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Aggregation and denaturation of antibodies: a capillary electrophoresis, dynamic light scattering, and aqueous two-phase partitioning study

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Abstract

Protein denaturation and aggregation are well-known problems in the pharmaceutical industry. As the protein aggregates, it loses its biological activity and creates problems in its administration to patients. In this paper, we explore the use of aqueous two-phase systems, capillary zone electrophoresis, and dynamic light scattering for the monitoring of protein denaturation and aggregation. Our studies focus on human IgG and HSA. Capillary zone electrophoresis was used to monitor changes in the charge to size ratio of the proteins upon denaturation and dynamic light scattering was used to detect the presence of any aggregates and to monitor the size of the proteins. The information obtained from aqueous two-phase partitioning is similar to that obtained from capillary zone electrophoresis. The simplicity of aqueous two-phase system and its low cost (compared to the other analytical techniques) suggest that it can be routinely used for the quality control of some pharmaceutical preparations.

Keywords: Dynamic light scattering; Aqueous two-phase systems; Partitioning; Aggregation; Denaturation; Immunoglobulin G

1. Introduction

In the biotechnology industry, protein aggregation is encountered routinely during purification, refolding, sterilization, shipping, and storage processes because of the presence of chemical and physical stresses. The presence of aggregates in a protein formulation causes a decrease in the efficiency of the drug and potential problems in its intravenous administration. Even though the protein preparation may be aggregate-free after the last polishing step has been completed, aggregates can form during storage. Aggregation levels as low as 1% over a 2-year shelf life can render a product clinically unacceptable [1]. Therefore, monitoring aggregation is an essential quality control practice for any protein-based pharmaceutical.

Both, native and denatured proteins may aggregate. Aggregation of native proteins is common upon relatively long storage periods. For example, it is a well-known fact that small amounts of dimers and oligomers form when antibodies are stored. Aggregation of native proteins may also occur during their freeze drying, a common polishing step in any bioseparations process. Aggregation of denatured proteins is extremely common and is of particular importance in the recovery of genetically engineered proteins present as inclusion bodies in the cell cytoplasm. Inclusion bodies are solubilized using high concentrations of denaturants and are then diluted to allow the proteins to refold. During the refolding process aggregation occurs, decreasing the recovery of the active protein.

Some common techniques used to measure the extent of denaturation and/or the formation of aggregates are circular dichroism, infrared spectroscopy, differential scanning calorimetry, NMR spectroscopy, fluorescence spectroscopy, capillary electrophoresis, second derivative UV spectroscopy, mass spectroscopy, and light scattering. It has become common knowledge that in order to provide accurate information on protein aggregation the use of some form of light scattering is essential. A technique that has been routinely used to measure hydrophobicity and charge of proteins and to determine their isoelectric point, but whose use in studies of protein stability has been quite limited [2–4], is aqueous two-phase systems. Because of its simplicity and its sensitivity to protein size and charge, aqueous two-phase system offers an alterna-

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tive to other more expensive and cumbersome analytical techniques.

We believe that a single analytical technique cannot provide a complete picture of protein denaturation and aggregation. Therefore, we have chosen to use capillary electrophoresis, dynamic light scattering, and aqueous two-phase partitioning to study the denaturation and aggregation of IgG. Our choice of protein reflects its current and potential uses as diagnostic tools and in treatment of human diseases [5]. The information supplied by these three methods complements each other well and provides a good assessment of the state of the proteins as they denature and aggregate. In the following paragraphs we present some background information on each of the three techniques.

Although capillary electrophoresis is mainly a separations technique, there has been a growing interest in its use to monitor changes in protein structure. There have been some studies about the use of capillary electrophoresis to monitor protein folding and protein aggregation [6–10]. Dai and Krull [7] investigated the conformational changes of a monoclonal antibody exposed to high temperatures for various periods of time. In another example, Stellwagen et al. [8] compared the folding and stability of a wild type, mutant, or post-translationally modified horse heart ferricytochrome *c*, bovine pancreatic ribonuclease, and egg white lysozyme using capillary zone electrophoresis. Finally, Righetti and Verzola [6] presented some evidence that suggests that capillary electrophoresis can be used to monitor protein aggregation.

