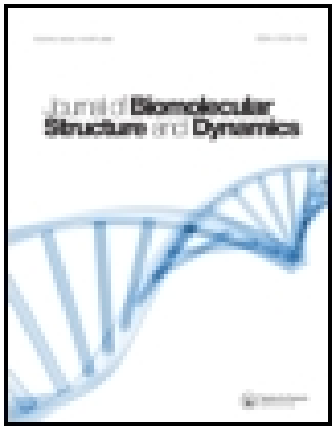

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Luisa A. Ferreira[a], Pedro P. Madeira[b], Leonid Breydo[c], Christian Reichardt[d], Vladimir N. Uversky[befg] & Boris Y. Zaslavsky[a]

[a] Cleveland Diagnostics, 3615 Superior Ave., Suite 4407B, Cleveland, OH 44114, USA

[b] Laboratory of Separation and Reaction Engineering, Department of Chemical Engineering, University of Porto, Dr. Roberto Frias St., 4200 465 Porto, Portugal

[c] Department of Molecular Medicine, Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA

[d] Department of Chemistry, Philipps University, Marburg, Germany

[e] Institute for Biological Instrumentation, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia

[f] Faculty of Science, Biology Department, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, Kingdom of Saudi Arabia

[g] Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia

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Role of solvent properties of aqueous media in macromolecular crowding effects

Luisa A. Ferreira[a], Pedro P. Madeira[b], Leonid Breydo[c], Christian Reichardt[d], Vladimir N. Uversky[b,e,f,g]* and Boris Y. Zaslavsky[a]*

[a]*Cleveland Diagnostics, 3615 Superior Ave., Suite 4407B, Cleveland, OH 44114, USA;* [b]*Laboratory of Separation and Reaction Engineering, Department of Chemical Engineering, University of Porto, Dr. Roberto Frias St., 4200 465 Porto, Portugal;* [c]*Department of Molecular Medicine, Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL 33612, USA;* [d]*Department of Chemistry, Philipps University, Marburg, Germany;* [e]*Institute for Biological Instrumentation, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia;* [f]*Faculty of Science, Biology Department, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, Kingdom of Saudi Arabia;* [g]*Laboratory of Structural Dynamics, Stability and Folding of Proteins, Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia*

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Analysis of the macromolecular crowding effects in polymer solutions show that the excluded volume effect is not the only factor affecting the behavior of biomolecules in a crowded environment. The observed inconsistencies are commonly explained by the so-called soft interactions, such as electrostatic, hydrophobic, and van der Waals interactions, between the crowding agent and the protein, in addition to the hard nonspecific steric interactions. We suggest that the changes in the solvent properties of aqueous media induced by the crowding agents may be the root of these "soft" interactions. To check this hypothesis, the solvatochromic comparison method was used to determine the solvent dipolarity/polarizability, hydrogen-bond donor acidity, and hydrogen-bond acceptor basicity of aqueous solutions of different polymers (dextran, poly(ethylene glycol), Ficoll, Ucon, and polyvinylpyrrolidone) with the polymer concentration up to 40% typically used as crowding agents. Polymer-induced changes in these features were found to be polymer type and concentration specific, and, in case of polyethylene glycol (PEG), molecular mass specific. Similarly sized polymers PEG and Ucon producing different changes in the solvent properties of water in their solutions induced morphologically different α-synuclein aggregates. It is shown that the crowding effects of some polymers on protein refolding and stability reported in the literature can be quantitatively described in terms of the established solvent features of the media in these polymers solutions. These results indicate that the crowding agents do induce changes in solvent properties of aqueous media in crowded environment. Therefore, these changes should be taken into account for crowding effect analysis.

Keywords: macromolecular crowding; solvatochromic comparison; aqueous two-phase system; partition; solvent properties

Introduction

It is generally accepted that protein folding, protein/protein interactions, and other biochemically important processes *in vivo* may differ from those in dilute solutions commonly used in laboratory experiments (Elcock, 2010; Nakano, Miyoshi, & Sugimoto, 2014; Phillip & Schreiber, 2013; Zhou, Rivas, & Minton, 2008). One of the reasons is believed to be the high overall concentrations of biological macromolecules that may occupy up to 40% of the cellular volume (Elcock, 2010; Nakano et al., 2014; Phillip & Schreiber, 2013; Zhou et al., 2008). The term "macromolecular crowding" is used to stress that the influence of high macromolecule concentrations results from the steric interactions of crowding agents with the biomolecules of interest. The crowding molecules are supposed to be inert toward the protein or nucleic acid under study. They physically occupy a significant fraction of the solution volume, leaving only restricted space available to biomolecules, hence the term "excluded volume effect" is often used (Elcock, 2010; Nakano et al., 2014; Phillip & Schreiber, 2013; Zhou et al., 2008).

According to Elcock (see Ref. (Elcock, 2010)),

> there is the question of whether truly inert crowding agents exist that could be used in experiments to provide a direct read out of excluded volume effects only, or whether it is inevitable that all crowding agents will also cause additional effects that must be considered.

The experimental data accumulated and reviewed in the literature show that the excluded volume effect is not the only factor affecting the behavior of biomolecules in a crowded environment (Elcock, 2010; Nakano et al., 2014; Phillip & Schreiber, 2013). In order to explain some experimental observations inconsistent with the excluded volume effect, it was suggested that there are

*Corresponding authors. Email: boris.zaslavsky@cleveland-diagnostics.com (B.Y. Zaslavsky); vuversky@health.usf.edu (V.N. Uversky)

