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Effects of polymer hydrophobicity on protein structure and aggregation kinetics in crowded milieu

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Abbreviations and Textual Footnotes: CD, circular dichroism; IDP, intrinsically disordered protein; PEG, polyethylene glycol; UV, ultra violet; ThT, thioflavin T.

Abstract

We examined the effects of water-soluble polymers of various degrees of hydrophobicity on folding and aggregation of proteins. The polymers we chose were polyethylene glycol (PEG) and UCON (1:1 copolymer of ethylene glycol and propylene glycol). Presence of additional methyl groups in UCON makes it more hydrophobic than PEG. Our earlier analysis revealed that similarly sized PEG and UCON produced different changes in the solvent properties of water in their solutions and induced morphologically different α -synuclein aggregates (Ferreira *et al.* Role of solvent properties of aqueous media in macromolecular crowding effects. *Journal of Biomolecular Structure and Dynamics*. In press, (1)). In order to better understand molecular mechanisms defining behavior of proteins in crowded environment, we tested the effects of these polymers on secondary and tertiary structure and aromatic residue solvent accessibility of 10 proteins (five folded proteins, two hybrid proteins; i.e., protein containing ordered and disordered domains, and three intrinsically disordered proteins, IDPs) and on aggregation kinetics of insulin and α -synuclein. We found that effects of both polymers on secondary and tertiary structures of folded and hybrid proteins were rather limited with slight unfolding observed in some cases. Solvent accessibility of aromatic residues was significantly increased for the majority of the studied proteins in the presence of UCON but not PEG. PEG also accelerated protein aggregation into amyloid fibrils, whereas UCON promoted aggregation to amyloid oligomers instead. These results indicate that even a relatively small change in polymer structure leads to significant change in the effect of this polymer on protein folding and aggregation. This is an indication that protein folding and especially aggregation are highly sensitive to presence of other macromolecules, and excluded volume effect is insufficient to describe their effect.

Keywords: Macromolecular crowding; solvent properties; intrinsically disordered protein; protein structure; protein aggregation;

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Introduction

Protein folding is a complex process that proceeds via a stochastic search for the most stable conformation corresponding to the natively folded state (2). In many cases, a natively folded state does not exist or is never reached, and a protein remains fully or partially unfolded. Structures of both folding intermediates and a final folded state are known to be strongly dependent on the environment. In addition to other factors such as pH and the presence of salts, presence of other biopolymers has a significant effect on protein folding *in vivo*. In fact, in the intracellular environment, macromolecules are present at overall concentrations of up to 300–400 mg/ml (3). Earlier studies on the effects of biopolymers on the behavior of proteins were usually focused on either strong protein-polymer interactions (specific binding, charge-charge interactions) or macromolecular crowding. Macromolecular crowding is generally believed to be primarily driven by excluded volume effect that works by decreasing the effective volume available for the proteins in the cell and thus increasing the effective protein concentration and promoting more compact protein conformations. However, the careful analyses of the macromolecular crowding effects in polymer solutions revealed that the excluded volume effect is not the only factor affecting the behavior of proteins 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 non-specific steric interactions (4-8). It is believed that these interactions are similar to preferential solvation of proteins by organic solvents (9).

Recently, we showed that changes in the solvent properties of aqueous media induced by the crowding agents might represent the molecular basis for these “soft” interactions (1). This conclusion was based on the analysis of the solvatochromic properties of specific dyes sensitive to the solvent dipolarity/polarizability, hydrogen-bond donor acidity, and hydrogen-bond acceptor basicity of aqueous media in solutions of different polymers (Dextran, PEG, Ficoll, UCON, and polyvinylpyrrolidone) with the polymer concentration up to 40%; i.e., in the concentration range typically used to model crowding conditions *in vitro* (1). This analysis revealed that solvent properties were affected by high polymer concentrations indicating that the crowding agents do induce changes in solvent properties of aqueous media in crowded environment (1). Here we extended this research and examined the effects of two polymers with

similar structures and different hydrophobicities on structural properties and aggregation propensities of several proteins.

Most studies of the effects of crowding agents on protein folding have used hydrophilic polymers such as PEG, dextran, and Ficoll that primarily affect proteins via steric exclusion (8, 10). Here we used two polymers, PEG (MW 4400) and its derivative UCON (MW 5400). UCON is a 1:1 copolymer of ethylene glycol and 2-propylene glycol. Thus, the structures of these polymers are very similar except for the presence of an additional methyl group on every other monomer unit of UCON making it more hydrophobic (see Figure 1). This makes it a good test case to see the effect of a modest change in polymer hydrophobicity on protein behavior in crowded environment. We previously reported that the effects of UCON and PEG on the solvent properties of aqueous media are quite different (1). We found that even this modest change is sufficient to significantly influence protein folding and especially aggregation.

Materials and methods

Materials

Recombinant α -synuclein was a gift from Dr. Munishkina (University of California Santa Cruz) and Dr. Rhoades (Yale University). Recombinant human insulin was from Akron Biotech (Boca Raton, FL). A commercially available mixture of core histones H2A, H2B, H3 and H4 from calf thymus (Calbiochem) was used without additional fractionation. UCON 50-HB-5100 (MW 4 kDa) was from Dow Corning. PEG (MW 4.5 kDa) was from Sigma. All other proteins and chemicals were from Sigma, Fisher Scientific or VWR Scientific.

Methods

Circular dichroism (CD)

Far-UV CD (195–260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at room temperature. A solution of protein (110 μ l, 1 mg/ml) was placed into a 0.2 mm pathlength cell, and the CD spectra were acquired with 20 nm/min scan speed at 0.2 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Three spectra were accumulated and averaged for each sample. The same buffer was used for CD measurements and for protein aggregation experiments.

