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Scaling Behaviors of α-Zein in Acetic Acid Solutions

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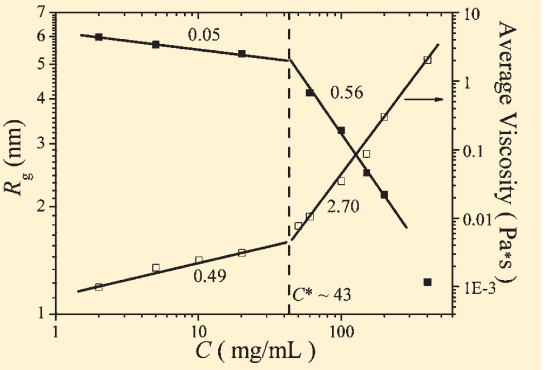
ABSTRACT: Whether the concentration scaling behavior of a protein solution is similar to that of neutral polymer solutions, polyelectrolyte solutions, or neither still remains unclear. In this paper, the structure and rheological properties of α-zein in acetic acid solutions have been investigated by small-angle X-ray scattering (SAXS), rheology, and circular dichroism (CD) measurements. Through the investigation of the radii of gyration, the secondary structure, and the solution viscosities of α-zein in acetic acid solutions as a function of α-zein concentration, we observed two distinct scaling regions with an identical threshold. This critical concentration is close to the bulk density of the α-zein in very dilute solution. The scaling relationships are close to the theoretical predictions for polyelectrolyte solutions, but obvious discrepancies still exist. The phenomena presented here may be widely present in other protein solutions, which can stimulate more attentions on the understanding of the scaling behaviors of protein solutions.



INTRODUCTION

Polymer solutions often show different scaling behaviors upon the increase of concentration. Scaling relationships of some general physical parameters in polymer solutions, such as the sizes of polymers and viscosities of polymer solutions as a function of polymer concentration have been theoretically well addressed and experimentally widely verified for neutral polymers and polyelectrolyte solutions.[1−3] Proteins, a very important class of biomacromolecules, have attracted a lot of research work during the last few decades.[4−6] However, few works were focused on the scaling behavior of proteins in solutions. Thus, the study of scaling behavior of protein solutions may facilitate the understanding of physical properties of protein solutions, which are usually complicated by the diversity and versatile features of proteins.

Generally, the scaling relationships of the size of a protein (expressed by the radii of gyration, R_g) and the viscosity (η) of the protein solution as a function of the protein concentration (C) can be written as

$$R_g \propto C^{\kappa} \quad (1)$$

$$\eta \propto C^{\lambda} \quad (2)$$

where κ and λ are the scaling exponents. The scaling exponents have been well addressed for several typical polymer solutions. For example, in neutral polymer solutions, λ is 1 in dilute solution ($C < C^*$); κ is $-1/8$ and λ becomes 15/4 in semidilute solutions ($C > C^*$)[1] with C^* the overlap concentration.[7] In polyelectrolyte solutions, λ is 1/2 in dilute solution, κ is $-1/4$ and λ becomes 5/4 in semidilute solutions.[2,3] For the concentration dependence of these two parameters, besides the scaling relationships, other equations have also been reported. A two-order polynomial equation has been used to interpret the viscosity in polymer solutions[8] or in hard-sphere colloid solutions.[9] However, to our best knowledge, the concentration-dependent power laws in pure protein solutions have not been studied in detail. It is necessary to conduct researches to provide fundamental understanding of the scaling behaviors in protein solutions.

We selected α-zein protein in acetic acid (AcOH) solutions as a model system because α-zein is a subtype of an important storage protein from corn, which has remarkably hydrophobic surface with self-aggregation proneness in particular. α-Zein is water insoluble but can be dissolved in various organic solvents, such as aqueous ethanol solutions, AcOH, dimethylformamide, etc. Among them, AcOH is a commonly used good solvent to isolate zein from corn extract.[10,11] Additionally, corn extracts, which mainly consist of α-zein, have broad potential applications in fibers, adhesives, coatings, ceramics, inks, cosmetics, textiles, chewing gums, etc.[12] α-Zein has two major components with molecular weights of 19 (known as Z19) and 22 kDa (Z22), as well as trace amount of their dimer. Structure models of α-zein have been reported in a number of publications,[13−16] but the native (crystal) structure of α-zein is still unavailable in Protein Data Bank (PDB). It is generally accepted that α-zein has a typical helix secondary structure and an asymmetric globular folding contour. The reported dimensions of the globular contour of α-zein and its conformations in solutions are quite divergent. Tatham et al. found that α-zein was either an equiaxial ellipsoid with size of 9.9 × 0.35 nm or a rod with size of 15.3 × 6.9 nm for 8.0 mg/mL α-zein in 70% (v/v) aqueous methanol solution.[13] Matsushima et al. observed an elongated rectangular prism size in 15.9 × 1.9 nm for 8.0 mg/mL α-zein in 70% (v/v)