A full explanation of the fundamental principles of dynamic light scattering is outside the scope of this paper. We suggest the reader refer to one or more of the numerous references strictly devoted to this topic available in books [11], websites [12], journals [13], etc. for details. Dynamic light scattering is mainly used to determine particle size. This is achieved by measuring the diffusion coefficient of the particles in a liquid solution and then using the diffusion coefficient to determine the size. As dynamic light scattering is used to determine particles size it has become very useful to investigate protein aggregation. For example, Rosenqvist et al. [14] used dynamic light scattering to study the aggregation kinetics of human IgG and Joessang et al. [15] used dynamic light scattering to study the kinetics of heat-induced aggregation of human IgG.

Aqueous two-phase system is a separations and analytical technique that exploits the uneven distribution of solutes between two polymers in solution or a polymer–salt solution at thermodynamic equilibrium. Aqueous two-phase systems are sensitive to both the size and charge of the molecules being partitioned. Although there is a myriad of polymers and salts that can be used to generate aqueous two-phase systems, the most widely used ones are poly(ethylene)glycol (PEG)–dextran (Dx) and PEG–sodium phosphate. For a complete understanding of aqueous two-phase partitioning the reader is referred to Bermudez and Forciniti [16], Forciniti [17], and Forciniti et al. [18]. The simplicity of the method and its low cost (compared to the other analytical techniques) provide a nice alternative to study protein denaturation.

Proteins can be denatured in a variety of ways. We chose to use urea, which is a chemical that is commonly used for this purpose. It is known that urea binds to the carbonyl groups of the polypeptide chain, disrupting helical structures. Urea is also a chaotropic compound that disrupts water structure, making water more “hydrophobic”. The literature is flooded with information on protein denaturation by urea. For example, as far back as 1978 Lapanje et al. [19] did a thermodynamic study on the isothermal interaction of human IgG with urea, and more recently, Gonzalez-Jimenez and Cortijo [20] used urea to denature unlabeled human serum albumin and a similar albumin labeled with acrylodan. This method of denaturation has become well-known and well-understood within our current knowledge of the denaturation
process.

To facilitate the interpretation of our results with the antibody we performed the same studies with a “simpler” protein, human serum albumin (HSA). HSA has a molecular weight of ∼69,000 and a pI of ∼4.9. This protein is not as complex as an antibody; therefore, interpreting its behavior under denaturing conditions should be easier. However, even for such a simple protein, interpretation of the results must be done with caution.

2. Experimental

2.1. Materials

Human IgG, HSA, acrylamide, urea, decamethonium bromide (DMB), borax, boric acid, PEG, Dx, hydrochloric acid, and sodium chloride were all purchased from Sigma Chemical (St. Louis, MO). Phosphate buffer (pH 8) and all capillary electrophoresis materials (capillaries, vials, coolant, etc.) were purchased from Beckman Coulter (Fullerton, CA).

2.2. Methods

Capillary zone electrophoresis was performed on a Beckman Coulter P/ACE MDQ Glycoprotein System equipped with a UV detector and 32 Karat (version 4.0) Software (Fullerton, CA). All experiments were performed in an uncoated 120 cm by 75 μm i.d. capillary using a run buffer of 50/50 0.1 M borate/0.02 M phosphate containing 100 mM DMB, which was added to reduce the electroosmotic flow and prevent protein interactions with the capillary wall [21]. The temperature was maintained at 22 °C, a separation voltage of 27 kV was applied, and samples were injected for 30 s under an applied pressure of 0.5 psi and detected at 200 nm. Thorough rinsing and equilibration of the capillary was performed both before and after each run with the same buffer used as the run buffer. The experimental running conditions were held constant for all experiments.

