1. Materials and methods

1.1. Materials.

Bovine serum albumin(BSA), choline chloride (ChCl), urea, glycerol (gly) and ficoll 70 (F70) were purchased from Sigma-Aldrich, U.S.A. All chemicals were of high purity and of analytical grade. Additionally, other crowding agents such as polyethylene glycol (PEG) 12 kDa (P12), PEG 20 kDa (P20), were purchased from Fluka Analyticals. All chemicals were of high purity and of analytical grade.

1.2. Synthesis and characterization of deep eutectic solvent (DES)

1.2.1. Synthesis of DES

Two DESs namely ChCl-urea and ChCl-glycerol were synthesized for the present work by following the protocol delineated by Zhu et al.[1,2] The process involved mixing starting materials - one hydrogen bond acceptor (ChCl) and hydrogen bond donor (urea/glycerol) over a magnetic stirrer at 80 °C until a homogenous, clear solution was obtained. The ChCl and urea/glycerol were mixed in the molar ratio 1:2 respectively. The chemicals structures of these DES are also included in scheme 1.

1.2.2. Characterization of DES by FTIR

The FTIR spectra of synthesized ChCl-urea and ChCl-glycerol are presented in our earlier paper.[3]

1.4. Spectroscopic Measurements. Absorption, fluorescence, far and near UV-CD measurements of BSA in presence and absence of varying concentration of DES and crowders were performed. Absorption spectra were recorded on Shimadzu UV-1800 (Japan) spectrophotometer with the highest resolution (1 nm) using 1 cm path length quartz cuvette. The

steady-state fluorescence experiments conducted on Cary eclipse spectrofluorimeter from Varian optical spectroscopy instruments, Mulgrave, Victoria (Australia). The emission spectra were recorded at a constant room temperature (25 °C) using a Peltier device, while the excitation wavelength was 295 nm with a slit width of 5 nm for both excitation and emission. The CD spectra were recorded on Jasco-185 spectrophotometer (USA) equipped with a Peltier system with an accuracy of ±0.1 °C was employed. Far and near-UV CD spectra were observed in the range 200-250 nm and 250-350 nm, respectively. The response time of 1 s and 1 nm bandwidth was used with a scan speed 50 nm/min. All spectra were averaged of three scans. All the spectra presented here are obtained after subtracting respective blank solutions. The MRE values were calculated according to the following equation

$$MRE = \frac{\theta_{obs}\ (mdegree)}{10\ \times n \times l\ \times C_p}$$

Where, θ is the observed value obtained from the instrument.

n, number of amino acid residues,

l, pathlength of cuvette in cm,

and Cp is the molar concentration of protein.

1.5. Thermal Fluorescence Spectroscopy. Thermal unfolding studies of BSA in the absence and presence of different concentrations of DES and crowders were conducted over a temperature range 15 to 95 °C at 5 °C/min of heating rate using same Cary eclipse spectrofluorimeter. The excitation wavelength was set at 295 nm. All thermal unfolding transitions were analyzed by assuming the two-state unfolding mechanisms.

1.6. Dynamic light scattering (DLS) measurement. The changes in the hydrodynamic diameter (d_H) of BSA under different conditions were executed at 25 °C using Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK) with a light source composed of He-Ne laser (4 mW), operating at a 633 nm wavelength along with emitting vertically polarized light at a 90°scattering angle. The instrument was accompanied with a thermostatic sample chamber. The detection range is known to be from 0.1 nm to 10 μm. All samples were prepared in buffer filtered through 0.2 μm pore size millipore syringe filter. From the time dependent fluctuations in the scattering intensity

of light, the translational diffusion coefficient was measured by using the instrumental software and the value of d_H was attained by means of the Stokes-Einstein equation.

