Supplementary Information for the manuscript:

**“Phase equilibria, solvent properties, and protein partitioning in aqueous polyethylene glycol-600-trimethylamine N-oxide and polyethylene glycol-600-choline chloride two-phase systems”**

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**S1. Partitioning experiments**

Stock solutions of all the components of the aqueous two-phase systems were prepared in deionized (DI) water. A mixture of polymers was prepared by dispensing appropriate amounts of the aqueous stock solutions into a 1.2 mL microtube using a Hamilton Company (Reno, NV, USA) ML-4000 four-probe liquid-handling workstation. Appropriate amounts of stock solution of polymer, trimethylamine N-oxide (TMAO) or choline chloride and sodium phosphate buffer (NaPB), pH 7.4, and water were added to give the required composition for the final system (after the sample addition – see below) with total weight of 0.5g.

An automated instrument for performing aqueous two-phase partitioning, the Automated Signature Workstation, ASW (Analiza, Inc., Cleveland, OH, USA), was used for the partitioning experiments. The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company, Reno, NV, USA) integrated with a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT, USA) and a UV-VIS microplate spectrophotometer (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). Solutions of all compounds including proteins were prepared in water at concentrations of 0.5–5 mg/mL depending on the compound solubility. Varied amounts (e.g. 0, 5, 10, 15, 20 and 25 μL) of compound solution and the corresponding amounts (e.g. 25, 20, 15, 10, 5 and 0 μL) of water were added to a set of the same polymer/TMAO or choline chloride/NaPB, pH7.4, mixture. The systems were then vortexed in a Multipulse vortexer and centrifuged (Jouan, BR4i, Thermo Fisher Scientific, Waltham, MA, USA) for 60 min at 3500×g at 23oC to accelerate phase settling. The top phase in each system was removed, the interface discarded, and aliquots from the top and bottom phases were withdrawn in duplicate for analysis.

For the analysis of proteins partitioning, aliquots of 30 µL from both phases were transferred and diluted with water up to 70 µL into microplate wells. Then, the microplate was sealed, shortly centrifuged (2 min at 1500 rpm) and following moderate shaking for 45 min in an incubator at 37ºC, 250 µL of o-phthaldialdehyde reagent was combined. After moderate shaking for 4 min at room temperature, fluorescence was determined using a fluorescence plate reader with a 360 nm excitation filter and a 460 nm emission filter, with a sensitivity setting of 100-125.

For the analysis of the dinitrophenylated amino acids partitioning, aliquots of 60 - 120 µL from both phases were diluted up to 600 µL in 1.2 mL microtubes, water was used as diluent. Following vortexing and a short centrifugation (12 min), aliquots of 250 µL were transferred into microplate wells, and the UV-VIS plate reader was used to measure optical absorbance at wavelengths previously determined to correspond to maximum absorption, 362 nm. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 240–500 nm range. In all measurements the same dilution factor was used for the upper and lower phases and correspondingly diluted pure phases were used as blank solutions.

***S2*. Solvatochromic studies**

The solvatochromic probes 4-nitroanisole, 4-nitrophenol and Reichardt’s carboxylated betaine dye were used to measure the dipolarity/polarizability, π\*, HBA basicity, β, and HBD acidity, α, of the media in the separated phases of ATPS and individual solutions of choline chloride, polyethylene glycol dimethyl ether, and polypropylene glycol prepared in deionized water. Aqueous solutions (ca. 10 mM) of each solvatochromic dye were prepared and 5-15 μL of each was added separately to a total volume of 500 μL of a given phase of ATPS or individual stock solution. A strong base was added to the samples (~5 μL of 1 M NaOH to 500 μL of a given phase) containing Reichardt’s carboxylated betaine dye to ensure a basic pH. A strong acid (~10 μL of 1 M HCl to 500 μL of the phase) was added to the samples containing 4-nitrophenol in order to eliminate charge-transfer bands of the phenolate anion that were observed in some solutions. The respective blanks without dye were prepared separately. The samples were mixed thoroughly in a vortex mixer and the absorption spectra of each solution acquired. To check the reproducibility, possible aggregation and specific interactions effects, the position of the band maximum in each sample was measured in five separate aliquots. A UV-VIS microplate reader spectrophotometer SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) with a bandwidth of 2.0 nm, data interval of 1.0 nm, and high resolution scan (~0.5 nm/s) was used for acquisition of the UV–Vis molecular absorbance data. The absorption spectra of the probes were determined over the spectral range from 240to 600 nm in each phase. Blanks were scanned first to establish a baseline. The wavelength of maximum absorbance in each phase was determined as described by Huddleston et al. [1] using PeakFit software package (Systat Software Inc., San Jose, CA, USA) and averaged. Standard deviation for the measured maximum absorption wavelength was ≤0.4 nm for all dyes in all solutions examined.

The behavior of the probes (4-nitrophenol, and Reichardt’s carboxylated betaine dye) in several solvents (water, n-hexane, methanol) was tested in the presence and absence of HCl (for 4-nitrophenol) and NaOH (for the betaine dye) at different concentrations of the probes, acid or base, and the maximum shifts of the probes were compared to reference values found in the literature and were within the experimental errors in all cases (data not shown).

The results of the solvatochromic studies were used to calculate π\*, β and α as described by Marcus [2].

*Determination of the solvent dipolarity/polarizability π\**.

π\* value was determined from the wave number (*v*1) of the longest wavelength absorption band of the 4-nitroanisole dye using the relationship:

π\* = 0.427(34.12 − *v*1) (1)

*Determination of the solvent hydrogen-bond acceptor basicity β.*

β values were determined from the wave number (*v*2) of the longest wavelength absorption band of the 4-nitrophenol dye using the relationship:

β = 0.346(35.045 − *v*2) − 0.57π\* (2)

*Determination of the solvent hydrogen-bond donor acidity α*

α values were determined from the longest wavelength absorption band of Reichardt’s betaine dye using the relationship:

α = 0.0649E*T*(30) − 2.03 − 0.72π\* (3)

The E*T*(30) values are based on the solvatochromic pyridinium N-phenolate betaine dye (Reichardt’s dye) as probe, and are obtained directly from the wavelength (λ, nm) of the absorption band of the carboxylated form, as .

E*T*(30) = (1/0.932)×[(28591/λ−3.335] (4)

REFERENCES

[1] J.G. Huddleston, H.D. Willauer, R.D. Rogers, The solvatochromic properties, α, β, π\*, of PEG-salt aqueous biphasic systems, Physical Chemistry Chemical Physics, 4 (2002) 4065-4070.

[2] Y. Marcus, The properties of organic liquids that are relevant to their use as solvating solvents, Chemical Society Reviews, 22 (1993) 409-416.