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aqueous ethanol solution.[14] The structure model for Z19 proposed by Forato et al. contains helix and sheet with hairpin tertiary fold,[15,17] whereas in the model by Monmany et al.,[16] Z19 has a triple superhelix tertiary fold. By using multiple experimental approaches to study Z19,[18,19] Cabra et al. found that the conformation and secondary structure distribution of Z19 showed strong dependence on pH but was insensitive to temperature variation. The effect of pH on the structure, rheological properties, and antioxidant activity of α-zein was also studied by Zhang et al.[20] It was found that both solvent and temperature had significant impact on the secondary and tertiary structures of zein on the basis of circular dichroism (CD) measurements.[21] Kim and Xu have reported that the aggregate structure of zein changed with the composition of ethanol/water mixed solvent.[22] Therefore, concentration, temperature, pH, and solvent may all influence the structure of zein. In addition, different sources of zein protein also present divergence in protein sequences[23,24] which may also cause the difference in zein protein structure as reported by different groups. Since many variables may affect the conformation of zein in solutions, it is necessary to find some general rules regarding the physical properties of zein solutions, and scaling relationships may be the most feasible way.

In this work, we carried out a set of experiments combining small-angle X-ray scattering (SAXS), circular dichroism (CD), and rheological measurements to study how the conformation and the secondary structure of α-zein in acetic acid solutions, as well as the viscosities of the zein solutions change as a function of zein concentration. We also predicted atomic model for α-zein native structure, which was used as a reference in understanding the structure variation of α-zein protein. The combined analysis of protein structure models with experimental measurements and the scaling behaviors in protein solutions have been thoroughly discussed.

EXPERIMENTAL DETAILS

Materials. The α-zein of biochemical-grade purity was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). The sample contains two identical proteins present in approximately equal amounts of Z22 and Z19 of α-zein, and there are small amount of their dimer (around 39 kDa) and β-zein components, as characterized in our previous work.[25] AcOH (ACS reagent grade, > 99.7%) for SAXS and rheology experiments as well as glacial AcOH (99.99%) for CD measurements were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

Small-Angle X-ray Scattering (SAXS). Zein AcOH solutions were prepared under ambient temperature with the concentration of α-zein ranging from 2.0 to 400.0 mg/mL. These solutions were measured using SAXS performed at the BioCAT 18-ID beamline of Advanced Photon Sources, Argonne National Laboratory. The wavelength of X-ray radiation was 1.033 Å, and a short exposure period of 1 s was used to acquire the scattering data. Samples were loaded to the flow cell, a cylindrical quartz capillary of 1.5 mm diameter equipped with a brass block (thermostatted with a water bath), at a constant rate (10 μL/s) during X-ray exposure to minimize radiation damage. The q range of 6.0×10^{-3} to 0.37 $Å^{-1}$ was set for α-zein solutions no more than 20 mg/mL, whereas the q range of 2.8×10^{-3} and 0.17 $Å^{-1}$ was used for higher zein concentration solutions. The final SAXS profiles were obtained through the average of 15 measurements followed by the subtraction of solvent background.

Circular Dichroism (CD) Measurements. The far-UV CD measurements were carried out on AVIV model 400 spectrophotometer with 14DS UV−vis detector. The wavelength scans from 180 to 250 nm, a range covered all signals from secondary structure. α-zein AcOH solutions of different protein concentrations were loaded in 0.1 mm quvettes and the scan lasted for 0.2 s for each sample under ambient conditions. All samples were clear and stable throughout the measurements. The final spectral profiles were averaged over 8 replicas after subtraction of solvent background.

Rheological Measurements. The static rheological measurements of α-zein AcOH solutions were carried out using a strain-controlled Advanced Rheometric Expansion system (ARES) (TA Instruments, New Castle, DE). The solutions were loaded into cone plates of 50 mm in diameter, and a small amount of mineral oil was used to prevent solvent evaporation from the plate edge. The shear rate range of 1−1000 s^{-1} was used in the measurements. All of the measurements were carried out at room temperature.

Protein Native Structure Prediction. In addition to the above experimental measurements of α-zein AcOH solutions, we also carried out bioinformatics search to accumulate more information of α-zein under native state. Since no crystal structure of α-zein protein is currently available, we predicted the native structure of α-zein using one of the top-ranked protein structure prediction servers, the I-TASSER automatic protein structure prediction server.[26,27]

RESULTS

Native Structure Models of α-Zein. Before the presentation of experimental results, we first predicted the full-atomic native structures of α-zein. According to the source and the molecular weight of α-zein protein, the sequence fragments shown in the report by Tatham and co-workers[13] and the average amino acid composition,[18,23] through the screening in NCBI protein sequence database,[28] we identified the most possible sequences for these two proteins (Z19 and Z22). Both of them were derived from a α-zein protein with 225/226 amino acids (gi|157780972) whose composition has been thoroughly analyzed.[29] They are

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>gi|226499666, 205 residues, Mw is 22.4 kDa, known as Z22
>gi|157780920, 171 residues, Mw is 18.7 kDa, known as Z19