ANS Fluorescence

Protein solution (26.7 μ l, 3 mg/ml, 0.1 mg/ml final concentration) was mixed with buffer (final concentration: 10 mM Hepes, pH 7.5, final volume 400 μ l) containing either no polymer or 10% of the respective polymer by weight. The solution was incubated for 15 min at 24 $^{\circ}$ C and then ANS fluorescence was measured. Excitation wavelength was 380 nm and emission spectrum was recorded in 400-600 nm range at 100 nm/min. Excitation and emission slits were at 2.5 nm. Emission spectra were recorded in triplicate and averaged. Emission spectra were integrated, and their integrals are presented in Table 2.

Intrinsic fluorescence quenching

Protein solution (13.3 μ l, 3 mg/ml, 0.05 mg/ml final concentration) was mixed with buffer (final concentration: 10 mM Hepes, pH 7.5, 0.1 M NaCl, final volume 400 μ l) containing different concentrations of acrylamide (0 – 0.5 M). The solution was incubated for 1 h at 24 $^{\circ}$ C, and intrinsic protein fluorescence was measured. Excitation wavelength was 280 nm, and emission spectrum was recorded in 295-350 nm range at 50 nm/min. Excitation and emission slits were at 5 nm. Emission spectra were recorded in triplicate and averaged. The measurements were performed in duplicate for each sample. The data resulting from the quenching of the intrinsic fluorescence were analyzed using the Stern-Volmer equation (Eq.1),

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \tag{1}$$

where F_0 is the fluorescence emission in the absence of acrylamide, F is the emission at a specific concentration of acrylamide, K_{sv} is the dynamic quenching constant (Stern-Volmer constant), and Q is the concentration of acrylamide.

Protein aggregation assays

Conditions for protein aggregation were optimized for each protein. Aggregation of insulin (0.5 mg/ml) was conducted in glycine buffer (20 mM, pH 2.5) at 37 $^{\circ}$ C. The protein was dissolved directly in this buffer and incubated for 5 min prior to the start of the reaction. Aggregation of α -synuclein (0.25 mg/ml) was conducted either in 40 mM acetate buffer, pH 3.5 in the presence of 0.1 M NaCl at 37 $^{\circ}$ C or in 20 mM Hepes, pH 7.5 in the presence of 0.1 M NaCl and 0.025 mg/ml heparin sulfate at 40 $^{\circ}$ C. α -Synuclein was initially dissolved in 50 mM

NaOH at 50 mg/ml, incubated in this solution for 1 min, diluted to 4 mg/ml with water, and the resulting solution added into the final reaction buffer.

Protein aggregation in the automated format was carried out in a reaction volume of 0.1 ml in black, flat-bottomed 96-well plates in the presence of 5 μ M ThT. Two Teflon 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 μ l) was split into three wells (100 μ l 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 3-8 minutes using 444 nm excitation and 485 nm emission filters. Data from replicate wells were averaged before plotting fluorescence vs time. The data were fit to a sigmoidal equation (Eq. 2) using Origin (OriginLab, Northampton, MA). The equation for this fit was:(11, 12)

$$F=A+B/(1+\exp(k(t-t_m))) \quad (2)$$

where A is the initial level of ThT fluorescence, B is the difference between the final level of ThT fluorescence and its initial level, k is the rate constant of amyloid accumulation (h^{-1}) and t_m is the midpoint of transition. The lag time (t_l) of amyloid formation was calculated as $t_l = t_m - 2/k$. The parameters derived from this equation are: yield of amyloid (B), lag time (t_l), and elongation rate (k) of amyloid. Initiation rate was defined as the inverse of lag time. Although eq. 2 gave good fits for the ThT kinetic profiles, the expression is strictly an empirical means of deriving kinetic parameters from the data and does not necessarily reflect the underlying complex kinetic scheme.

Electron Microscopy

10 μ l aliquots of protein solutions (0.1-0.3 mg/ml) were taken from the 96-well plates at the end of the incubation (40-80 hours) and adsorbed onto 200 mesh formvar/ carbon-coated nickel grids for 3 min. The grids were washed with water (20 μ l), stained with fresh 2% uranyl acetate solution for 1 min and washed with water again. The samples were analyzed with a JEM 1400 transmission electron microscope (JEOL) operated at 80 kV.

Results

The polymers we chose were polyethylene glycol (PEG) and UCON (1:1 copolymer of ethylene glycol and propylene glycol with monomer units distributed randomly). Addition of propylene glycol monomer units increased the polymer hydrophobicity without altering other polymer properties significantly. We selected five folded proteins (insulin, β -lactoglobulin B, α -lactalbumin, lysozyme and RNase B), two hybrid proteins; i.e., proteins contacting both ordered and intrinsically disordered regions (trypsinogen, α -chymotrypsinogen A), and three typical intrinsically disordered proteins, IDPs (α -casein, histones, α -synuclein).

Folded proteins used in this study are commercially available proteins with a variety of secondary structures. Insulin is a small, primarily α -helical protein (MW 5.5 kDa). We tested it at both pH 2.5 where it's monomeric and at pH 7.5 where it's hexameric (13). β -Lactoglobulin B is a β -sheet rich protein (MW 18 kDa) present at high concentrations in milk (14). α -Lactalbumin, a regulatory subunit of lactose synthase, is a primarily α -helical calcium-binding protein containing four disulfide bonds (MW 14 kDa) (15, 16). Hen egg white lysozyme, a glycoside hydrolase, is a primarily α -helical globular protein (MW 14 kDa) (16). It shows a strong sequence and structural similarity to α -lactalbumin (17). An important structural difference between these two proteins is the existence of easily accessible molten globule denaturation intermediates for α -lactalbumin but not for hen egg white lysozyme (18). Ribonuclease B is an endonuclease with specificity for pyrimidine linkages. It's a glycoprotein (MW 14.7 kDa) composed of a mixture of α -helices and β -sheets (19). Charges of these proteins at physiological pH vary significantly. Insulin, β -lactoglobulin B and α -lactalbumin have a pI in the 4.5-5.5 range and are negatively charged at neutral pH. Lysozyme has a pI of 11.3 and is highly positively charged at neutral pH while ribonuclease B has a pI of 9.3 and is thus positively charged as well.