"soft" interactions, such as electrostatic, hydrophobic, and van der Waals interactions between the crowding agent and the protein, in addition to hard nonspecific steric interactions (Nakano et al., 2014; Phillip & Schreiber, 2013). This hypothesis allows one to explain the experimental data by a balance of attractive as well as repulsive crowding agent/protein interactions (Benton, Smith, Young, & Pielak, 2012; Knowles, LaCroix, Deines, Shkel, & Record, 2011; Nakano et al., 2014; Phillip & Schreiber, 2013; Wang, Sarkar, Smith, Krois, & Pielak, 2012). It is generally ignored that there is a third component in all crowded solutions – water, which is known to be important for all the biochemical processes (protein folding, aggregation, protein/protein interactions, etc.) (Ben-Naim, 2003). The commonly used macromolecular crowding agents include dextran, Ficoll, polyethylene glycol (PEG), and polyvinylpyrrolidone (PVP), though proteins, such as albumin or lysozyme, are sometimes used as well. It was reported recently that under crowding conditions, there is an overlapping of hydration shells for the crowding agent implying that water in the solution is affected by the agent (King, Arthur, Brooks, & Kubarych, 2014). This finding agrees with the suggested water restructuring in the presence of low molecular weight osmolytes and in the presence of crowding agents as an important factor in enhancement of protein stability (Canchi & Garcia, 2013; Politi & Harries, 2010; Sukenik, Sapir, Gilman-Politi, & Harries, 2013). There is also a vast literature on the effects of small osmolytes on protein structure and stability where osmolytes effects on protein–water interactions are discussed (see, e.g., in Ref. (Canchi & Garcia, 2013)) but this literature is beyond the scope of the present discussion.

It is known that the dielectric and thermodynamic properties of water in aqueous solutions of polymers, such as dextran, Ficoll, PEG, and PVP, change significantly relative to those in pure water (Arnold, Herrmann, Pratsch, & Gawrisch, 1985; Zaslavsky, 1994). Furthermore, according to the theoretical analysis, confinement of water molecules in a hydration shell around the hydrophobic interface produces a thin layer of water molecules characterized by low correlation, entropy, dielectric constant, and slow reorientation of their intrinsic molecular dipoles (Despa, Fernandez, & Berry, 2004). This hydrophobe-structured water with the hindered rotational motion of water molecules and decreased dielectric constant can enhance the effective forces between charged groups (Despa et al., 2004). Solvent polarity of aqueous media in solutions of dextran, Ficoll, and PEG was shown to change depending upon the polymer type and concentration (Zaslavsky, 1994). Using the solvatochromic comparison method, Kim et al. demonstrated that PEG can also affect the hydrogen-bond donor (HBD) acidity of water (Kim et al., 2002).

It is well known that all the aforementioned synthetic polymers in different combinations may form aqueous two-phase systems (ATPS) (Zaslavsky, 1994). These systems arise in aqueous solutions of two particular polymers, for example, dextran and poly(ethylene glycol) or dextran and Ficoll, above certain concentration thresholds. Two immiscible phases are formed with one phase containing predominantly one polymer, and the other phase containing predominantly the other, while both containing 70–90% water. It is well established that phase separation occurs because of different effects of the two polymers on the water structure (Zaslavsky, 1994). The solvent properties of aqueous media in the two phases are different (Madeira, Reis, Rodrigues, Mikheeva, & Zaslavsky, 2010). These differences are determined primarily by the polymer composition of the phases. The solvent properties of different solvents may be studied by the approach developed by Taft, Kamlet, and others (Kamlet, Abboud, & Taft, 1977; Kamlet & Taft, 1976; Taft & Kamlet, 1976). This approach is based on using a set of solvatochromic dyes with the wavelength positions of their UV–visible absorption maximum shifting depending on different solvent properties. This approach was used to quantify the solvent's dipolarity/polarizability, HBD acidity, and hydrogen-bond acceptor (HBA) basicity in the phases of ATPS (Madeira et al., 2010) as well as in aqueous solutions of PEG of different molecular mass (Kim et al., 2002). It was demonstrated that partitioning of organic compounds and proteins in ATPS can be described and even predicted by a linear combination of different solute/water interactions using solvatochromic solvent features of aqueous media in the phases (Madeira et al., 2010). Therefore, we suggest that the macromolecular crowding effect may be related to the crowding agent influence on the solvent features of aqueous media.

The purpose of this study was to explore how different macromolecular crowding agents affect the solvent properties of aqueous media in their solutions. We explored here the solvent features of aqueous media in solutions of several polymers (dextran, PEG, Ucon, Ficoll, and PVP) of different molecular masses and different concentrations using a set of solvatochromic probes. It was also crucial for the purpose of this study to examine whether the solvatochromic dyes used are able to bind to the polymers studied.

Materials and methods

Materials

Polymers

Dextran-75 (Dex-75; lot 119945), mass-average molecular mass (MW) ~75 kDa was purchased from USB (Cleveland, OH, USA), dextran-40 (Dex-40; lot 1387316 V), MW ~

40 kDa was purchased from Sigma-Aldrich (St. Louis, MO, USA). PEG 10,000 (PEG-10 K; lot 043K2522), MW ~10 kDa; and PEG 4450 (PEG-4.5 K; lot 11608 EB), MW ~4.45 kDa; were purchased from Sigma-Aldrich. PEG 600 (PEG-600; lot 47171728), MW ~600 Da was purchased from EMD (Billerica, MA, USA). Ucon 50-HB-5100 (lot SJ1955S3D2), MW = 3930 Da was purchased from Dow to Chemical (Midland, MI, USA). Ficoll-70 (Ficoll-70; lot 128K1136), MW ~70 kDa and PVP 40 (PVP-40; lot WXBB3898V), MW ~40 kDa were purchased from Sigma-Aldrich. All polymers were used without further purification.

Solvatochromic dyes

The solvatochromic probes 4-nitrophenol (spectrophotometric grade) was purchased from Sigma and 4-nitroanisole (GC > 99%) was supplied by Acros Organic (New Jersey, USA). Reichardt's carboxylated betaine dye sodium {2,6-diphenyl-4-[4-(4-carboxylato-phenyl)-2,6-diphenylpyridinium-1-yl])phenolate} was synthesized according to the procedure reported previously (Reichardt, Harbusch-Görnert, & Schäfer, 1988).