Z22    MAAKIFSILMLLALSACVANATIFPQYSQAPIAALLPPYLPSMTASVCENPALQPYRLQQ 60
Z19    -----------------AATATIFPQCSQAPIASLLPPYLSPVVSSVCQNPILEPYRIQQ 43
Z22sd CCHHHHHHHHHHHHHHHHHCEECCCCCCCCCCCCCCCCCCHHHHHCCCHHHHHHHHHH
Z19sd -----------------CCCCEECCCCCCCCCCCCCCCCCCHHHHHCCCCHHHHHHHHH
Z22ac eeeebbbbbbbbbbbbbbbbbbbbbbbebbbbebbeebbeebbbbbbbbbbbbbbbbb
Z19ac -----------------eeebebbeebeeeebbebbeebbeebbbbbbbbbbbbbbbbb

Z22    AIATSNLPLSPLFFQQS----PALSLVQSLVQTIRAQQLQQLVLPVISQVALANLSPYSQ 116
Z19    AIAAGILPLSPLFLQQSSALLQQLPLVHLLAQNIRAQQLQQLVL--------ANLAAYSQ 95
Z22sd HHHHHHHHHHHHHHHC----HHHHHHHHCCCHHHHHHHHHCCCCCHHHHCCCHHHHH
Z19sd HHHHHHCCCHHHHCCHHHHHHHHHHHHHHHHHHHHHHHHH--------CCCHHHHH
Z22ac bbbebbbeeeeeebbbe----ebbebbbbbbeeeebeebbbebbebbbbbbbebbebbbb
Z19ac bbbbbebeeebbebebeebbeebbebbeebbbeeebeebeebbe--------bbeebbee

Z22    QQQFLPFNQFAALNPSAYFQQQILLPFGQLATTSPASFLTQQQLLPFYQQFVANPATLLQ 176
Z19    QQQFLPFNQLAALNSASYLQQQ-QLPFSQLPAAYP------QQFLPFNQLAALNSPAYLQ 148
Z22sd HCCCCCCCHHHHCCHHHHHHHCCCCCCHHHHCCCCCCCCHHHCCCCCCHHHCCCHHHHH
Z19sd HHHCCCCCHHHHCCHHHHHHHHHCCCCHHHHHHHH------HHCCCHHHHHHCHHHHHH
Z22ac bbebbeeeebbebbbebbbebbebbbbebbbeeeeebbbebeebbebeeeebbebbbbbbe
Z19ac beebbebebbbebbbbbbbeeeebeeeebbebbee------bbbebbbbbebbeebbbeb

Z22    LQQLLPFVQLALTNPAAFYQQHIIG-GALF 205
Z19    QQQLLP--QLLP-----FYQHAAPNAGTLL 171
Z22sd HHHCCCCHHHHHCCHHHHHHCCCCC-CCCC
Z19sd HHHCCC--CCCC-----HHHHCCCHHHCCC
Z22ac bbebbebebbbbbbbbbeeebebbb-beee
Z19ac eebbee--bbeb-----beebbeeeeeeee
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In the above graphic, the sequence alignment was made using ClustalW,[30] Z22 and Z19 are the sequences of the two proteins, -sd and -ac are sequences based prediction of secondary structure

Table 1. Number of Each Kind of Residues Contained in Z22 and Z19 Sequences[a]

composition	Z22	Z19	composition	Z22	Z19
Leu	36	34	Cys	2	2
Gln	34	33	ARG	2	2
Ala	28	25	His	1	2
Pro	20	19	Glu	1	1
Ser	16	13	Lys	1	0
Phe	15	8	helix	119	107
ILE	10	7	coil	84	62
Val	9	5	strand/turn	2	2
Thr	9	3	buried	141	96
Asn	8	8	exposed	64	75
Tyr	7	7	Exp*	33	35
Gly	3	2	Gln*	8	5
Met	3	0	Gln**	23	20

[a] Asp and Trp are absent in both sequences. Exp* and Gln* (Gln**) are the number of exposed hydrophobic residues and the number of glutamine in loop or turn region, respectively. Gln* was calculated from the sequence while Gln** is based on Z22 and Z19a structure models.

by PSI-PRED[31] and solvent accessibility using SSpro,[32] respectively. In the secondary structure prediction, C, E and H represent the residues in coil, beta-strand/turn and helix status respectively. In the solvent accessibility prediction, e and b represent residues in exposed (solvent accessible) or buried status with a threshold of 25% exposure.[32] Based on sequence composition analysis shown in Table 1, around half of all solvent accessible residues in α-zein protein are nonpolar residues (Exp*), causing α-zein to be insoluble in water. There are 58% of Z22 and 62% of Z19 residues containing helix secondary structure, which agree well with previous reports.[16] In addition, both proteins contain a large amount of glutamine, and the glutamine-rich "turns" or "loops", an important feature of zein,[14,16] can also be observed here. However, only one-fourth of glutamine residues are exactly located in loops, and most of the rest of the glutamine residues are in the vicinity of the loop regions.