We also used two hybrid proteins and three IDPs with different charge/hydrophobicity ratios (20). Trypsinogen and chymotrypsinogen A are the precursor forms of the respective proteolytic enzymes. They contain both folded and disordered regions and can thus be considered hybrid proteins (21, 22). α -Caseins are highly phosphorylated, mostly disordered proteins (MW 22-25 kDa) (23). Core histones are lysine-rich, highly charged IDPs that bind to DNA as octamers (24). They exist in a variety of oligomeric states ranging from monomer (MW 14 kDa) to octamer depending on the assay conditions with octamers being prevalent at neutral pH (25-27). α -

Synuclein is an intrinsically disordered, highly amyloidogenic protein. Conformational flexibility of this protein depends on pH with more compact and flexible conformation at low pH and more extended conformation at neutral pH (6, 28, 29) due to higher protein charge at neutral pH. We used α -synuclein at two pH values: pH 3.5 and pH 7.5. Far UV CD spectra of IDPs (Fig. 2) confirmed that they are predominantly unfolded in the assay conditions. Histones, trypsinogen and chymotrypsinogen were positively charged at neutral pH (pI values 9-11) while α -casein and α -synuclein were negatively charged (pI \sim 4.6).

Secondary structure perturbation by polymers detected by far UV CD spectroscopy.

In order to assess the effect of polymers on the protein secondary structure, we used far-UV CD spectroscopy. All experiments were conducted at neutral pH (10 mM Hepes, pH 7.5) with the exception of trypsinogen, insulin and α -synuclein. Assays for trypsinogen were conducted at pH 3.0 (10 mM glycine) in order to prevent its cleavage by trace amounts of trypsin; insulin was assessed at both pH 2.5 (10 mM glycine) and pH 7.5; and α -synuclein was evaluated at pH 3.5 (10 mM citrate) and pH 7.5. A range of polymer concentrations (0 – 10%) was used. However, for the sake of presentation the spectra of all proteins are shown here in three sets of conditions: without polymers, in the presence of 10% PEG and in the presence of 10% UCON (Fig. 2). More detailed dependences of CD spectra on polymer concentrations are shown for select proteins in the Supplemental Information (Fig. S1).

Effects of polymers on the protein secondary structure were quite variable. Far-UV CD spectra of several folded proteins (insulin at pH 7.5, lysozyme, ribonuclease B) and of both hybrid proteins (α -chymotrypsinogen A and trypsinogen) were unchanged in the presence of both polymers (Fig. 2, Fig. S1). Spectral minimum of β -lactoglobulin B shifted from 214 to 217 nm in the presence of UCON (Fig. 2C) indicating an alteration of the secondary structure although we could not attribute this change to any specific structural transition. Spectra of insulin at pH 2.5 and of α -lactalbumin at pH 7.5 did change significantly with the addition of UCON (Fig. 2). CD spectra of these proteins are typical for primarily α -helical proteins with peaks at 208 and 222 nm. In the presence of UCON, the peak at 222 nm progressively decreased in intensity with increasing UCON concentrations, while intensity of the peak at 205 nm increased. This shift is consistent with UCON-induced partial loss of the protein secondary structure. As for IDPs, spectra of α -synuclein at either pH 3.5 or 7.5 did not change significantly in the presence of both

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3 polymers. The CD spectrum of histones in the presence of PEG (but not UCON) showed the
4 shift of the peak minimum from 202 to 204 nm and increase in the intensity of the shoulder at
5 224 nm (Fig. 2), indicating possible gain of ordered secondary structure. For α -casein, a similar
6 but even larger spectral change was observed in the presence of UCON. These changes indicate
7 an increased degree of protein folding in the presence of the respective polymers.
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11 Overall, in the presence of PEG, spectral changes consistent with the excluded volume effect
12 (protein folding) were observed for histones. CD spectra of other proteins remained essentially
13 unchanged. We have also observed that UCON promoted partial loss of secondary structure in
14 two folded proteins (insulin at pH 2.5 and α -lactalbumin) and gain of secondary structure in α -
15 casein. We have also attempted to evaluate effects of polymers on the secondary structure of the
16 proteins by deconvolution of the CD spectra using the K2D algorithm on the Dichroweb website
17 (30). Based on this analysis, we observed (Table 1) some structural changes in the presence of
18 polymers for several proteins: insulin at pH 2.5, β -lactoglobulin B, α -lactalbumin, α -casein,
19 histones and α -synuclein at pH 3.5. This result was similar to the result of the visual examination
20 of the spectra.
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32 *Tertiary structure perturbation by polymers detected by near UV CD spectroscopy*

33 We confirmed our observations by examining the effect of polymers on tertiary structure of
34 these proteins with the near-UV CD spectroscopy. Since IDPs do not have a stable tertiary
35 structure, near-UV CD spectra of only folded and hybrid proteins were examined. Near-UV CD
36 spectra arise from aromatic amino acid residues in a chiral environment, and decreased spectral
37 intensity is a an indication of the loss of tertiary structure. Spectra of most proteins remained
38 essentially unchanged in the presence of both polymers (Fig. 3, Fig. S2). The intensities of the
39 spectra of insulin at pH 7.5 and β -lactoglobulin B increased slightly in the presence of both
40 polymers. Intensities of the spectra of α -lactalbumin, lysozyme and chymotrypsinogen have
41 decreased in the presence of both polymers, an indication of possible loss of some tertiary
42 structure (Fig. 3, Fig. S2). The effect was especially pronounced for α -lactalbumin. Changes in
43 the spectrum fine structure in the presence of polymers were observed for chymotrypsinogen
44 although these changes were relatively minor and couldn't be readily interpreted. No significant
45 difference between the effects of PEG and UCON on near UV CD spectra was observed for
46 either of these proteins.
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Structural changes in the presence of polymers assessed by ANS binding and quenching of intrinsic protein fluorescence by acrylamide