Other chemicals

Recombinant human α-synuclein was expressed in *E. coli* BL21 (DE3) cells and purified as described previously (Yamin, Glaser, Uversky, & Fink, 2003). The purity of protein was confirmed by SDS PAGE and mass spectrometry. All salts and other chemicals used were of analytical-reagent grade. Deionized water was used for preparation of all solutions.

Methods

Salvatochromic studies

The solvatochromic probes 4-nitroanisole, 4-nitrophenol, and Reichardt's carboxylated betaine dye were used to determine the solvent dipolarity/polarizability π^*, HBA basicity (β), and HBD acidity (α) of the media in the polymer solutions.

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 polymer solution. All aqueous polymer solutions were prepared in .01 M sodium phosphate buffer (NaPB), pH 7.4 by weight. NaPB was prepared by mixing appropriate amounts of sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$). A strong base was added to the samples (~5–15 µL of 1 M NaOH to 500 µL of the polymer solution) 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 solution) 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 blank solutions without dye were prepared separately. The samples were mixed thoroughly in a vortex mixer and the absorption spectra of each solution were acquired. To check the reproducibility, possible aggregation and specific interactions effects, the position of the band maximum in each polymer solution was measured in two separate aliquots from each of three separately prepared polymer solutions of a given concentration. UV–vis microplate reader spectrophotometer SpectraMax Plus384 (Molecular Devices, Sunnyvale, CA, USA) with a bandwidth of 2.0 nm, data interval of 1 nm, and high resolution scan (~.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 240 to 600 nm in each polymer solution in .01 M NaPB, pH 7.4. The spectral response from appropriate blank was subtracted before data analysis. The wavelength of maximum absorbance in each solution was determined using the PeakFit software package (Systat Software Inc., San Jose, CA, USA) and averaged. Standard deviation for the measured maximum absorption wavelength was ≤.4 nm for all dyes in all polymer solutions examined.

The behavior of the dyes (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 dyes, and the maximum absorption wavelengths of the dyes were compared to the reference values reported in the literature and were found to be 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 (1993).

Determination of the solvent dipolarity/polarizability π^*

The π^* values were determined from the wavenumber ($\nu_{(1)}$) of the longest-wavelength absorption band of 4-nitroanisole using the relationship:

$$\pi^* = .427(34.12 - \nu_{(1)}) \quad (1)$$

Determination of the solvent HBA basicity β

Each β value was determined from the wavenumber ($\nu_{(2)}$) of the longest-wavelength absorption band of 4-nitrophenol using the relationship:

$$\beta = .346(35.045 - \nu_{(2)}) - .57 \cdot \pi^* \quad (2)$$

Determination of the solvent HBD acidity α

The values of the parameter α (the solvent HBD acidity) were determined from the longest-wavelength absorption band of the Reichardt's carboxylated betaine dye using the relationship:

$$\alpha = .0649 \cdot E_T(30) - 2.03 - .72 \cdot \pi^* \quad (3)$$

The $E_T(30)$ values are based on the solvatochromic pyridinium *N*-phenolate betaine dye (Reichardt's dye) as a probe and are obtained directly from the wavelength (λ, nm) of the absorption band of its carboxyl-substituted derivative as follows:

$$E_T(30) = (1/.932) \times [(28591)/\lambda - 3.335] \quad (4)$$

The determined wavelength used in Equation (4) and the determined wavelength used in Equations (1) and (2) (converted to wavenumber) correspond to the maximum of the longest-wavelength solvatochromic absorption band of each probe in each solution. Standard deviation for the measured maximum absorption wavelength was ≤.4 nm for all dyes in all polymer solutions examined. In order to check the reproducibility, possible aggregation and specific binding effects, the position of the band maximum in each polymer solution was measured in two separate aliquots from each of three separately prepared polymer solutions of a given concentration. Therefore, we believe that the maximum wavelength was determined with high accuracy and precision.

Aqueous two-phase systems

Preparation of ATPS dextran-PEG and Ficoll-Ucon and partitioning of the Reichardt's betaine dye in these systems was performed as previously described (Madeira et al., 2010). The protocols are described in more detail in the Supporting Information.

Protein aggregation

Aggregation of α-synuclein (.5 mg/ml) was conducted in 20 mM Hepes, pH 7.5 in the presence of .1 M NaCl, and .025 mg/ml heparin sulfate. α-synuclein was initially dissolved in 5 mM NaOH at 4 mg/ml, incubated in this solution for 1 min and diluted into the final reaction buffer. Protein aggregation was carried out for four days in a reaction volume of .1 ml in black, flat-bottomed 96-well plates in the presence of 5 μM ThT. Aggregation was analyzed in the absence or presence of various concentrations of PEG 4450 (MW ~ 4.45 kDa) or Ucon 50-HB-5100 (MW 3930 Da). Polymer concentrations used in this study were 5 or 15% for PEG and 2 or 15% for Ucon. Two Teflon or polyethylene balls (2.38 mm diameter, Engineering Laboratories, Oakland, NJ) were placed into each well of a 96-well plate. The reaction mixture containing protein and ThT (320 μl) was split into three wells (100 μl into each well), the plates were covered by Mylar septum sheets (Thermo), and incubated with continuous orbital shaking at 280 rpm in an Infinite M200 Pro microplate reader (Tecan). The kinetics was monitored by top reading of fluorescence intensity every 6 min using 444 nm excitation and 485 nm emission filters (data not shown).

Electron microscopy

5 μl aliquots of protein solutions were adsorbed onto prewashed 200 mesh formvar-/carbon-coated nickel grids for 5 min. The grids were washed with water (20 μl), stained with 2% uranyl acetate for 2 min, and washed with water again. The samples were analyzed with a JEM 1400 transmission electron microscope (JEOL) operated at 80 kV.