HSA and IgG were prepared as 4 mg/ml samples in the 50/50 0.1 M borate/0.02 M phosphate run buffer. Urea was added to each of the protein solutions to give samples that were 8, 6, 4, and 2 M in urea. Run buffers containing the same amounts of urea as the samples were also prepared. Each sample was run twice as well as repeated at later times to ensure consistent and reproducible results.

In order to account for the increase in solution viscosity, literature values for solutions of urea at 22 °C were obtained from Singh and Ram [22] and Kawahara and Tanford [23] and then these values were used to calculate apparent and modified mobilities for each protein. Acrylamide, a neutral analyte that does not interact with the capillary walls, was run under the same experimental conditions as the proteins to provide a direct measure of electroosmotic flow. The modified mobility of acrylamide was then subtracted from the modified mobility of each protein sample to calculate the true mobility of each protein.

The dynamic light scattering experiments were performed on a fiber-optic quasi-elastic light scattering (FOQELS) instrument from Brookhaven Instruments Corp (Brookhaven, NY). The FOQELS uses an 800 nm solid state laser (70 mW) with a fixed scattering angle of $Q = 155^{\circ}$ and a digital auto-correlator. The delay time intervals are not linearly spaced which allows broad distributions to be sampled properly. The same samples used in the capillary electrophoresis experiments were poured into a 4.5 ml rectangular plastic cuvette and placed inside a constant temperature holder which is capable to control the temperature in the range of 5–75 °C on steps of 0.1 °C. The temperature was maintained at either 22.5 or 25 °C. Data was taken and collected in 3 min intervals for a total of 12 min for each sample. Each sample was run twice as well as repeated at later times to ensure consistent and reproducible results. The method of cumulants was used to analyze the data. When a distribution of sizes is present, the effective diameter can be obtained by averaging the intensity-weighted diameters. In FOQELS, a multi-modal size distribution analysis is available through a non-negatively constrained least squares approach.

Preparation of the aqueous two-phase systems and partitioning of the proteins was done as follows: (1) urea was dissolved in percentage of 50/50 0.1 M borate/0.02 M phosphate buffer, pH ∼8.7, at concentrations of 2, 4, 6, and 8 M; (2) solid PEG 1450 and Dx 71,000 were added to each solution to reach concentrations of 14%, for each polymer; (3) the pH of each buffer solution was brought down to pH 8 with 3 N HCl; (4) 1 mg of each protein was dissolved in 1 ml of each buffer containing urea; (5) 1 ml of protein solution were added to each aqueous two-phase system; (6) the systems were shaken for 15 min on a rotary shaker at 20 rpm and then centrifuged for 25 min at 10,000 × g and 20 °C to ensure complete phase separation; and (7) approximately 1 g samples were removed from each phase, properly diluted, and absorbance readings at 280 nm were taken. The final concentrations of urea in the tubes were: 0, 8.64, 17.28, 25.9 and 34.6% (w/w). All experiments were done in duplicate.

3. Results

HSA and IgG were exposed to urea concentrations ranging from 2 to 8 M in the absence of a reducing agent. Although most proteins are completely denatured in 8 M urea, it is generally accepted that there is a critical, protein-dependent, urea concentration at which proteins completely unfold. For HSA the concentration is ∼5 M [24], whereas for IgG is ∼3 M [19]. It is also known, from the copious work on inclusion bodies, that a high concentration of urea eliminates or minimizes protein aggregation, whereas a low concentration of it favors aggregation [25]. What is, by far, less clear is the magnitude in the charge change of the protein upon denaturation. For example, there are several reports in which capillary electrophoresis has been used to monitor protein denaturation but all the observations have been interpreted only according to changes in the size of the protein. This has been done in spite of evidence that shows a substantial change in the isoelectric point of some proteins upon denaturation [24]. The fact that the electrophoretic mobility is far more sensitive to charge than to molecular size suggests that researchers may have oversimplified the interpretation of their results.