Fluorescence emission of 8-anilinonaphthalene-1-sulfonate (ANS) significantly increases and undergoes a blue shift upon binding to exposed hydrophobic surfaces of proteins. Partial denaturation or aggregation of proteins results in significant increase in ANS binding and fluorescence, and thus ANS fluorescence can be used to monitor changes in the protein tertiary structure (31, 32). We examined ANS fluorescence (Table 2) in the presence of all the proteins in this study (at 0.1 mg/ ml) both in the absence and presence of polymers (10% of either PEG or UCON). We found that addition of polymers (in the absence of proteins) resulted in significant increase in ANS fluorescence intensity. Fluorescence of ANS also increased in the presence of several proteins (in the absence of polymers) although no blue shift of fluorescence was observed. Addition of proteins to ANS in the presence of polymers didn't have significant additional effects on ANS fluorescence. Casein, trypsinogen and chymotrypsinogen in the presence of UCON were the exceptions as addition of these proteins resulted in increased ANS fluorescence. These results indicate that while proteins bind ANS to variable extent, presence of much higher polymer concentrations results in ANS preferentially binding to polymers. However, a few proteins (especially α -casein) in the presence of UCON did present sufficient exposed hydrophobic surface to have an effect on ANS fluorescence. This observation confirms significant structural changes observed for α -casein in the presence of UCON by other methods (Fig. 2).

Intrinsic protein fluorescence is primarily due to their tyrosine and tryptophan residues. Solvent accessibility of these aromatic residues can be probed by analyzing the efficiency of quenching of their fluorescence by small molecules. Acrylamide is a common chemical quencher. Recent studies have shown that quenching by acrylamide can occur via through-space interactions (i.e., a type of orbital interaction resulting from direct spatial overlap of two orbitals), and acrylamide does not need to come into direct contact with the fluorophore (33). Thus, rate of quenching of intrinsic protein fluorescence by acrylamide is a measure of proximity of the aromatic residues to the protein surface and hydrophobicity of the environment of these residues.

In order to assess the effect of PEG and UCON on surface accessibility of aromatic residues and hydrophobicity of their environment, we have examined quenching of intrinsic protein fluorescence by acrylamide in the presence of these polymers. We found that for most proteins, both polymers increased acrylamide quenching rates, indicating increased exposure of aromatic residues to solvent. Furthermore, in all cases studied, the effect of UCON was much stronger than that of PEG (Table 3, Fig. 4). For several proteins (β -lactoglobulin B, α -lactalbumin, and α -chymotrypsinogen A) PEG either had no effect on the efficiency of quenching or decreased it, whereas the presence of UCON consistently increased quenching rates, often by a significant margin. IDPs were especially sensitive to the presence of polymers, although small folded proteins (insulin and RNase B) were highly sensitive as well. As expected, insulin was more accessible to acrylamide at pH 2.5 (where it is monomeric and partially unfolded) than at pH 7.5 (where it is oligomeric and primarily α -helical). Overall, these results confirmed the observation based on far UV CD data that UCON promotes protein unfolding much more effectively than PEG and extended it across the full range of proteins we examined.

Lack of specific interactions between proteins and polymers

Detection and analysis of protein interactions with other macromolecules generally depends on the choice of the appropriate technique to be used. To prove the lack of such interactions is much more difficult because it is always possible that the particular technique employed may be insufficiently sensitive. The only indirect approach enabling one to prove the lack of specific protein-polymer interactions seems to be analysis of protein partitioning in aqueous two-phase systems (ATPS) formed by pairs of different polymers (34). It has been shown that the logarithms of partition coefficients of 12 different proteins including α -chymotrypsinogen, lysozyme, trypsinogen, RNase B, and β -lactoglobulin B in Ficoll-PEG and Dextran-UCON ATPS are linearly interrelated as(35):

$$\log K_i^{Dex-UCON} = b + a*\log K_i^{Ficoll-PEG} \tag{3}$$

where $K_i^{Dex-UCON}$ and $K_i^{Ficoll-PEG}$ are partition coefficients for i^{th} protein in dextran-UCON and Ficoll-PEG ATPS, correspondingly; a and b are constants. It should be mentioned that the partition coefficient of a protein in any given ATPS is commonly defined as the ratio of the protein concentration in the upper phase to that in the lower phase.

One should keep in mind that coefficients a and b in Eq.3 depend upon the solvent features of the ATPS under comparison and therefore cannot be predicted. Eq. 3 is an empirical relationship first established for partitioning of compounds of the same chemical nature in different organic solvent-water biphasic systems. In ATPSs this equation holds for different biopolymers, such as proteins and nucleic acids, and small organic compounds (1, 34). The fulfillment of the relationship described by Eq.3 for different proteins in ATPSs formed by different pairs of polymers is generally accepted as indication that the proteins fitting the relationship do not participate in direct interactions with the phase forming polymers, and that their distribution between the coexisting aqueous phases in each of two ATPSs under comparison is driven solely by differences between protein-water interactions in the two phases (1, 34). Therefore, the data obtained for the proteins examined in our study were used with the purpose to check if there are direct interactions between the protein of interest and UCON or PEG. The solvent properties of the aqueous media in the phases of ATPS cannot be classified in terms of “bad” and “good” solvent, but may be quantified in terms of the solvent ability to have dipole-dipole interactions, solvent ability to serve as a hydrogen bond donor and as a hydrogen bond acceptor. This issue, however, is beyond the scope of the present study.