Results and discussion

Polymer interactions with the solvatochromic dyes

There are numerous methods and tools to examine the existing interactions between different compounds in solution; however, it is close to impossible to prove experimentally the lack of such interactions. No matter what experimental technique is employed, it is always possible that the sensitivity of the technique is insufficient. The empirical Collander relationship applied to a given substance in ATPS formed by various pairs of polymers was previously suggested as a reliable test for lack of solute/polymer interactions in ATPS (Madeira, Teixeira, Macedo, Mikheeva, & Zaslavsky, 2008).

The so-called Collander linear solvent regression equation describes an empirical relationship between distribution coefficients of solutes in different organic solvent/water two-phase systems as (Hansch & Leo, 1995):

$$\log D_i^j = a_{jo} \cdot \log D_i^o + b_{jo} \quad (5)$$

where D_i is the distribution coefficient of *i*-th solute in the two-phase system "*j*" or "*o*," and a_{jo} and b_{jo} are constants for the given type of solutes of *same chemical nature* (Hansch & Leo, 1995). Both coefficients and the b_{jo} value in particular depend on the chemical nature of the compounds being partitioned. This dependence results from differences between specific solute/solvent interactions for various compounds in different organic solvents under comparison (Hansch & Leo, 1995; Zaslavsky, 1994).

It was established previously that the partition coefficients of solutes in ATPS formed by different pairs of nonionic polymers are typically interrelated according to Equation (5) (Madeira et al., 2008, 2013; Zaslavsky, 1994). Both coefficients a_{jo} and b_{jo} are constant and

independent of the nature of the solute being partitioned (from simple organic compounds to proteins and nucleic acids). This finding implies two possible explanations (Madeira et al., 2008, 2013; Zaslavsky, 1994): (1) All the compounds, independent of their chemical nature, bind to the phase-forming polymers in a similar manner, which is extremely unlikely or (2) The solutes being partitioned do not specifically bind to the polymers but are differently affected by aqueous solvent media in two phases due to the differently changed properties of the solvent media in these phases. This explanation agrees with the measurements of various solvent characteristics in the phases of ATPS and serves as a basis for different analytical applications of aqueous two-phase partitioning (Madeira et al., 2010, 2013; Zaslavsky, 1994).

Analysis of the partition coefficients for the solvatochromic dyes presented in Table S1 shows that they all fit the Collander relationship reported previously (Madeira et al., 2008; Zaslavsky, 1994). The data from Table S1 are plotted in Figure 1 for ATPS formed by different pairs of polymers, dextran-PEG, and Ficoll-Ucon. The linear relationship in Figure 1 can be described as follows:

$$\log K_j^{Ficoll-Ucon} = .0_{(\pm.02)} + 1.19_{(\pm.035)} \cdot \log K_j^{Dextran-PEG} \quad (5a)$$

$N = 25$; $r^2 = .9806$; SD = .10; $F = 1163$

where $K_j^{\text{Ficoll-Ucon}}$ and $K_j^{\text{Dextran-PEG}}$ are the partition coefficients for the j-th compound in the dextran-PEG and Ficoll-Ucon ATPS; N is the number of compounds examined (10 proteins, 8 free and dinitrophenylated amino acids, 7 organic compounds, including the solvatochromic dyes used here); r is the correlation coefficient; SD is the standard deviation; and F is the ratio of variance.

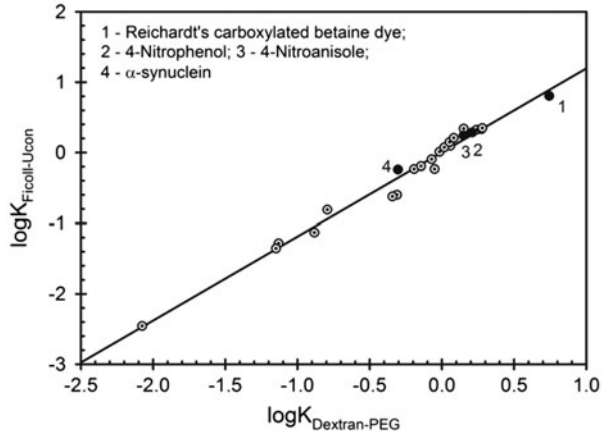

Figure 1. Partition coefficients K, experimentally measured for all the compounds in dextran-PEG, plotted against K-values for the same compounds in Ficoll-Ucon ATPS (see in Table S1).

This experimental observation confirms that the dyes employed here do not interact with the polymers and can be used as solvatochromic probes for characterization of solvent features of aqueous media in the polymer solutions. This analysis also revealed that the recombinant human α-synuclein used in our study to look on the effects of PEG and Ucon on protein aggregation does not bind to these polymers too.

It was shown previously that the relationship described by Equation (5) exists for ATPS formed by similar polymers of different molecular masses and those of different polymer concentrations for a given pair of polymers (Madeira et al., 2008, 2013; Zaslavsky, 1994). Hence, the established Collander relationship (Equation (5a)), indicating the lack of solvatochromic dye/polymer interactions for ATPS formed by dextran, PEG, Ucon, and Ficoll of the particular molecular masses, may be extended over all ATPS formed by similar polymers of different molecular masses and at different polymer concentrations. Therefore, we conclude that the solvatochromic dyes under discussion can be used for the analysis of solvent properties of aqueous media in solutions of polymers used as crowding agents.

Solvent properties of aqueous media in solutions of crowding agents

It should be mentioned that the original Kamlet-Taft methodology requires the use of several different solvatochromic dyes in order to compensate for idiosyncratic results obtained with a single dye by averaging the values obtained with the different dyes used. This issue is discussed in detail by Ab Rani et al. (2011). The set of the dyes used here was previously used for analysis of solvent properties of media in coexisting phases of ATPS, and it was demonstrated that the data obtained allow one to predict the partition behavior of simple organic compounds and proteins in ATPS (Madeira et al., 2008, 2013; Zaslavsky, 1994). An additional equally important factor affecting solute partitioning in ATPS was found to be the electrostatic properties of the aqueous media in the coexisting phases. Unfortunately, these properties cannot be quantified by solvatochromic dyes. Therefore, the description of the polymer-induced changes in the solvent properties of aqueous media is admittedly incomplete. On the other hand, it was demonstrated that the difference between the relative hydrophobic character of the coexisting phases in ATPS may be quantified in terms of the parameters derived from the use of the solvatochromic dyes (π^*, β, and α) (Madeira et al., 2012).