To better understand the conformation of α-zein in solutions, we then submitted both sequences for full-atomic structure prediction. The models predicted by I-TASSER server were shown in Figure 1. α-Zein proteins have typically tandem helix structures in fold state which is consistent with the other reported structure models.[13,14,16] Z22 has a globular shape folding with 13 fragments of helixes and dimension of 5.8 × 5.0 × 3.4 nm, an oblate disk shape. Z19a has an oblate ellipsoid conformation with 10 fragments of helixes and dimension of 5.9 × 4.5 × 2.3 nm. The extended state of Z19b was also shown, which has a size of 22.7 × 1.2 × 0.7 nm and can be considered as a string of helices. The asymmetric Z19 models with an axial ratio is 2.6 for Z19a and 32.4 for Z19b are quite close to the reported values of Momany et al.[16] Based on the predicted models, we can calculate the R_g of these models through

$$R_g^2 = \frac{1}{N_a}\sum_{i=1}^{N_a} |r_i - r_{cm}|^2 \quad (3)$$

In this equation, r_i and r_{cm} are the coordinates of the ith atom (except hydrogen atom) and the geometrical center of the protein, N_a is the number of atoms in the protein. The R_g values of Z22, Z19a, and Z19b are 2.4, 2.3, and 7.0 nm, respectively. With the help of the structure models, in both Z22 and Z19a structure models, more than 60% of glutamine residues are located in loops, which again, confirmed the existence of glutamine-rich loops in α-zein.

It is noteworthy that the structure models shown here are not the exact native structures of α-zein Z19 and Z22, but they are consistent with the existing reports in the distribution of secondary structures which include the glutamine-rich loops and the globular folding of protein models, suggesting that the full atomic models predicted here are quite reliable. Therefore, we take these models as references to study the protein conformation variation as a function of α-zein concentration in AcOH solutions.

X-ray Scattering Intensity Profiles. The scattering intensity profiles from SAXS of α-zein AcOH solutions in logarithmic scale were presented in Figure 2. In solutions of α-zein concentrations from 2.0 to 6.0 mg/mL, the slopes at large q range changed from −1.16 to −1.39. Further increase of zein concentration led to the decrease in slope until reaching the value of −0.04 in +400 mg/mL α-zein solution. Since the X-ray scattering profiles in this q range reveal the shape of zein in solutions, the scaling exponent in this range is not far from the value expected for a rod-like molecule which has an exponent of −1.[33] The decrease of the slope in large q range for concentrated solutions suggested that the interface between protein and AcOH become less significant or large-sized aggregates formed upon concentration increase. In the small q range, the slopes increase when more α-zein was added into the AcOH solutions. It changes from −0.65 to −0.80 in solutions with zein concentration up to 20 mg/mL, suggesting that only minor zein aggregation existed in these solutions. However, at higher zein concentrations (i.e., above 20 mg/mL), the slope increased sharply from −1.67 to −2.80, revealing that proteins may form large aggregates with the further increase of α-zein concentration. Such a large change in the slopes also indicated that the aggregation of α-zein in AcOH solutions may experience a rod-like (slope of −1), a random walk (slope of −2), or a partial disk packing (slope of −3) manner. A rod like aggregation may occur when Z22 or Z19 has extended conformations. A random walk aggregation takes place when structure motif with secondary structure in α-zein connected by random coils randomly aggregated, and a partial disk packing occurs when α-zein still holds native conformation with oblate ellipsoid shape which is close to oblate disk in geometrical shape, as suggested from the predicted protein structure models.

Guinier Analysis. We determined the R_g of particles in solution using the Guinier plot:[34]

$$\ln \frac{I(q)}{I(q=0)} = -\frac{1}{3}(qR_g)^2 \quad (4)$$

The data points used for Guinier analysis satisfy $qR_g < 1.57$, a region safe for Guinier fitting.[35] The fitting and the concentration dependence of R_g were shown in Figure 3a. With the increase of AcOH concentration, $I(q)$ increased in small q range, which may result from the aggregation of protein to form large aggregates.[36] The upward increase of $I(q)$ in very dilute solution (i.e., 2 mg/mL solution) may be due to the trace amount of Z19 and Z22 dimer as observed in SDS-PAGE.[25]

We extracted the R_g information from the Guinier plot and presented the concentration dependence of R_g in Figure 3b. The R_g values monotonously decreased from 5.96 to 1.23 nm as zein