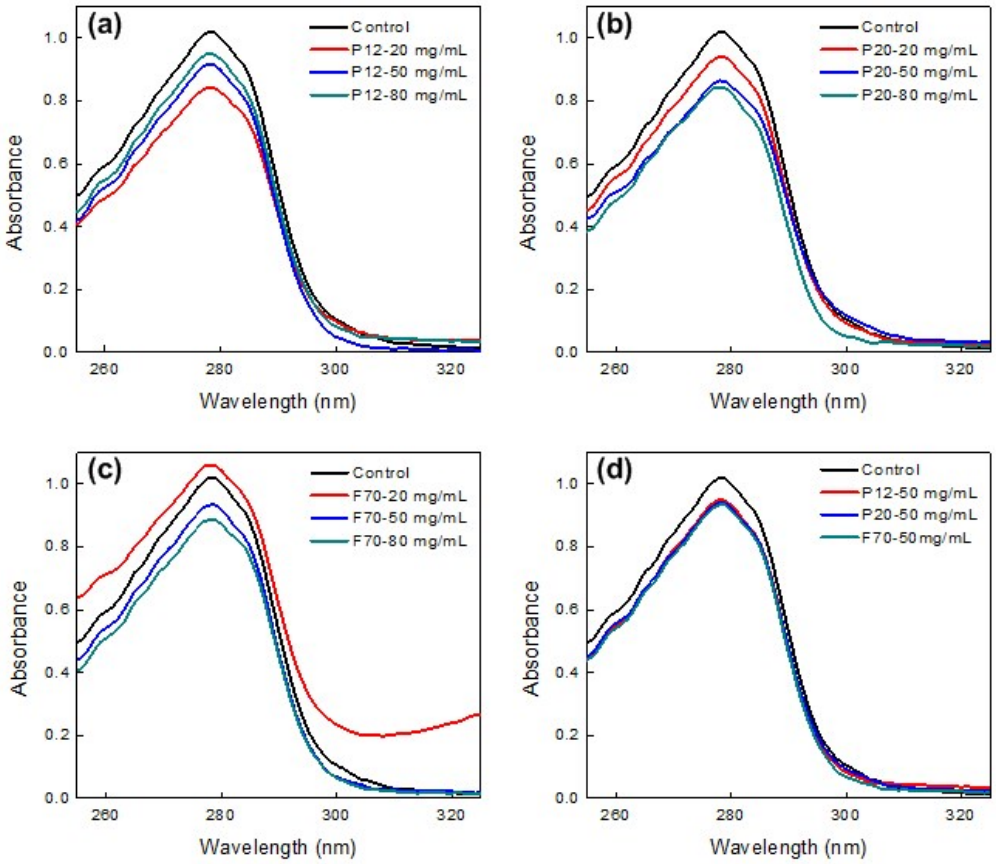

Fig.S1. UV-visible spectra of BSA in absence and presence of (a) P12 (b) P20 (c) F70 and (d) 50 mg/mL concentration of different crowding agents in phosphate buffer of pH 7.Comparing the effect of crowders on protein BSA**.**

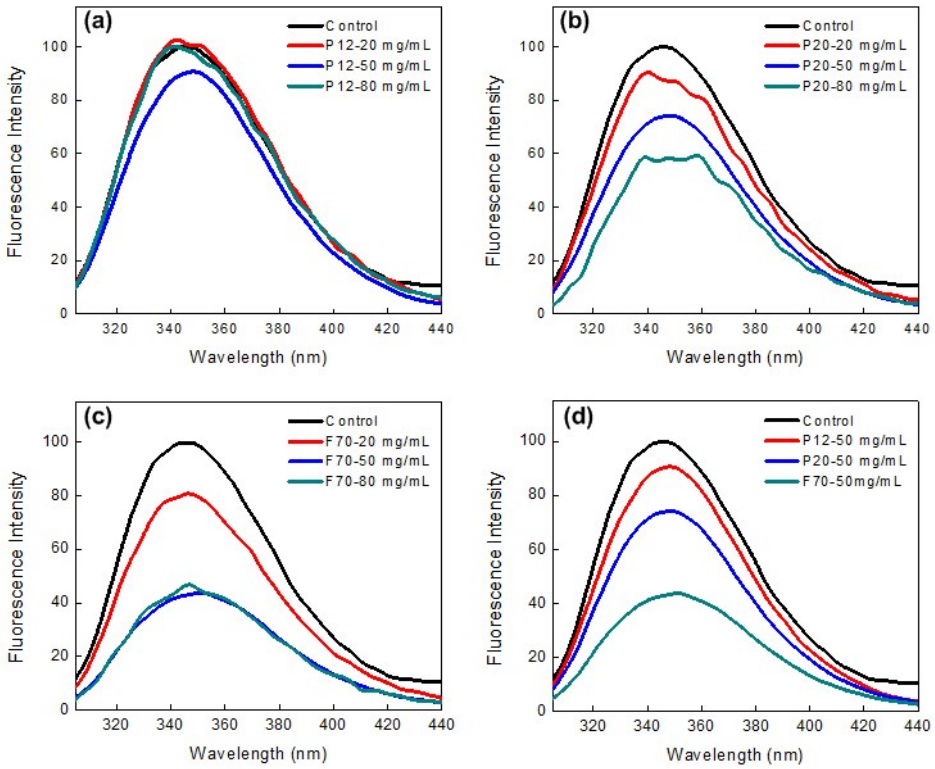

Fig.S2. Emission spectra of BSA in absence and presence of (a) P12 (b) P20 (c) F70 and (d) 50 mg/mL concentration of different crowding agents in phosphate buffer of pH 7.Comparing the effect of crowders on protein BSA**.**