It should be kept in mind that the results obtained are to be viewed as relative estimates of the solvent

properties in the solutions under study. The results obtained are listed in Supplementary Materials (Tables S2–S4) and illustrated graphically in Figures 2–4. The data presented in Table S2 indicate that the solvent dipolarity/polarizability (π^*) of aqueous media increases with the polymer concentration for all polymers examined except Ucon. The polymer effect on the solvent dipolarity/polarizability (π^*) characterizing the interactions of aqueous media with solute dipoles and induced dipoles decreases in the following sequence: PVP-40 > dextran-40 = dextran-75 = Ficoll-70 > PEG-10 K = PEG-4.5 K > PEG-600 > Ucon, at both concentrations of 30 and 40%.

The data presented in Table S3 and illustrated in Figure 3 indicate that the solvent HBA basicity (β) of aqueous media increases with the polymer concentration for all polymers examined. The polymer effect decreases in the sequence: PVP-40 = Ucon > PEG-10 K = PEG-4.5 K > PEG-600 > Ficoll-70 > dextran-40 > dextran-75, at both concentrations of 30 and 40%. It should be noted that this sequence is different from the one found for the polymer effect on the solvent dipolarity/polarizability.

The data presented in Table S4 and illustrated in Figure 4 show that the solvent HBD acidity (α) of aqueous media decreases with the polymer concentration for all polymers examined. The polymer effect decreases in the sequence: Ucon > PEG-4.5 K ≥ PEG-10 K > PEG-600 > PVP-40 > Ficoll-70 > dextran-75 = dextran-40, at both concentrations of 30 and 40%. It should be noted that the sequence is almost similar to the one determined for the polymer effect on the solvent HBA basicity (β) with only difference in the PVP position.

In order to analyze the possible role of the established influence of polymers on the solvent properties of aqueous media in the crowding effects of these polymers, it is important to consider how significant

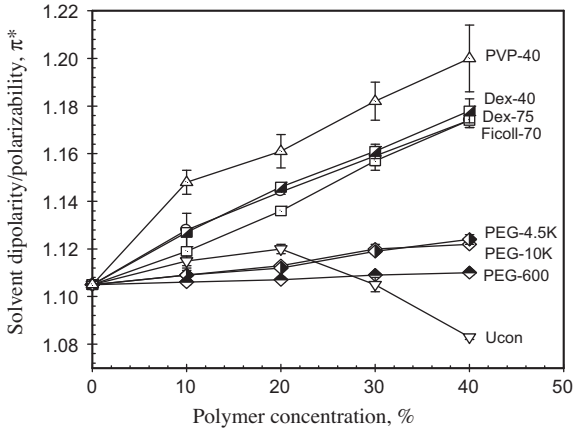

Figure 2. Solvent dipolarity/polarizability (π^*) of aqueous media as a function of polymer concentration in solutions of different polymers (lines are added for eye-guidance only).

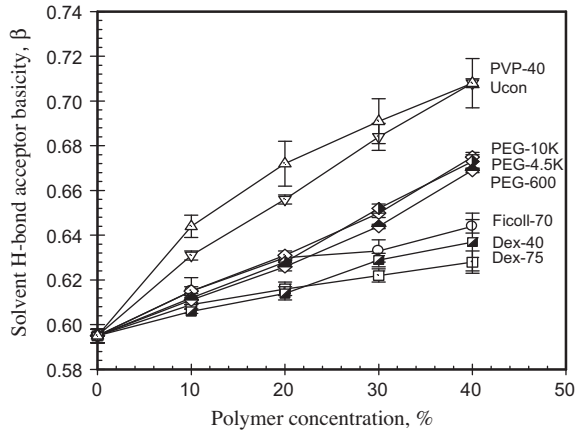

Figure 3. Solvent HBA basicity (β) of aqueous media as a function of polymer concentration in solutions of different polymer (lines are added for eye-guidance only).

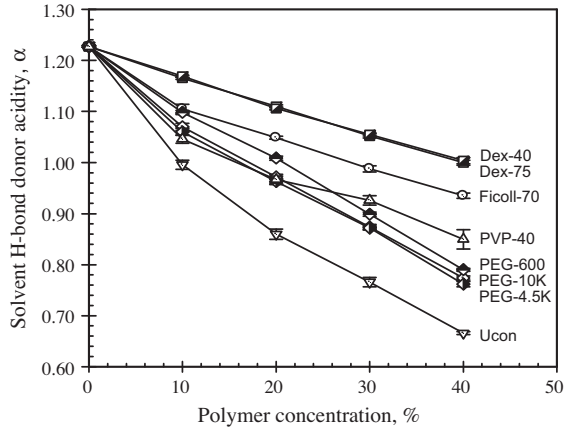

Figure 4. Solvent HBD acidity (α) of aqueous media as a function of polymer concentration in solutions of different polymers (lines are added for eye-guidance only).

these effects are. The dipolarity/polarizability parameter, π^*, of aqueous media in 40% Ucon solution (see Table S2) differs from that of the polymer-free media by .022. The difference between the π^* values for such organic solvents as methanol and ethanol is .06 (Kamlet, Abboud, Abraham, & Taft, 1983); that is, three times larger. In the aqueous two-phase system formed by dextran-75 and Ficoll-70 (Madeira et al., 2008, 2013; Zaslavsky, 1994), however, the difference between the π^* values for the coexisting phases amounts only to .003, and this difference affects the distribution of small compounds and proteins between the two phases.