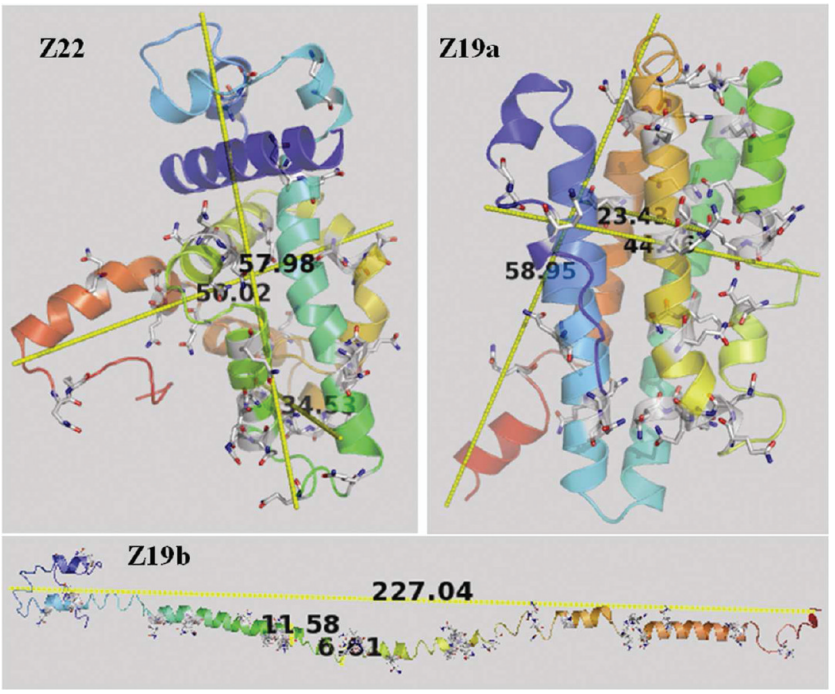

Figure 1. Cartoon illustrations of protein structure models. Z19a and Z19b are models for Z19 in folded and extended states, respectively. Glutamine residues were shown in stick to highlight their locations. Size measurements (unit, Å) were presented along the principal axis and the secondary axis was normal to the principal axis in each model. Plots were made using Pymol.

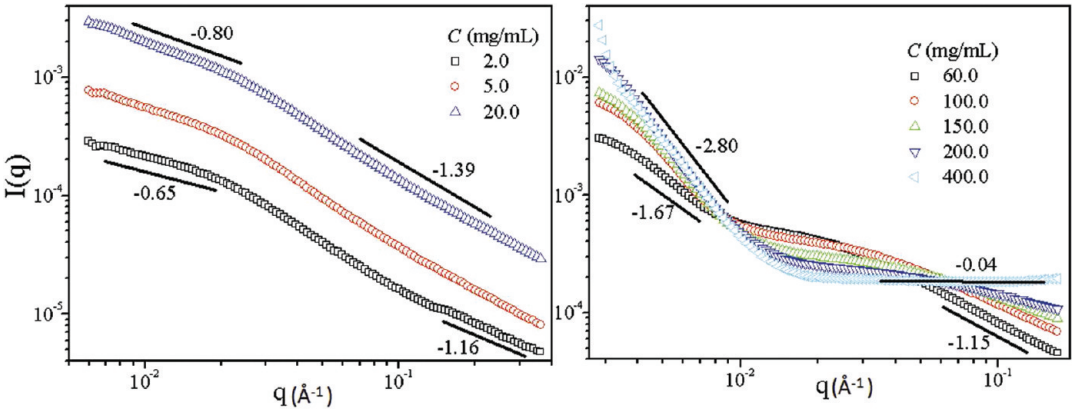

Figure 2. Scattering intensity profiles of zein/AcOH solutions with different zein concentrations. Scaling exponents were obtained through the best-fit in log−log plot, and the solid lines are used to guide the eyes.

concentration increased and bypass the size in native structures of ∼2.3 nm. Meanwhile, we collected the reported R_g values from SAXS measurements of α-zein aqueous methanol (70% methanol) solution[13] and aqueous ethanol (70% ethanol) solutions[14] and put in the same plot for comparison. It is clear that α-zein has a larger size in AcOH than in aqueous ethanol/methanol solutions, which indicated α-zein was more swollen in AcOH than in other solvents. This agrees with the observation by Selling and Woods, where AcOH has a higher performance to dissolve zein than other alcoholic solvents.[10]

Interestingly, we found the R_g of α-zein had two distinct scaling regions of concentration which can be analogous to the threshold between dilute and semidilute regions in polymer solutions. The threshold concentration is around 43 mg/mL. We estimated the bulk density of protein in dilute solution through $(3M/4\pi)N_AR_{g0}^{3}$,[7] with N_A the Avogadro's number, M the molecular weight which takes an average of Z19 and Z22, and R_{g0} is the radii of gyration of the α-zein at very dilute solution. The estimated bulk density of α-zein is 39.7 mg/mL by taking the R_{g0} from 2 mg/mL solution. The threshold concentration between two distinct scaling regions is very close to the bulk density of α-zein in very dilute solution, suggesting that the threshold can be regarded as C^*, the overlap concentration between dilute and semidilute solutions.

When the concentration of α-zein in AcOH solution is lower than C^*, R_g only slightly decreased with increasing concentration, and the scaling exponent is close to 0. It is suggested that α-zein dissolved in isolated form, and the intermolecular interaction could be negligible. Above C^*, the dimension of protein greatly decreased upon the increase of protein concentration. This may