A realistic picture of the effect of the denaturant on protein structure and on protein aggregation can be obtained by using a battery of techniques that offer supplementary information. As electrophoretic mobilities depend on the charge-to-size ratio of the analyte, it is impossible to determine changes in either size or charge by using capillary zone electrophoresis. Therefore, we measured sizes and size distributions at the various urea concentrations by using dynamic light scattering, which for dilute samples yields hydrodynamic sizes. This allowed us to uncouple possible changes in charge from changes in size; and therefore, to better rationalize the results. In addition, we also measured the distribution of the proteins in aqueous two-phase systems containing urea. As the partition coefficient depends on both charge and size of the analyte, it should yield information similar to capillary electrophoresis but at a much lower cost.

Before mobilities can be extracted from capillary zone electrophoresis experiments, the electroosmotic flow must be either eliminated or quantified. In our view, the electroosmotic flow cannot be totally eliminated, even by the addition of electroosmotic flow retardants or by using coated capillaries. Therefore, the electroosmotic flow was measured by monitoring the mobility of acrylamide, a neutral marker that does not interact with the walls of the capillary. Two modified mobilities (relative to the viscosity of water) of acrylamide are presented in Fig. 1. One corresponds to experiments where the running buffer pH was held constant at 8.7, whereas the other corresponds to experiments where the running buffer pH was not adjusted (Table 1). The variation in pH is the result of the addition of urea to the run buffers, which causes an increase in their pH because of its basic nature. The electroosmotic flow mobility is a function

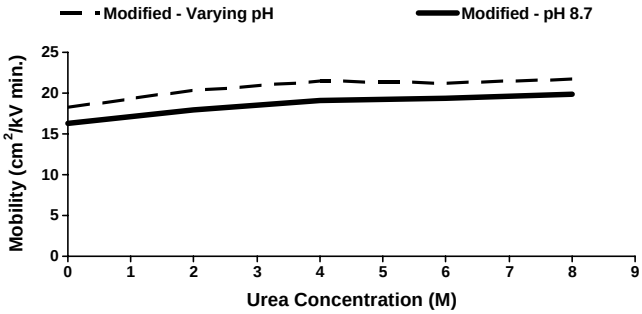

Fig. 1. Modified (relative to the viscosity of water) mobility of acrylamide vs. urea concentration. The solid line corresponds to samples whose pH has been maintained constant. The dashed line corresponds to samples whose pH depends on the urea concentration as shown in Table 1.

of the potential on the capillary surface and the viscosity and dielectric constant of the buffer. It has been found that the electroosmotic flow increases non-linearly with the buffer's pH and is directly proportional to the product of the dielectric constant of the solvent and the zeta potential. As the zeta potential increases with increasing pH [26], the electroosmotic flow should increase with an increase in urea concentration when the pH is not adjusted, as observed. The mobility observed in experiments where the pH was not adjusted was higher than in those experiments whose pH was adjusted. This is a consequence of a faster electroosmotic flow at higher pHs. A similar increase in the mobility of acrylamide was observed when the pH of the buffer was adjusted. When the pH of all running buffers containing urea was the same, the increase in the mobility of acrylamide, as the urea concentration increases, is due to an increase in the dielectric constant of the medium. Chitra and Smith [27] reported an increase in dielectric constant of urea solutions from 78.5 (pure water) to 96.4 (7.8 M urea), or an increase of about 23%. The observed mobilities of acrylamide increase from 16.2 cm^2/kV min (buffer) to 19.9 cm^2/kV min (8 M urea) or by 22.8%, which agrees well with the expected increase due to an increase in dielectric constant. Because of insufficient data, the mobilities were not adjusted by a change in the dielectric constants. However, this does not present a problem because, as the mobilities of the analytes are adjusted by electroosmotic flow, any variation in dielectric constants is cancelled out.