Similar relationship was reported (1) for 19 small organic compounds and seven different proteins including α -synuclein, RNase B, and β -lactoglobulin B in dextran-PEG and Ficoll-UCON ATPS. It follows from the constant values of coefficients a and b in Eq. 3 that either all different proteins and organic compounds examined interact with the polymers used for forming a given ATPS in identical manner or that there is no specific interactions between proteins and small compounds and phase-forming polymers at all. It was concluded (34) that there is no direct protein-polymer interactions for the proteins fitting the relationships described by Eq. 3, and that the unequal distribution of proteins in ATPS occurs due to different interactions of proteins with aqueous media with different solvent properties in the coexisting phases. This conclusion was confirmed (36-38) by demonstrating that the partition coefficient of a solute (including proteins) may be described quantitatively in terms of quantified solvent properties of the coexisting phases in ATPS, and even predicted with 90-95% accuracy.

Therefore, for six out of ten proteins studied here there is no doubt that these proteins do not participate in any direct interactions with PEG or UCON.

Effects of polymers on the protein aggregation kinetics

Effects of crowding agents on protein aggregation have been extensively investigated in the past. It has been shown that aggregation of many amyloidogenic proteins and peptides is accelerated by the presence of crowding agents (28, 39-42). This acceleration has been attributed to the excluded volume effect (43, 44), although the existence of weak interactions between proteins and polymers have been proposed as well (3, 4, 45, 46). On the other hand, aggregation of stable folded proteins, such as lysozyme, has been inhibited by crowding agents presumably due to monomer stabilization (41).

We have examined the effect of polymers on kinetics of fibril formation by insulin and α -synuclein, at the conditions previously determined to be favorable for the conversion of these proteins to amyloid fibrils. Aggregation of insulin was examined at pH 2.5, where this protein is primarily monomeric. For α -synuclein two sets of conditions were used: pH 3.5 and pH 7.5. Aggregation of α -synuclein at pH 7.5 was conducted in the presence of 25 μ g/ml heparin sulfate, a negatively charged polysaccharide used to accelerate protein aggregation (11, 47-49). In these conditions, proteins efficiently converted to amyloid fibrils with the lag phase of several hours (Fig. 5). Thioflavin T fluorescence was used to assess the protein aggregation kinetics. In control experiments (Fig. S4) we determined that polymers don't have a significant effect on the fluorescence of this dye in the absence of proteins.

From kinetic data we found that aggregation of both insulin and α -synuclein was significantly accelerated in the presence of PEG with initiation rate increasing 5-10 fold (Fig. 5). ThT fluorescence decreased about 3-fold for fibrils grown in the presence of PEG (Fig. 5, Fig. S3). But we also found (Fig. S4) that addition of either PEG or UCON to preformed fibrils significantly decreases fluorescence of ThT bound to them. Thus, lower ThT fluorescence of fibrils in the presence of polymers is likely due to competition between polymers and fibrils for ThT binding and thus not related to the mechanism of aggregation. Electron microscopy showed somewhat shorter fibrils in the presence of PEG for both proteins but overall fibrillar morphology was preserved (Fig. 6).

Using a combination of kinetic data and electron microscopy we found that UCON directed aggregation of both insulin and of α -synuclein towards oligomeric structures. UCON completely inhibited aggregation of insulin to amyloid fibrils even at the lowest polymer concentration tested (Fig. 5). Electron microscopy has shown that oligomeric aggregates were formed instead

(Fig. 6). Effect of UCON on aggregation of α -synuclein was weaker (Fig. 5, Fig. S3), and some amyloid fibrils were still observed at low polymer concentrations. However, at higher UCON concentrations (10-15%) only α -synuclein oligomers were observed (Fig. 6).

Discussion

Here we have examined the effects of two polymers of different hydrophobicity (Fig. 1) used as crowding agents on protein structure and aggregation. We found that with a few exceptions effects of both less hydrophobic (PEG) and more hydrophobic (UCON) polymers on the secondary and tertiary structures of proteins were relatively minor. These exceptions included α -lactalbumin and chymotrypsinogen that appeared to convert to a molten globule-like conformation in the presence of both polymers as seen from significant decrease of intensity of near-UV CD along with smaller changes in the far-UV CD (Fig. 2, 3, Fig. S1, S2). Both of these proteins can be converted to molten globule-like states at neutral pH by thermal or guanidine-dependent denaturation(18, 50). Polymers also appeared to induce some ordered secondary structure in IDPs (PEG for histones and α -synuclein, UCON for α -casein), although these effects were not reflected in changes in the accessibility of aromatic residues in these proteins to acrylamide. On the other hand, for most proteins both polymers had a significant effect on the solvent accessibility of the hydrophobic core measured by the quenching of the intrinsic fluorescence. For most proteins, Stern-Volmer constants increased in the presence of PEG and increased even further in the presence of UCON. The effects of UCON on IDPs were especially large. This could be an indication that some proteins (especially IDPs) in the presence of UCON (and to some extent PEG) undergo a structural transition that does not alter their secondary or tertiary structure but increases exposure of buried hydrophobic residues.

Effects of polymers on protein aggregation were even more dramatic. While the presence of PEG decreased the lag phase of fibril formation and somewhat altered the fibril morphology, in the presence of UCON, fibril formation was almost completely inhibited, and various oligomers were formed instead. PEG may be acting on aggregating proteins via excluded volume effect by increasing the effective protein concentration and stabilizing compact conformations(8). Effect of UCON could be due to other factors such as changes in the solvent properties of aqueous media in solution(1). It appears that aggregation intermediates are more sensitive to both

crowding and other effects of polymers. This is not unexpected due to their oligomeric nature and higher abundance of exposed hydrophobic surfaces.