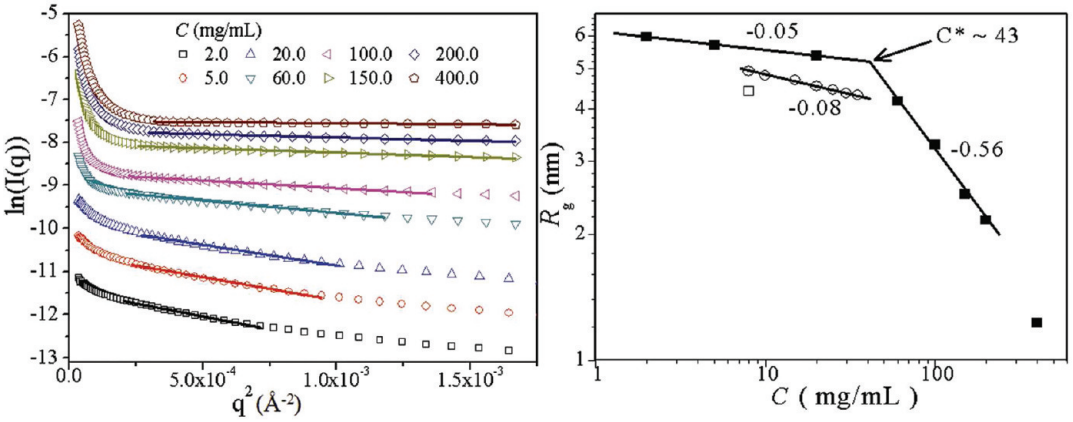

Figure 3. Guinier plots for SAXS scattering intensity profiles of α-zein in AcOH with various α-zein concentrations (a), and the concentration dependence of protein radius of gyration (R_g) in log−log plot (b). In (b), solid squares are measurements of α-zein in acetic acid solutions, the empty square is the measurement in aqueous methanol solution by Tatham et al.,[13] whereas the empty circles are from α-zein in aqueous ethanol solutions collected by Matsushima et al.[14]

lead to two consequences: (1) if each protein is still isolated, increasing protein concentration suppresses the volume (plus hydration layer) and (2) proteins are greatly unfolded and the intermolecular interpenetration occurs. The former case is normally seen in colloidal packing, while the latter one is often observed in polymer or polyelectrolyte solutions. In the latter case, R_g may be contributed by not only the whole protein but also some compact domains or structure motifs. In the 400.0 mg/mL solution, the R_g of zein protein in the solution decreased to 1.2 nm, a size much smaller than that of the native structure models (~2.3 nm), suggesting that the protein must be unfolded when protein concentration is high and the latter consequence would be followed. Since the R_g of zein molecules in the 400.0 mg/mL solution is so small that it is very unlikely contributed by isolated zein proteins, we got the scaling exponent of −0.56 in concentrated solutions without this point (it is −0.63 with this point). This scaling exponent is significantly larger than that of −1/8 in the semidilute neutral polymer solutions,[1,37] but close to that of polyelectrolyte solutions $(-1/2)$[3].

Aggregation of Zein Proteins. According to scattering theories, the scattering intensity in a monodispersed and spherically symmetric solution can be expressed by the product of the form factor $P(q)$ and the structure factor $S(q)$ as[38]

$$I(q) = \frac{\Delta\rho^2}{\Omega} P(q)S(q) \tag{5}$$

where $\Delta\rho$ is the difference in electron density between the solute and the solvent, Ω is proportional to the concentration of solute C, which equals the number of particles per unit mass of solute. In dilute solution, interparticle interactions can be neglected, and $I(q)_{C\to 0}$ can be regarded as purely contributed from P(q) [S(q)=1 in this case]. Therefore, the structure factor can be obtained through

$$S(q) = \frac{I(q)}{I(q)_{C\to 0}} \tag{6}$$

In practice, the measurement in very dilute solution is almost impossible because the scattering signal is too weak. Rather, to investigate the extra aggregation of zein proteins upon concentration increase above a given concentrated C_0, the structure factor $S(q,C)$ can be calculated through[39]

$$S(q, C) = \frac{C_0 I(q, C)}{C I(q, C_0)} \tag{7}$$

Here, $S(q,C)$ is also known as the solution structure factor[4] which has a value larger than 1, suggesting an extra-aggregation in a given correlation length range; otherwise, particles are less aggregated in this range. We selected 2.0 and 60.0 mg/mL α-zein AcOH solutions as two references for the structure factor calculation in dilute and in concentrated solutions according to eq 7, and the results were presented in Figure 4. As shown at low q region, the increase of α-zein in solution always led to the formation of large aggregates, and the extra aggregation becomes more pronounced in solutions with higher concentration. In 20.0 mg/mL solution, the extra aggregation takes place in a broad range with correlation length ranging from 4.3 to 40.9 nm. In all solutions above C^*, a common less aggregated gap (depleted layer) between 38.1 and 42.0 nm can be observed. This gap shifts to larger q values with concentration increase. The depleted layer may be contributed by the excluded volume effect of protein[40] in complementary to the formation of large α-zein aggregates as indicated in very small q range. Meanwhile, increasing α-zein concentration suppressed the correlation length between α-zein aggregates, thus shifting the gap to a smaller length. This result agreed with the finding of gap shifting to larger q values.

CD Measurements. CD measurements of zein AcOH solutions were presented in Figure 5. Compared to the typical absorption of secondary structure in far-UV range, helix has negative absorption peak at 209 and 222 nm.[41] Our CD profiles show peaks ranging from 227 to 237 nm as the protein concentration increased from 10 to 150 mg/mL. By extrapolating the peak to infinite dilute solution, the peak can be allocated at 224 nm which is very close to the peak for helix under ideal situation (Figure 6). Other peaks in far-UV lower than 210 nm were almost submerged by solvent adsorption. This is consistent with the report by Selling et al., which is the only reported study of CD measurements on zein as we know, that AcOH cannot be a solvent for far-UV measurement.[21] However, our results suggested that AcOH is capable of being a solvent in far-UV measurement as long as the wavelength is larger than 210 nm and AcOH is of high purity.