The measured electroosmotic flow, or rather the modified mobility of acrylamide, compares well with values reported in the literature. Bello et al. [28] reported electroosmotic

Table 1
pH of various urea solutions

Urea concentration (M)	pH
0	8.78
2	8.81
4	8.97
6	9.08
8	9.18

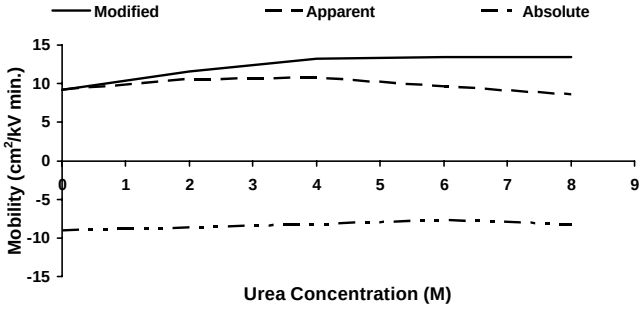

Fig. 2. Modified, apparent, and absolute mobilities of HSA vs. urea concentration. The pH at each urea concentration is shown in Table 1.

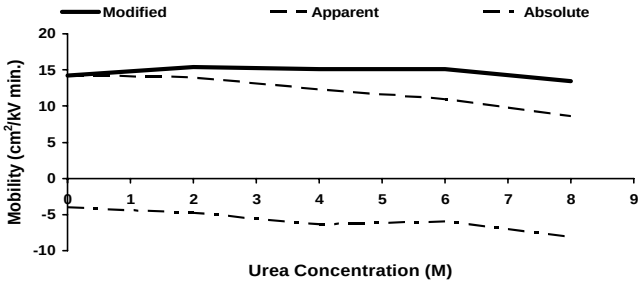

Fig. 3. Modified, apparent, and absolute mobilities of IgG vs. urea concentration. The pH at each urea concentration is shown in Table 1.

flow mobilities using neutral analytes for different applied voltages and solutions of different pH. They used a capillary similar to ours in their experiments; however, they did not specify its length. The data set obtained by Bello et al. using 5 mM acetic acid/7.5 mM Tris combined run buffer at pH 8 is the closest to our running conditions. They obtained an electroosmotic flow value of approximately 2.0×10^{-4} cm^2/Vs for that system that compares well with our values of 2.7×10^{-4} and 3.3×10^{-4} cm^2/Vs for the system held at constant pH and 3.0×10^{-4} and 3.6×10^{-4} cm^2/Vs for the system of varying pH.

Fig. 2 shows the apparent (includes electroosmotic flow), modified (relative to the viscosity of water) and absolute mobilities for HSA; the running buffer pH was not kept constant. The mobilities for IgG are shown in Fig. 3 (variable pH) and Fig. 4 (constant pH). As the effects of viscosity and electroosmotic flow have been taken into account, absolute

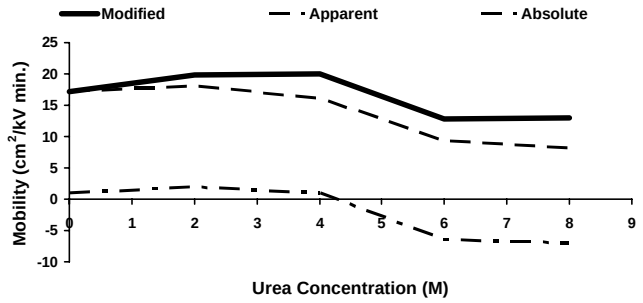

Fig. 4. Modified, apparent, and absolute mobilities of IgG vs. urea concentration. The pH is 8.7 for all urea concentrations.

mobilities are a measure of the physical-chemistry characteristics of the molecules; and therefore, are the quantities to be considered in this work. In each of the plots all the absolute mobilities are negative. This is because the electroosmotic flow moves the fluid from the anode to the cathode but the negatively charged proteins move from the cathode towards the anode.

The mobility of HSA remains nearly constant for all urea concentrations. We would have expected the mobility of the protein to change as the urea concentration is increased because of changes in the size and charge of the protein. Candiano et al. [24] found that at 8 M urea the isoelectric point of HSA increases from 4.9 to 6.1 in the native state, which indicates that the net negative charge decreases. Therefore, the mobility of the denatured protein should increase. In addition, the protein is unfolding and thus becoming larger. This increase in protein size should slow down its mobility. Finally, the negative charge of the protein is also increasing because the pH of the solution is increasing with urea concentration; this should result in an increase in HSA mobility. All these effects seem to cancel one another out and hence the mobility remains nearly constant. In summary, it is possible that the absence of an abrupt transition in mobilities is due to a change in charge of the protein that is compensated for by an increase in its size. Therefore, the charge to mass ratio remains constant and hence so does the mobility.