Similarly, the polymer influence on the HBA basicity β of aqueous media is rather small. The difference between the β values for aqueous media in 40% dextran-75 solution and for polymer-free media is .033. Similar

difference for methanol and ethanol is .15 (Kamlet et al., 1983). On the other hand, the difference between the HBA basicity β for aqueous media in the coexisting phases of dextran-75-PEG-600 is just .005 (Madeira et al., 2008, 2013; Zaslavsky, 1994).

The polymer influence on the HBD acidity α of aqueous media is quite significant. The difference between the α values for aqueous media in 40% dextran-40 solution and for polymer-free media is .223, much larger than the difference between the α values for methanol and ethanol of just .10 (Kamlet et al., 1983). In polymer/polymer ATPS, the differences between the HBD acidity α of the aqueous media in the coexisting phases varies from 0 in Ficoll-70-PEG-6000 to .181 in dextran-75-Ucon ATPS, depending on the polymer and salt composition (Madeira et al., 2008, 2013; Zaslavsky, 1994).

It follows from the experimental data obtained here that nonionic polymers used as macromolecular crowding agents change the solvent properties of water in their aqueous solutions. If the data obtained in the studies of polymer/protein interactions are considered with regard to the aforementioned data, it becomes clear that the conclusions about polymer/protein interactions (Phillip & Schreiber, 2013) are commonly based on the deviation of the experimental data from one or the other model chosen by the respective authors. As an example, heats of mixing lysozyme or ovalbumin solutions with those of PEG were measured calorimetrically (Pico, Bassani, Farruggia, & Nerli, 2007). Heats of corresponding dilutions of these solutions were measured separately, and it was found that the sum of heats of dilutions was not equal to the heat of mixing. The difference observed was interpreted as evidence of protein/PEG interactions, though it may readily be explained by protein transfer from water to aqueous media with PEG-induced changes in the solvent properties (Pico et al., 2007). Similarly, the conclusion about PEG/lysozyme interactions was made by Bloustine, Virmani, Thurston, and Fraden (2006), based on deviation of the light scattering data from the water depletion model, while the same data may be explained by the effect of PEG-induced changes in the solvent properties of water. Same explanation may be applicable to the other data reported (Crowley, Brett, & Muldoon, 2008; Kulkarni, Chatterjee, Schweizer, & Zukoski, 2000). The studies of partition behavior of numerous different proteins in different polymer/polymer ATPS do not indicate protein/polymer interactions, though this possibility cannot be excluded for any particular protein.

It should be mentioned that the possible involvement of aqueous media in the crowding effects was discussed in the literature (Canchi & Garcia, 2013; Harada, Sugita, & Feig, 2012; Nakano et al., 2014; Politi & Harries, 2010; Sukenik et al., 2013). In order to test whether the changes of the solvent properties of aqueous media are relevant for macromolecular crowding effects, we analyzed the results of the studies where the numerical experimental data were reported.

The oxidative refolding of reduced, denatured hen egg white lysozyme was examined in the presence of bovine albumin, dextran-70 and Ficoll-70 (Zhou, Liang, Du, Zhou, & Chen, 2004). The refolding yield of lysozyme reported was examined in terms of solvent properties of aqueous media in dextran-75 and Ficoll-70 solutions (see Tables S2–S4) (Zhou et al., 2004). It has been shown previously (Madeira et al., 2010, 2012, 2014) that different properties of solutes in aqueous solutions may be expressed as linear combination of different solvent properties of the aqueous media. Therefore, we attempted to use the similar expression for protein folding/refolding in the presence of crowding agents. To this end, the data were fit with the simplest linear model based on previously reported linear relationships between a variety of solvation related parameters for various proteins and small organic compounds and the dipolarity, donor acidity, and acceptor basicity of aqueous media. The observed relationship shown graphically in Figure 5 can be described as follows:

$$\text{Yield}\,(\%) = 4200_{(\pm 902)} - 3200_{(\pm 653)} \cdot \pi^* - 440_{(\pm 152)} \cdot \alpha \tag{6}$$

$$N = 4;\ r^2 = .9849;\ \text{SD} = 4.2;\ F = 32.7$$

where yield is as indicated above; π^* and α are the solvent dipolarity/polarizability and solvent HBD acidity in aqueous polymer solution, respectively; N is the number

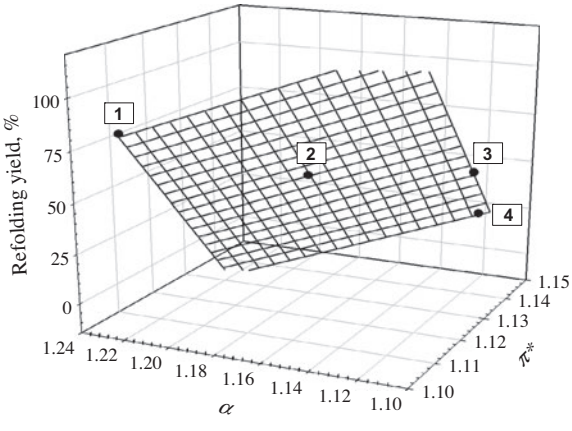

Figure 5. The refolding yield of denatured reduced lysozyme reported in Ref. (Zhou et al., 2004) as a function of the solvent dipolarity/polarizability (π^*) and HBD acidity (α) of aqueous media in the absence and presence of dextran and Ficoll. (1) Absence of polymer, (2) 10% dextran, (3) 10% Ficoll, and (4) 20% dextran.

of experimental data; all the other parameters are as defined above. The number of the experimental data is extremely small (the yields reported in Ref. (Zhou et al., 2004) in the presence of 10 and 20% dextran, 10% Ficoll, and in polymer-free solution were used), and hence the relationship (Equation (6)) cannot be viewed as sufficiently reliable. However, the fact that it exists at all, even though the ionic composition of the refolding media used differs from that used in our solvatochromic measurements, is of interest (Zhou et al., 2004).