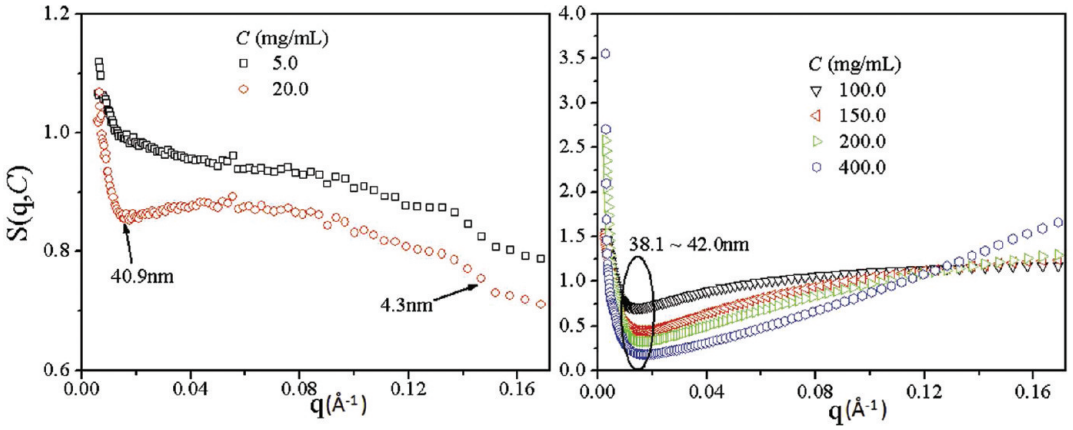

Figure 4. Concentration dependence of the structure factors from zein/AcOH solutions. Thresholds of correlation length accompany with extra-aggregation upon concentration increase were labeled using the relationship of $2\pi/q$.

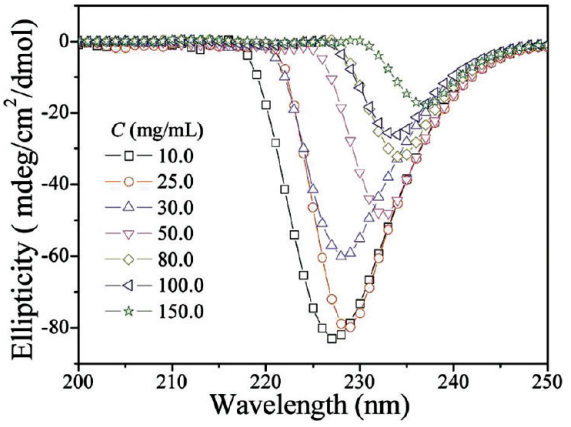

Figure 5. Circular dichroism (CD) measurements of zein/AcOH solutions at various zein concentrations.

Based on the plots of the peak position, the magnitude and the integral area from 217 to 250 nm of the adsorption peak against zein concentration, we found that the first two terms monotonically increase and the area decrease with the increase of zein concentration. All of the three trends suggest that helical secondary structure decrease as zein concentration increases. This result agrees with the SAXS experiments where the loss of secondary structure is concomitant with the unfolding of α-zein and the extra aggregation in broad range of correlation length upon the increase of zein concentration.

Rheology measurements. The shear rate and concentration dependence of the viscosities of α-zein AcOH solutions were shown in Figure 7. Most of the protein solutions exhibit Newtonian fluid where viscosity is independent of shear rate in a broad shear rate range from 10 to 100 s^{-1}. At a zein concentration above 150 mg/mL, the solution shows shear thinning at very high shear rate (>300 s^{-1}) and in a very low shear rate range from 2 and 8 s^{-1}. The shear thinning in concentrated solutions may result from high disperse phase fraction of α-zein aggregates.[42] We take the average viscosity in the plateau with a shear rate range from 10 to 100 s^{-1} and plot them against concentration. The viscosity of α-zein AcOH solutions shows two distinct scaling regions with a threshold concentration of 43 mg/mL. The threshold is identical to the one observed in the SAXS study.

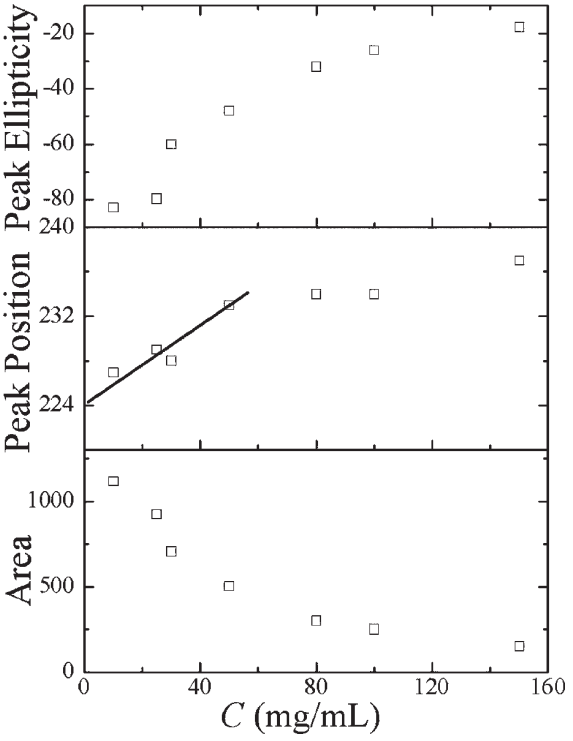

Figure 6. Peak intensities, the peak positions and the integrated areas of the absorption peaks in CD spectral profiles as a function of zein concentration.