Since our analysis of the partition of target proteins in various ATPSs containing polymers of interest revealed the lack of specific protein-polymer interactions, the most probable explanation for the different effects of similarly sized PEG and UCON on the structural properties and aggregation behavior of proteins is the presence of the polymer-induced changes in solvent properties of aqueous media in crowded environment. Overall, we found that increasing the hydrophobicity of PEG by adding a methyl group to every other monomer unit allowed the resulting polymer to affect proteins more effectively and inhibit protein aggregation to amyloid fibrils. This is an indication that protein folding and especially aggregation are highly sensitive to presence of other macromolecules, and excluded volume effect is insufficient to describe the effects of these biopolymers.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

Electronic Supplementary Information (ESI) available: **Figure S1.** Far UV CD spectra of insulin, RNase B, α -casein and α -synuclein in the presence of variable concentrations of PEG and UCON. **Figure S2.** Near UV CD spectra of folded and hybrid proteins in the presence of variable concentrations of PEG and UCON. **Figure S3.** Kinetic curves for aggregation of insulin and α -synuclein in the presence of variable concentrations of PEG and UCON. Aggregation was

monitored by following ThT fluorescence. All experiments were conducted in triplicate. **Figure S4.** Effect of polymers on ThT fluorescence alone (A) or in the presence of 5 μM α -synuclein fibrils (B). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Figure legends

Figure 1. Structures of PEG and UCON polymers. UCON polymer is composed of an equal proportion of ethylene glycol and propylene glycol monomer units distributed randomly.

Figure 2. Far UV circular dichroism spectra of proteins in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON.

Figure 3. Near UV circular dichroism spectra of natively folded and hybrid proteins in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON.

Figure 4. Intrinsic protein fluorescence quenching by acrylamide in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON.

Figure 5. Effect of polymers on protein aggregation kinetics. Aggregation of insulin (pH 2.5) and α -synuclein (pH 3.5 and 7.5) was monitored by following ThT fluorescence. All experiments were conducted in triplicate and the averages are shown. Dependence of kinetic parameters derived from these experiments (initiation and elongation rates of protein aggregation and ThT fluorescence intensity at the conclusion of the aggregation) on the polymer concentration is also shown.

Figure 6. Morphology of amyloid fibrils of insulin and α -synuclein grown in the presence of either PEG or UCON polymers. Fibrils of insulin at pH 2.5, α -synuclein at pH 3.5 and α -synuclein at pH 7.5 were grown in the presence of polymers. Scale bars correspond to 100 nm.

Table 1. Analysis of the protein secondary structure based on deconvolution of far UV CD spectra.

		α -helix, %	β -sheets, %	random coil, %
Insulin, pH 2.5	No polymer	62	6	32
	10% PEG	61	7	31
	10% UCON	69	4	27
Insulin, pH 7.5	No polymer	100	0	0
	10% PEG	100	0	0
	10% UCON	100	0	0
β -Lactoglobulin B	No polymer	21	30	49
	10% PEG	19	32	49
	10% UCON	12	39	48
α -Lactalbumin	No polymer	27	13	60
	10% PEG	25	15	60
	10% UCON	31	11	59
Lysozyme	No polymer	26	19	56
	10% PEG	30	14	56
	10% UCON	30	13	57
RNase B	No polymer	23	40	36
	10% PEG	23	40	36
	10% UCON	23	40	36
α -Chymotrypsinogen	No polymer	28	33	39
	10% PEG	28	33	40
	10% UCON	28	33	39
Trypsinogen	No polymer	23	23	54
	10% PEG	24	22	55
	10% UCON	23	22	55

α -Casein	No polymer	26	27	47
	10% PEG	22	27	50
	10% UCON	18	28	54
Histones	No polymer	8	41	50
	10% PEG	9	35	56
	10% UCON	8	41	51
α -Synuclein, pH 3.5	No polymer	8	41	51
	10% PEG	8	44	48
	10% UCON	8	46	47
α -Synuclein, pH 7.5	No polymer	7	51	42
	10% PEG	7	51	42
	10% UCON	7	51	42

Table 2. ANS fluorescence in the presence of proteins and polymers. Fluorescence emission spectrum (ex 380 nm, em 400-600 nm) was integrated, and the integral was normalized against the one obtained in the absence of protein.

	ANS fluorescence emission, %		
	No polymer	10% PEG	10% UCON
Insulin, pH 2.5	273	115	106
Insulin, pH 7.5	746	90	102
β -Lactoglobulin B	298	92	107
α -Lactalbumin	185	79	148
Lysozyme	101	84	103
RNAse B	99	87	98
α -Chymotrypsinogen	485	90	139
Trypsinogen	901	135	132
α -Casein	316	84	240
Histones	106	83	102
α -Synuclein, pH 3.5	406	162	115
α -Synuclein, pH 7.5	108	83	104

Table 3. Stern-Volmer constants (K_{SV} , M^{-1}) for acrylamide quenching of intrinsic protein fluorescence in the presence and absence of polymers.