Similarly, the yield of refolded rabbit muscle creatine kinase can be described in terms of solvent properties for dextran-70 and Ficoll-70 (see Figure 6) (Du et al., 2006), but not for PEG-2000 effect which may be assigned to different volume-excluded effects of PEG-2000 and those of dextran and Ficoll of the same molecular mass. It was established that the effects of PEG-600 and PEG-4500 on the solvent properties of aqueous media are different. Since the PEG-2000 effects were not examined, we could not include the PEG-2000 crowding effect data in our analysis. The relationship shown in Figure 6 can be described as follows:

$$\text{Yield}\,(\%) = 4000_{(\pm 1339)} - 2800_{(\pm 974)}\pi* - 600_{\pm 218}\alpha \quad (7)$$

$N = 5$; $r^2 = .8104$; SD = 6.3; $F = 4.2$

where yield is the yield of refolded rabbit muscle creatine kinase; all the other parameters are as indicated before. The yields reported in Ref. (Du et al., 2006) in the presence of 10 and 20% dextran, 10 and 20% Ficoll, and in the polymer-free solution were used. It should be noted that the protein refolding was analyzed in .05 M aqueous Tris–HCl, pH 7.5 (Du et al., 2006), and the ionic composition of the polymer solutions was already shown to affect the solvent properties of aqueous media (Miklos, Sarkar, Wang, & Pielak, 2011). Therefore, the observed relationship should be only viewed as a trend and not as a reliable correlation.

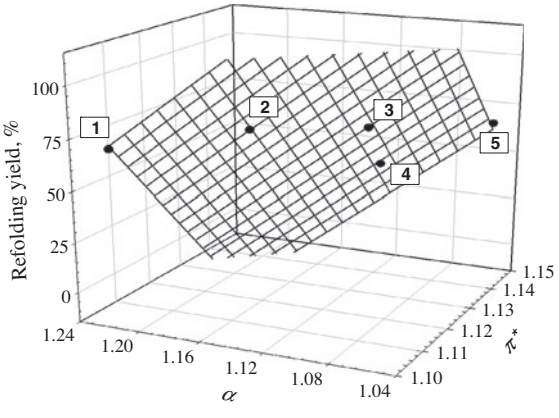

Figure 6. The refolding yield of denatured rabbit muscle creatine kinase as a function of the solvent dipolarity/polarizability (π^*) and HBD acidity (α) of aqueous media in the absence and presence of solutions of dextran and Ficoll (Du et al., 2006). (1) Absence of polymer, (2) 10% dextran, (3) 10% Ficoll, (4) 20% dextran, and (5) 20% Ficoll.

The relationships described by Equations (6) and (7) do not provide unambiguous experimental evidence, but they clearly support the assumption that polymer-induced changes in the solvent properties of aqueous media may play an important role in macromolecular crowding effects. We suggest that the solvent properties measured here by solvatochromic dyes represent one aspect of the structure of water in the solutions of crowding agents. The data accumulated so far do not allow one to answer the most important question – if the macromolecular crowding effect is the effect of agent-induced changes on the properties of aqueous media or a combination of size-exclusion effect together with the solvent restructuring effects. The issue is complicated not only by our current limited views of the water structure but also by the essentially complete lack of knowledge of relationship between specific properties of biological macromolecules and the solvent properties of aqueous media. At this time, we may suggest the combination of the two effects results in the experimentally observed changes in biomolecule behavior in crowded solutions. The relative importance of the two types of the effects may be specific for the protein or nucleic acid under analysis.

Protein aggregation is very sensitive to environmental conditions. It was suggested that high concentrations of inert polymers, that are used to mimic macromolecular crowding in *in vitro* experiments, may have a large influence on the behavior of biological macromolecules (Bismuto et al., 2002; Eggers & Valentine, 2001a, 2001b; Minton, 2000b), affecting protein–protein interactions in general (Martin et al., 2014; Minton, 2000a; Morar, Olteanu, Young, & Pielak, 2001) and could modulate both the rate and the extent of amyloid formation *in vivo* (Lansbury, 1999; Minton, 2000a). Accelerated *in vitro* aggregation and fibrillation in the presence of crowding agents have been reported for human apolipoprotein C-II (Hatters, Minton, & Howlett, 2002), α-synuclein (Breydo et al., 2014; Munishkina, Ahmad, Fink, & Uversky, 2008; Munishkina, Cooper, Uversky, & Fink, 2004; Munishkina, Fink, & Uversky, 2008; Shtilerman, Ding, & Lansbury, 2002; Uversky, Cooper, Bower, Li, & Fink, 2002), β-synuclein (Yamin et al., 2005), amyloid-β peptide (Lee, Bird, Shaw, Jean, & Vaux, 2012), human tau protein (Ma, Hu, Chen, & Liang, 2013), and human copper, zinc superoxide dismutase (Ma et al., 2013). Crowders of similar chemical nature are known to affect protein aggregation on a concentration-dependent manner (Uversky et al., 2002). Also, crowders of different chemical nature can modulate protein aggregation in a different manner (Assarsson, Linse, & Cabaleiro-Lago, 2014; Breydo et al., 2014;

Uversky et al., 2002). For example, in the case of the Aβ42 aggregation, accelerating effects were observed from the positively charged polymers, whereas no aggregation modulating effects were seen from the negative or neutral polymers (Assarsson et al., 2014). It was also shown that rigid and flexible polysaccharides influence protein aggregation via different mechanisms. Furthermore, it has been suggested that, in addition to excluded volume effects, changes in solution viscosity and nonspecific "soft" protein–polymer interactions might influence the structure and dynamics of proteins in crowded environments (Breydo et al., 2014).

To further clarify factors affecting protein fibrillation in crowded environments, we examined the effects of PEG and Ucon of similar size (~4.0 kDa) on aggregation of α-synuclein into amyloid fibrils under conditions closely resembling physiological. We want to emphasize here once again that the analysis of the partition of recombinant human α-synuclein in aqueous dextran-PEG and Ficoll-Ucon two-phase systems (namely, using the Collander solvent regression relationship between the proteins partition coefficients in different ATPSs) suggested that this protein does not specifically bind to the polymers used in our study (see Figure 1).