The mobility versus urea concentration plots for IgG without pH control are shown in Fig. 3. It is obvious that a clear transition between the native and the unfolded state does not exist. The mobility of IgG should increase with increasing urea concentration because: (1) the pH increases as the urea concentrations increases; and (2) the working pH is higher than the isoelectric point of IgG. In contrast, the size of the protein may increase as the urea concentration is increased, which implies that the mobility of the protein should decrease. The net effect is an increase in IgG's mobility because the effect of charge plays a larger role than size on mobility. However, it is impossible to say if there is a net increase in charge of the protein besides the one caused by an increase in pH.

The absolute mobility of IgG at constant pH shows an abrupt transition at a concentration of 4 M urea (Fig. 4). This is in reasonably good agreement with the reported transition value of 3 M urea [19]. However, it has been claimed that antibodies do not exhibit a sharp denaturation transition but rather a smooth one. As the pH is held constant, the abrupt change in mobility can only be due to an increase in the charge of the antibody that is not compensated for by an increase in its size. So, it is apparent that although conformational changes, as indicated by changes in size or in secondary structure content, may be smooth; the change in the charge of the protein is not. One odd feature about these data is that the mobility remains slightly positive until a 4 M concentration of urea is reached, which can never occur because the protein is negatively charged. However,

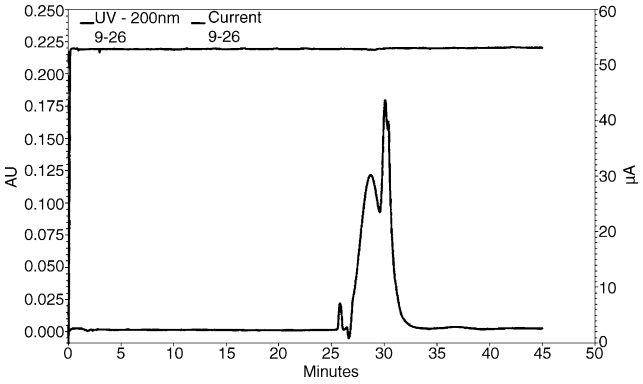

Fig. 5. Electropherogram of IgG in the absence of urea.

this merely reflects small errors in the measurement of the electroosmotic flow.

The electropherograms in Figs. 5 and 6 are for native and denatured IgG, respectively. The multiplicity of peaks reflects the heterogeneity of the antibody. IgG consists of four isotypes: IgG1, IgG2, IgG3 and IgG4 whose relative abundance in serum is 60–65, 20–25, 10, and 5%, respectively. A definitive identification of the peaks is not possible without resorting to, for example, mass spectroscopy. We have run pure IgG1, IgG2 and IgG3 and their mixture under the same electrophoretic conditions. The three proteins showed very close retention times and when seeded together they produce a broad peak. The negative peak observed just before the broad peak has been also found by Righetti and Verzola [6]. They argued that it may be due to the sequestering of ions from the buffer by large aggregates. However, the dynamic light scattering results do not confirm that hypothesis (see next paragraph). Moreover, the aggregates seem to decrease in number as the urea concentration increases, whereas the negative peak becomes more pronounced. Puzzling enough, no negative peak was observed when pure IgG1–3 were seeded individually or in a mixture. There is a distinct change in peak shape and size as the urea concentration increases, yet the number of peaks does not change. This suggests that even if aggregates are present under some