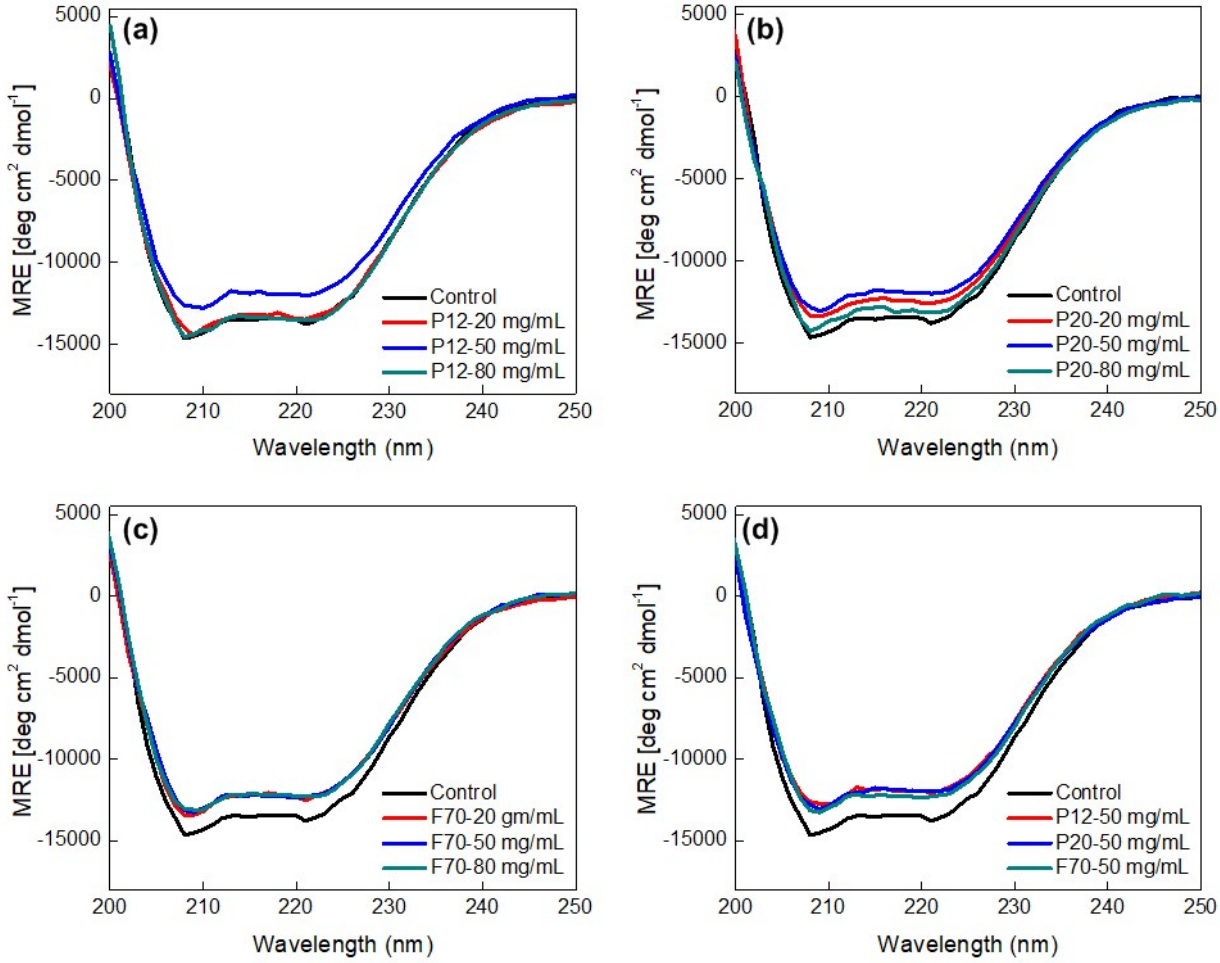

Fig. S3. Far UV-CD spectra of BSA in absence and presence of (a) P12 (b) P20 (c) F70 and (d) 50 mg/mL concentration of different crowding agents in phosphate buffer of pH 7.Comparing the effect of crowders on protein BSA**.**