	No polymer	10% PEG	10% UCON
Insulin, pH 2.5	13.5±2.2	15.1±3.9	23.3±0.1
Insulin, pH 7.5	7.7±2.4	12.1±1.4	19.6±3.5
β-Lactoglobulin B	8.4±0.1	5.3±0.3	9.3±0.9
α-Lactalbumin	7.4±0.9	7.1±1.3	8.6±1.5
Lysozyme	7.1±0.5	9.4±1.7	9.2±1.3
RNAse B	7.1±0.9	12.1±0.8	22.1±0.2
α-Chymotrypsinogen	4.6±1.0	4.5±0.1	6.9±0.5
Trypsinogen	5.7±0.7	9.9±0.7	11.5±2.7
α-Casein	8.7±0.6	17.3±0.8	13.3±3.6
Histones	7.7±0.4	9.2±2.1	19.1±1.1
α-Synuclein, pH 3.5	8.6±2	9.8±0.6	29.8±1.0
α-Synuclein, pH 7.5	10.3±2.2	10.6±1.8	29±0.6

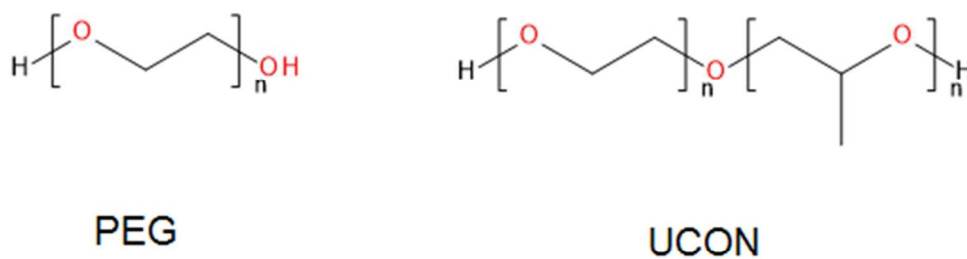


Figure 1

Figure 1. Structures of PEG and UCON polymers. UCON polymer is composed of an equal proportion of ethylene glycol and propylene glycol monomer units distributed randomly.
84x28mm (300 x 300 DPI)

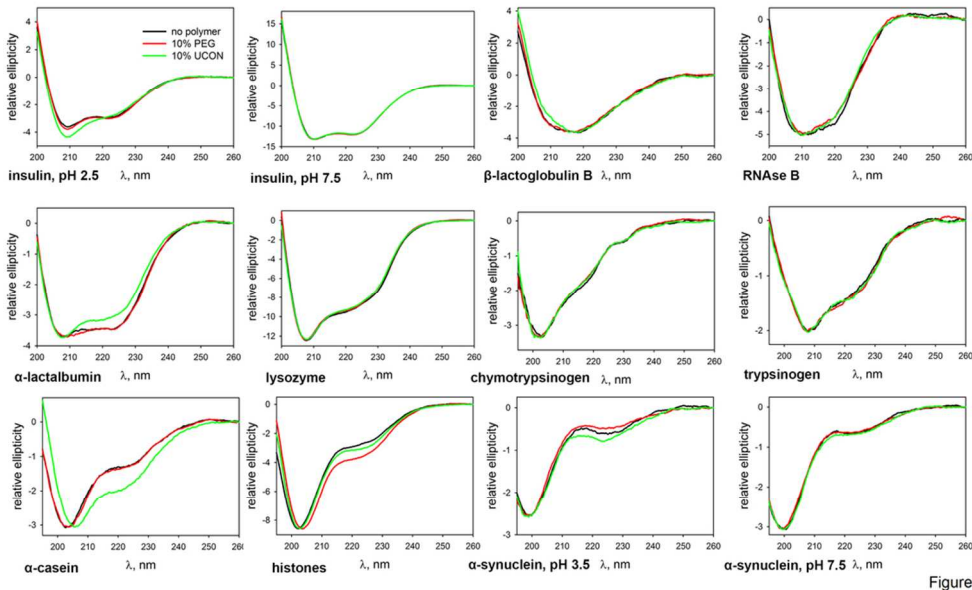


Figure 2. Far UV circular dichroism spectra of proteins in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON.
97x60mm (300 x 300 DPI)

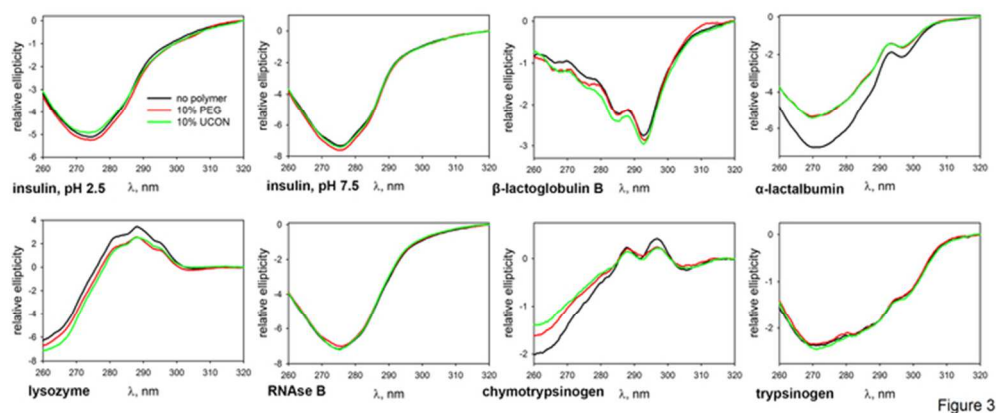


Figure 3. Near UV circular dichroism spectra of natively folded and hybrid proteins in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON.
63x25mm (300 x 300 DPI)

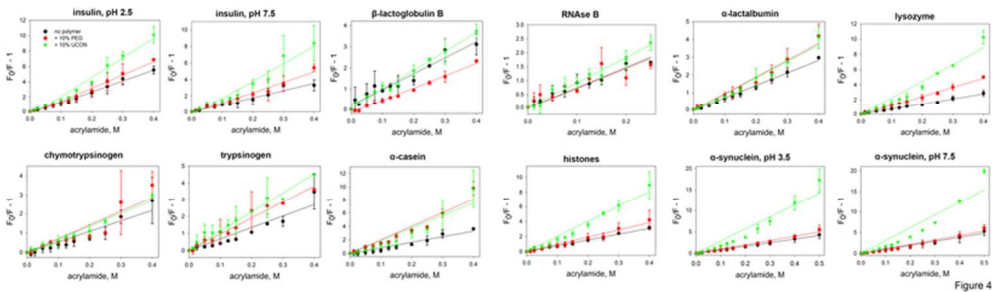


Figure 4. Intrinsic protein fluorescence quenching by acrylamide in the presence of PEG and UCON polymers. Black – no polymer additives, red – 10% PEG, green – 10% UCON. 69x20mm (300 x 300 DPI)