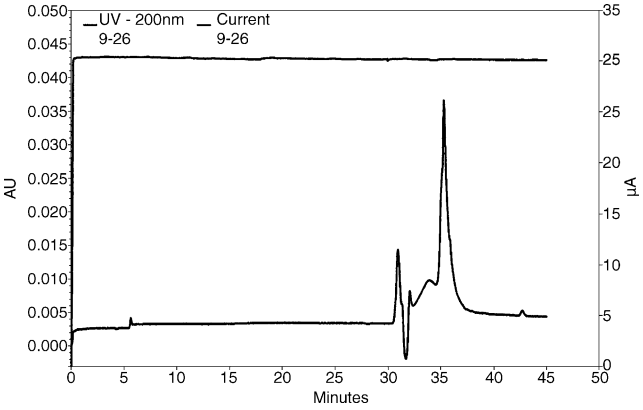

Fig. 6. Electropherogram of IgG in 8 M urea.

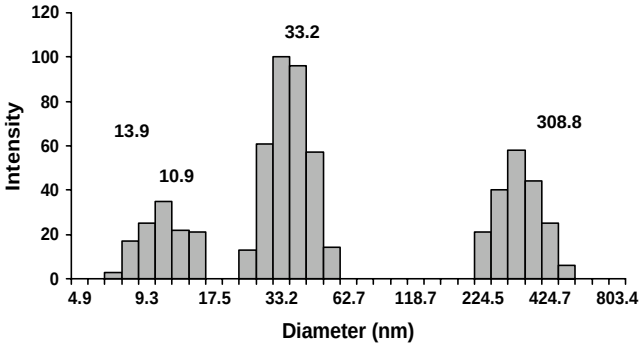

Fig. 7. Histogram showing the size distribution of an IgG solution in the absence of urea. The *y*-axis is the intensity average of the scattered light. The *x*-axis is the absolute size of the scatters obtained by a multimodal fitting of the time correlation function.

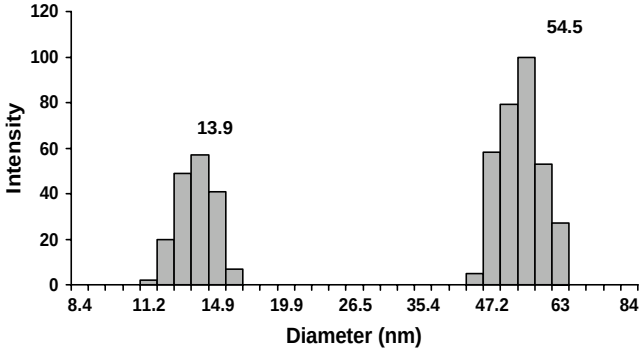

Fig. 8. Histogram showing the size distribution of an IgG solution in 8 M urea. The *y*-axis is the intensity average of the scattered light. The *x*-axis is the absolute size of the scatters obtained by a multimodal fitting of the time correlation function.

conditions but absent under others capillary electrophoresis is not able to capture those differences in the composition of the mixture. It is important to notice the transition that the protein goes through in between 0 and 8 M urea. The electropherograms for 2, 4 and 6 M urea all look similar to the one with no urea, with a few exceptions: (1) the third peak becomes more of a spike and remains that way until a urea concentration of 8 M is reached where it broaden; (2) the second peak is reduced to a hump and continues to flatten out as the urea concentration is increased; and (3) the first peak remains nearly the same until 8 M is reached where it grows in size. The elecropherograms for HSA change significantly as urea concentration is increased. The denaturation of HSA by urea is reflected in the electropherograms as a well defined split peak in the absence of urea that changes to a short and broad peak, which is a likely indication of aggregation. The change in the electropherograms occurs abruptly after a concentration of 2 M urea is reached. This is in disagreement with the value of 5 M that has been suggested as the highest urea concentration that HSA may tolerate. Although 5 M urea may be needed to observe changes in the secondary structure of HSA, lower urea concentrations may induce a change in tertiary structure. Aggregation of HSA upon addition of urea is expected because of the hydrophobic nature of this protein; i.e., it is not necessary to expose the hydrophobic amino acids buried in the interior of the protein to induce aggregation [29].