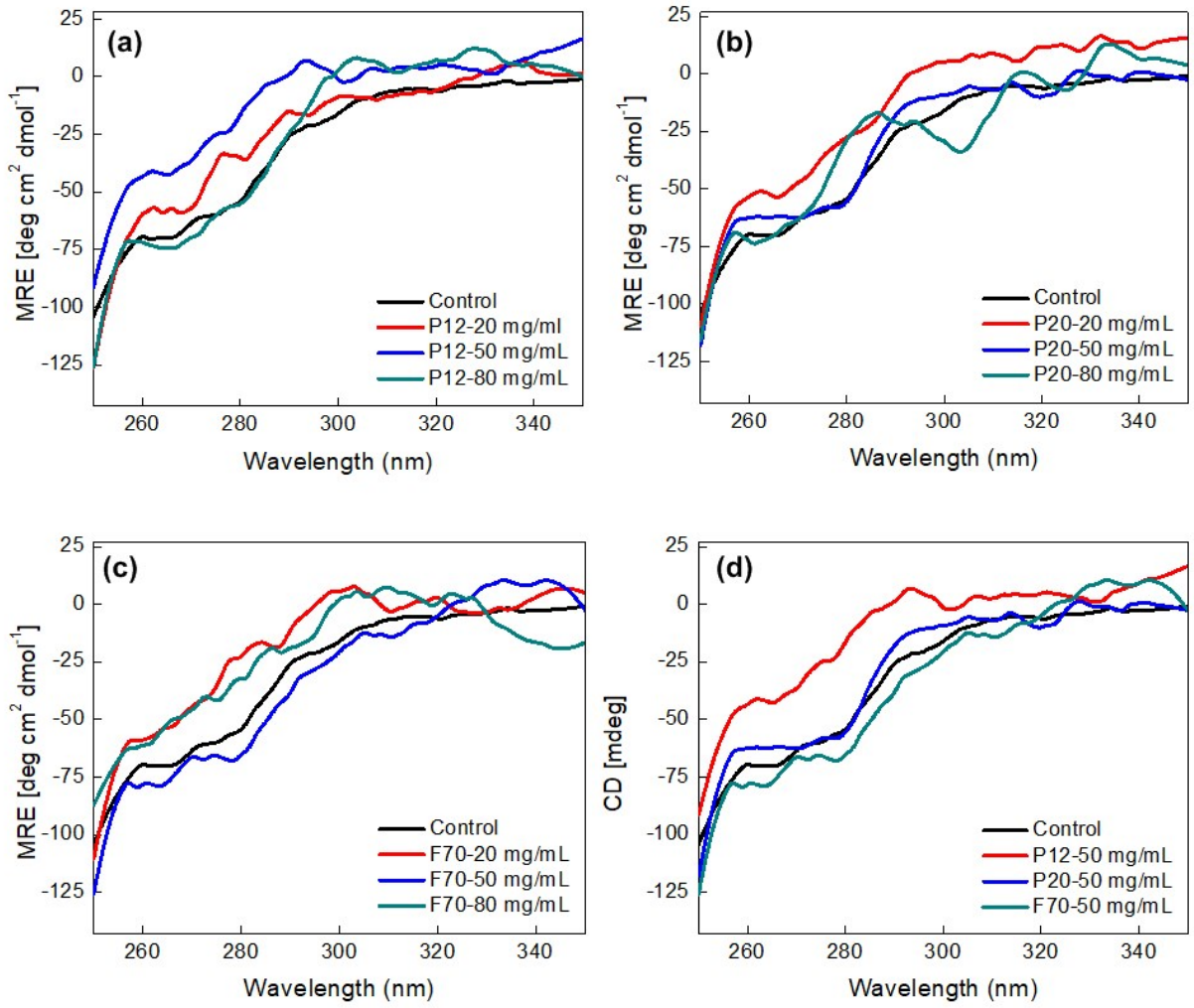

Fig. S4. Near UV-CD spectra of BSA in absence and presence of (a) P12 (b) P20 (c) F70 and (d) 50 mg/mL concentration of different crowding agents in phosphate buffer of pH 7.