Aggregation was conducted at neutral pH at 40 °C and in the presence of a low concentration (.025 mg/ml) of heparin sulfate, a negatively charged natural polysaccharide often used to accelerate protein aggregation. Under these conditions, α-synuclein efficiently converts to amyloid fibrils with the lag phase of several hours. Figure 7 shows that in the presence of PEG, fibrils were formed, although they became shorter at high PEG concentrations. In the presence of Ucon, however, fibril yield significantly decreased, and at higher Ucon concentrations, they disappeared entirely and were replaced by oligomeric aggregates.

It is important to note that the aforementioned astonishing difference in the effects of similar concentrations of similarly sized Ucon and PEG on aggregation of α-synuclein, where protein efficiently fibrillated in the presence of high PEG concentrations, whereas no amyloid-like fibrils were formed in the presence of Ucon, clearly shows that not all crowders are made equal and that their influence on protein aggregation cannot be attributed to the simple excluded volume effects. It is likely that the mentioned difference in the aggregation behavior of α-synuclein in the presence of similar concentrations of similarly sized PEG and Ucon can be

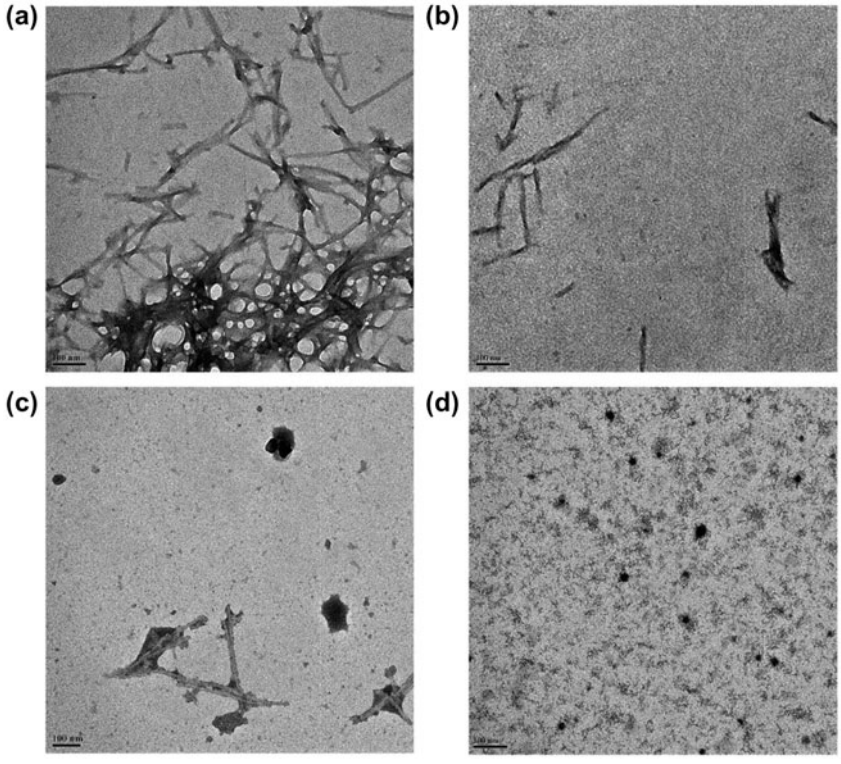

Figure 7. Morphology of amyloid fibrils of α-synuclein grown in the presence of either PEG 4450 (MW ~ 4.45 kDa) or Ucon 50-HB-5100 (MW of 3930 Da) polymers. The image of amyloid fibrils was obtained by electron microscopy. Fibrils were grown at pH 7.5 for four days in the presence of .025 mg/ml heparin sulfate and various polymer concentrations: 5% PEG (A); 15% PEG (B); 2% Ucon (C); and 15% Ucon (D). White bar at the left bottom corner of each panel correspond to 100 nm.

attributed to the different effects of these polymers on solvent properties of aqueous media in their solutions.

Proteins such as albumin, lysozyme, and others were used as crowding agents (Miklos et al., 2011; Sarkar, Lu, & Pielak, 2014). It is important therefore to examine whether these and other proteins may also affect the solvent properties of aqueous media. Further studies in this direction are currently in progress in our laboratories.

Conclusions

It is shown that macromolecular crowding agents change solvent properties of aqueous media in their solutions. The solvent dipolarity/polarizability, HBD acidity, and HBA basicity of aqueous media evaluated in solutions of crowding agents are agent-specific and dependent on agent concentration. Polymers, such as PEG and copolymer of ethylene glycol and propylene glycol (Ucon), of the same size but producing different changes in the solvent properties of water in their solutions induce morphologically different α-synuclein aggregate forms. Analysis of several examples from the literature shows that the effects of different crowding agents on protein refolding and stability may be described in terms of the solvent properties of the aqueous media in the solutions of crowding agents. These data suggest that crowding agent-induced changes in the solvent properties of aqueous media are important contributors to the macromolecular crowding effects.

Therefore, it is suggested that the so-called 'soft interactions' for a biological macromolecule with crowding agents may be viewed as the interactions between the macromolecule and aqueous media with solvent properties altered under the crowding agent influence.

Supplementary information

Electronic supplementary information available: S1. Description of ATPS used in this study; S2. Description of the peculiarities of partitioning experiments; Table S1. Distribution coefficients for proteins and organic compounds in aqueous dextran-PEG and Ficoll-Ucon two-phase systems; Table S2. Solvent dipolarity/ polarizability values determined for different concentration of aqueous solutions of crowding agents; Table S3. Solvent HBA basicity values determined different concentration of aqueous solutions of crowding agents; Table S4. Solvent HBD acidity values determined different concentration of aqueous solutions of crowding agents.

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Supplemental data

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ORCID

Vladimir N. Uversky http://orcid.org/0000-0002-4037-5857

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