The scaling exponent in dilute solutions of 0.49 well satisfies the Fuoss law[43] where the solution viscosity has a scaling of 1/2 against concentration increase of polyelectrolyte. The 1/2 is smaller than the one in neutral polymer solutions of scaling exponent 1, contributed to electrostatic repulsion and counterion regulation. In the second concentration region, the scaling exponent is 2.7 which is neither close to the scaling in polyelectrolyte solutions of 5/4 scaling nor in neutral polymer solutions with scaling of 15/4. Rather, it is quite close to the measurements by Selling et al.,[24] where the viscosity of zein in *N*,*N*-dimethylformamide (DMF) solution increases in a power fashion of zein concentration with power of 3.3. The scaling relationship is quite different from that of zein ethanol aqueous solutions, where the

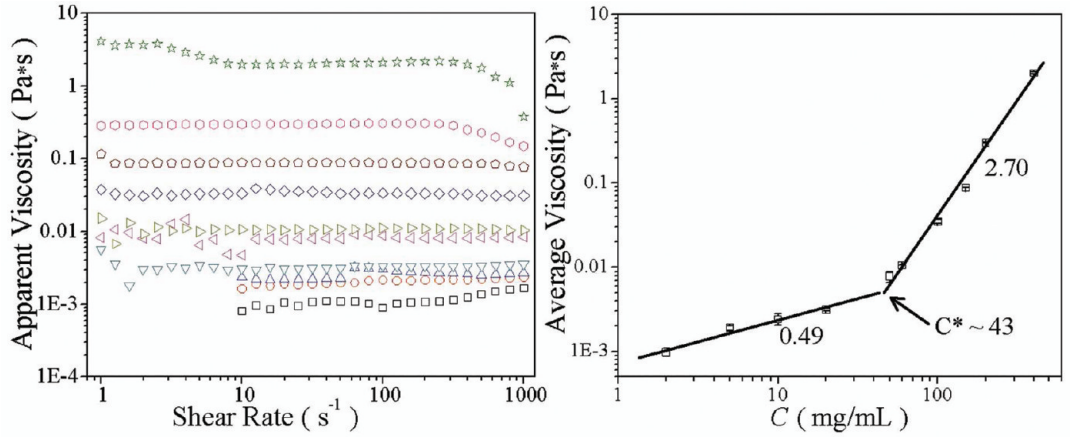

Figure 7. Viscosities of zein in AcOH solutions versus shear rate with zein concentrations of 2.0, 5.0, 10, 20, 50, 60, 100, 150, 200, and 400 mg/mL from bottom to top (left), and the logarithmic plot of viscosity against zein concentration in AcOH solutions (right). The solid lines are best fits to the range of points with a threshold.

Table 2. Comparison of Sacling Exponents in Different Zein Concentration Regions

models	neutral polymer	polyelectrolyte (free of salt)	our observation
κ (dilute)			−0.05
κ (semidilute)	−1/8	−1/4 (−1/2[a])	−0.56
λ (dilute)	1	1/2	0.49
λ (semidilute)	15/4	5/4	**2.70**

[a] Scaling exponent on the concentration dependence of correlation length.

logarithm of the solution viscosity linearly increases with zein concentration.[44]

DISCUSSION

Based on the results presented above, the concentration effect on α-zein AcOH solutions can be described as follows: in dilute region, zein protein keeps its individual shape and conformation, the majority of particles are made from single folded protein, and concentration variance in this region is sensitive to neither protein conformation nor solution viscosity; in the solutions with zein concentration higher than 43 mg/mL, proteins begin to overlap or even penetrate to each other due to crowding, concentration variation results in different degree of unfolding and aggregation, thus both the size of particles and solution viscosity show strong concentration dependency.

Consider the two distinct scaling regions for both the size of α-zein and the viscosity of the solutions, we summarized the scaling exponents from neutral polymer, polyelectrolyte solutions and our observation from α-zein solutions and shown in Table 2. Although three of the scaling exponents can be well explained through analogous to polyelectrolyte solutions, the viscosity scaling in the second concentration region still lacks convincing interpretation. This suggests more theoretical work should be done on such scaling behaviors in protein solutions to better understand the physical properties of protein solutions.

CONCLUSION

In summary, SAXS, CD, and rheology have been carried out on α-zein AcOH solutions over a wide concentration range. We found both the size of protein and the viscosity of solutions have two distinct scaling regions with an identical threshold concentration, and the threshold concentration is very close to the bulk density of α-zein in very dilute solution. Proteins can unfold and penetrate mutually with the increase of protein concentration. This finding is complementary to the traditional model for proteins in solutions where proteins are hardly or only slightly penetrable. Similar to the theoretically predicted scaling exponents for neutral polymers and polyelectrolyte solutions, the scaling behaviors in α-zein AcOH are closer to those in polyelectrolyte solutions. Meanwhile, no theory was able to interpret the scaling behaviors of α-zein in AcOH solutions, and further research is still needed to clarify the scaling behaviors of protein solutions.

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