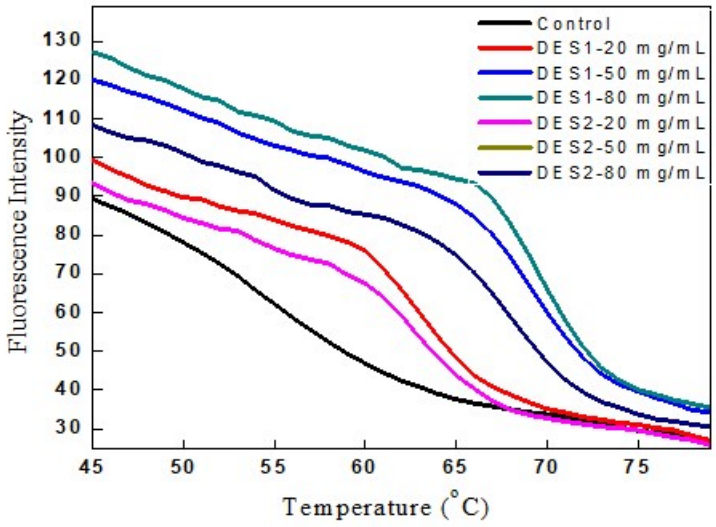

Fig. S5. The thermal transition curve of BSA in absence and presence of varying concentrations of DES1 and DES2 in phosphate buffer of pH 7.

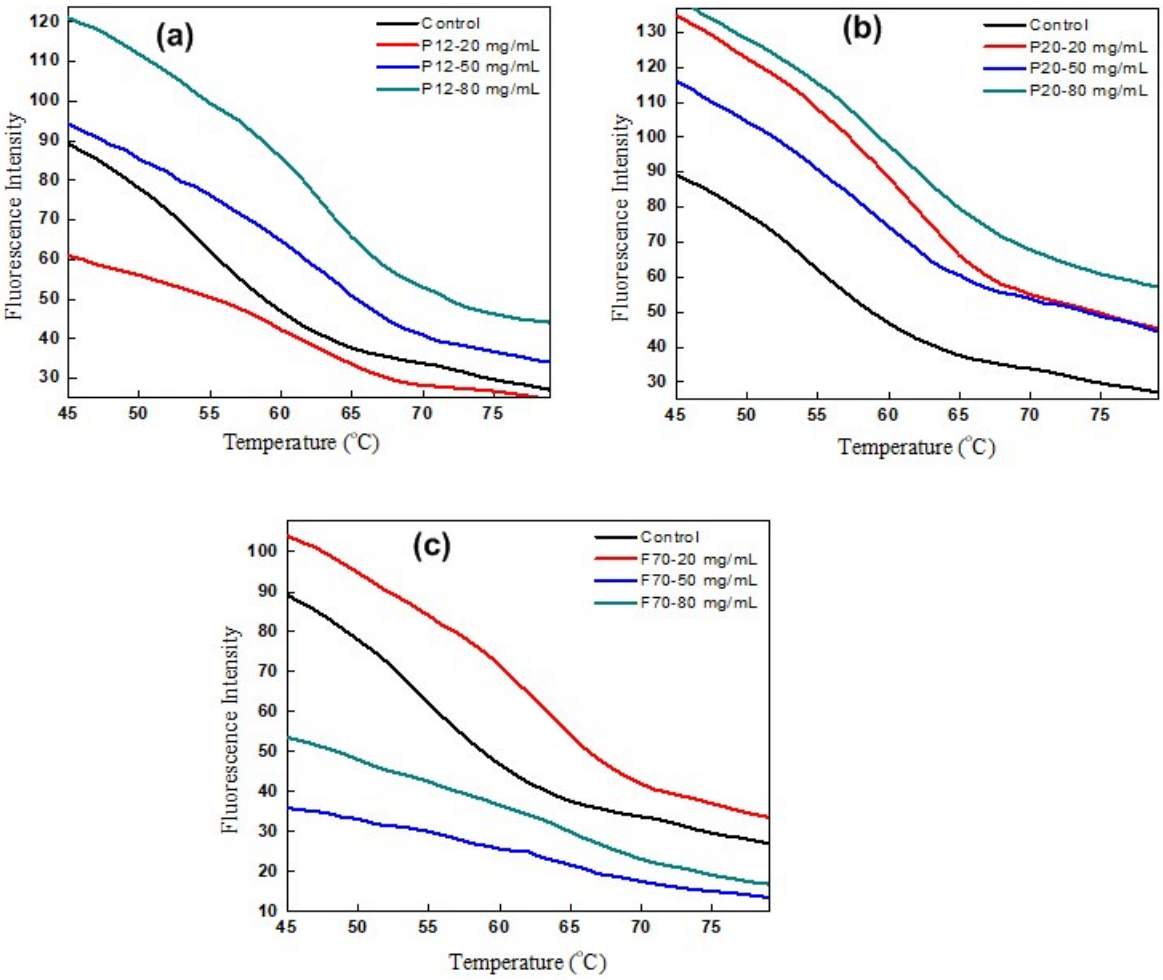

Fig. S6. The thermal transition curve of BSA in absence and presence of varying concentrations of (a) P12, (b) P20 and (c) F70 in phosphate buffer of pH 7.