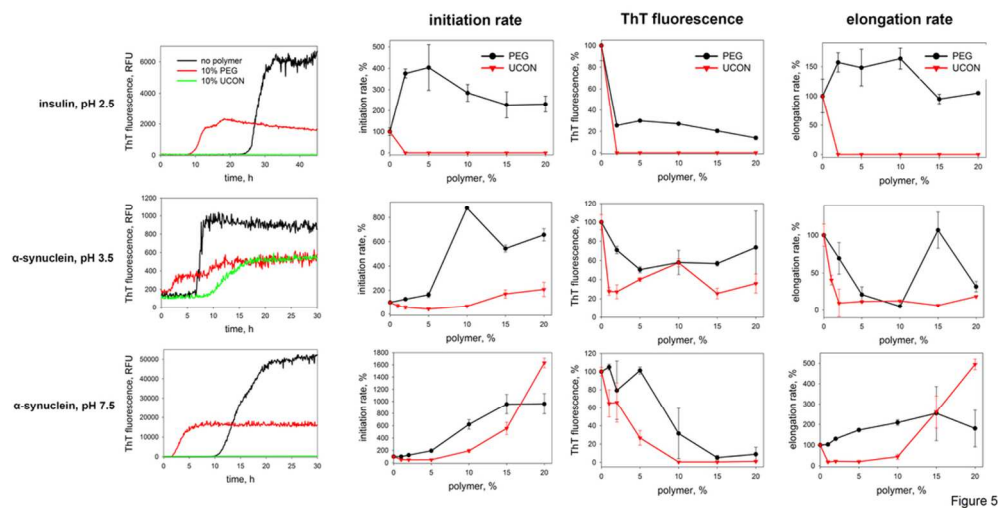


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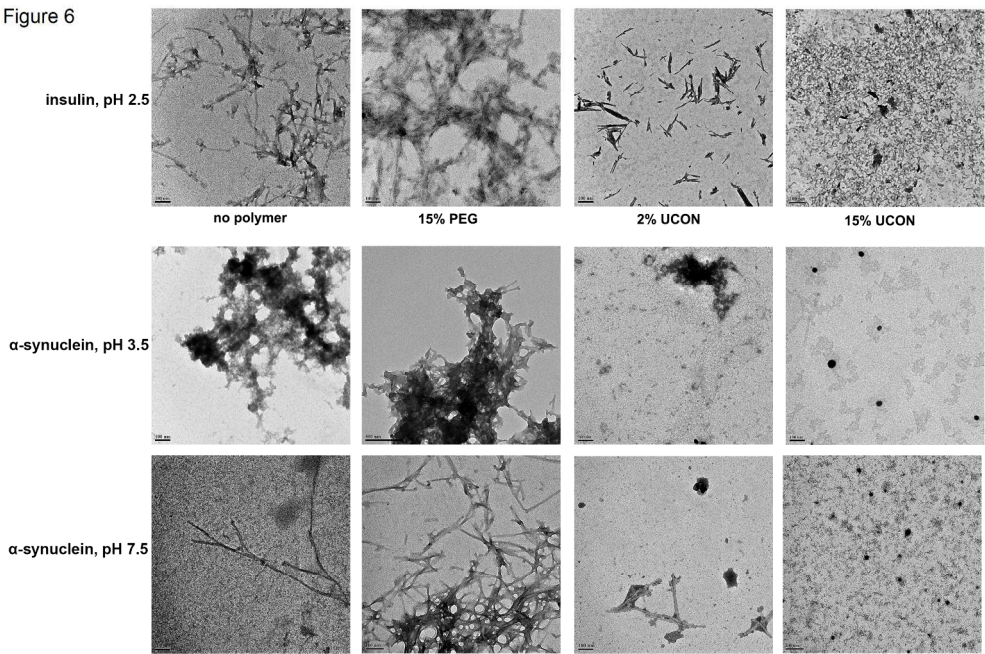
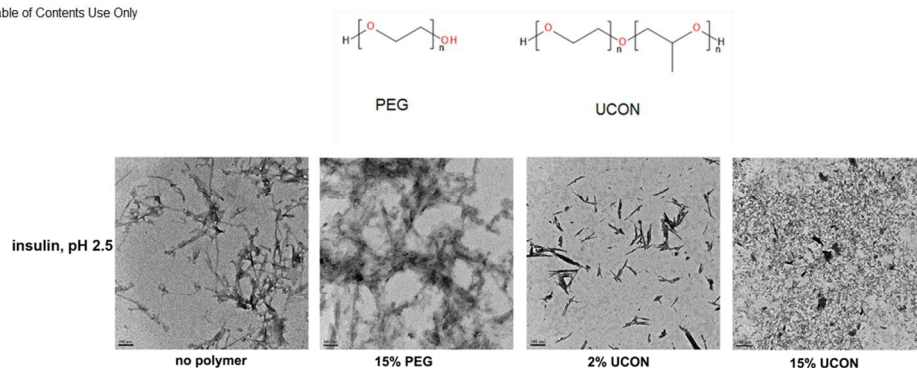


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207x139mm (300 x 300 DPI)

For Table of Contents Use Only



insulin, pH 2.5

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332x130mm (96 x 96 DPI)