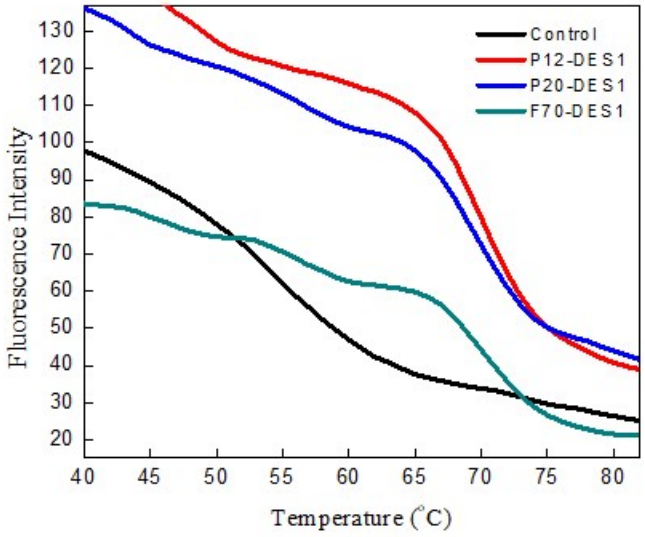

Fig. S7. The thermal transition curve of BSA in absence and presence of mixture of DES1 with different crowders (50 mg/mL each) in phosphate buffer of pH 7.

Table S1. The T_m values of BSA in the presence of varying concentrations of crowders/DES.

S.No.	Sample	Concentration (mg/mL)	T_m (°C)*
1.	Control	Native BSA	55.1
2.	P12	20	59.5
3.		50	56.5
4.		80	60.4
5.	P20	20	59.4
6.		50	56.5
7.		80	61.5
8.	F70	20	59.9
9.		50	60.6
10.		80	51.6
11.	DES1	20	62.1

12.		50	69.4
13.		80	69.8
14.	DES2	20	62.6
15.		50	65.7
16.		80	66.6

Table S2. The d_H value of BSA in the presence of varying concentrations of crowders/DES

S.no.	**Sample**	**Concentration (mg/mL)**	**d_H (nm)**
1.	Control	Native BSA	8.1
2.	P12	20	7.3
3.		50	6.5
4.		80	4.7
5.	P20	20	7.8
6.		50	6.8
7.		80	6.5
8.	F70	20	8.3
9.		50	6.5
10.		80	6.5
11.	DES1	20	7.4
12.		50	7.9
13.		80	8.9
14.	DES2	20	8.3
15.		50	8.2
16.		80	7.3

Table S3. The variation in the T_m value of BSA in the presence of crowded DES medium.

S. no.	Sample	T_m(°C)*
1.	Control	55.1
2.	DES1	69.4
3.	P12	56.5
4.	P12+DES1	70.5
5.	P20	56.5
6.	P20+DES1	70.8
7.	F70	60.6
8.	F70+DES1	68.1

Table S4. The d_H values of BSA in the presence of crowded DES media.

S. no.	Sample	d_H (nm)
1.	Control	8.1
2.	DES1	7.9
3.	P12	6.5
4.	P12+DES1	6.1
5.	P20	6.8
6.	P20+DES1	7.6
7.	F70	6.5
8.	F70+DES1	8.5

2. References

1. P. Zhu, Z. Gu, S. Hong and H. Lian, *Carbohydr. Polym.,* 2017,**177**, 217–223.
2. P. S. Saravana, T. C. Ho, S. J. Chae, Y. J. Cho, J. S. Park, H. J. Lee and B. S. Chun, *Carbohydr. Polym.,*2018,**195**, 622–630.
3. N. Yadav, K. Bhakuni, M. Bisht, I. Bahadur and P. Venkatesu, *ACS Sustain. Chem. Eng.,* 2020, **8**, 10151–10160.