A clearer picture of the changes in protein size and of the presence or absence of aggregates as well as their relative abundance is given by the dynamic light scattering results. Figs. 7 and 8 are histograms of the size and size distribution of IgG with and without urea. The diameters reported are intensity averages; i.e., the diffusion coefficients of the different scatters are weighted according to the magnitude of the intensity of the light that they scatter. It is also possible to calculate number, volume, and surface averages by assigning weights to the diffusion coefficients of the scatters by their number, volume or surface area, respectively. Number and volume yield information about the number of molecules present of a certain size. Intensity averages are reported here because they are extremely sensitive to the presence of aggregates. Number or volume averages of both histograms show that the amount of aggregates in the samples is <1%.

The reported size of native IgG is ~10 nm [29], which is comparable to the 10.9 nm, obtained in our experiments. Fig. 7 also shows small, most likely dimers, and larger aggregates present in the commercial IgG sample. However, the dimers and larger aggregates present only represent a small fraction of the total number of molecules (~1%), as confirmed by number average calculations. The presence of these aggregates is common and known to form during storage. A comparison between Figs. 7 and 8 shows that IgG denatures in the presence of 8 M urea as indicated by the increase in monomer size from 10.9 nm in its native state to 13.9 nm in an unfolded state. It is also clear from both figures that high concentrations of urea break apart the large aggregates.

Figs. 9 and 10 show the increase in monomer and aggregates sizes of both HSA and IgG, respectively, as the concentration of urea is increased. Both proteins maintain

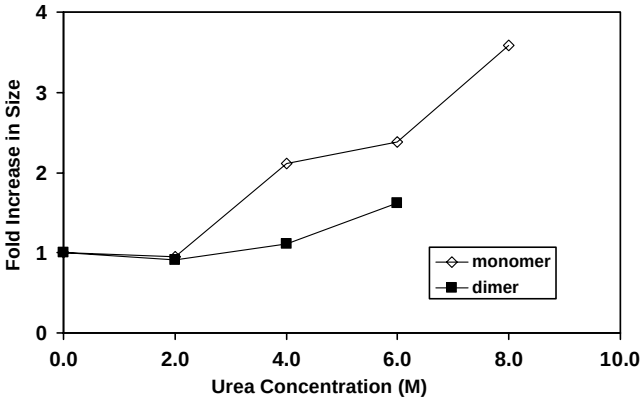

Fig. 9. Variations in the size of the monomer and dimer of IgG vs. urea concentration. The fold increase in the size of the protein (actual size/size under native conditions) is plotted against urea concentration.

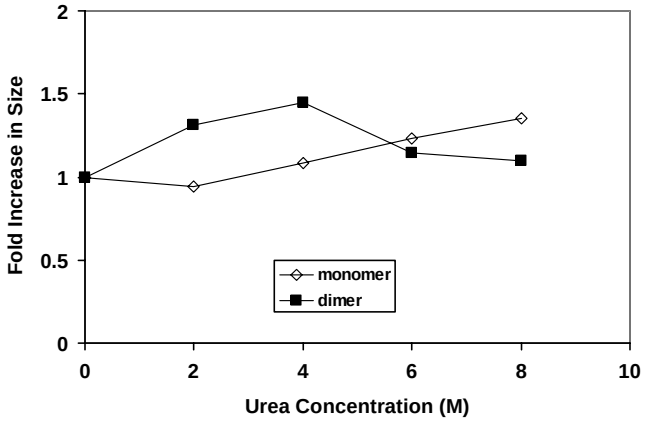

Fig. 10. Variations in the size of the monomer and dimer of HSA vs. urea concentration. The fold increase in the size of the protein (actual size/size under native conditions) is plotted against urea concentration.

their native size until the addition of just past 2 M urea. IgG shows a smooth increase in size, from ∼11 to ∼15 nm. This is consistent with observations made in the past. Lapanje et al. [19] found, using calorimetry data, a smooth transition for IgG from its native state to a fully denatured state when exposed to denaturing concentrations of urea. It must be noticed that it is only the size of the protein that undergoes a smooth transition. The size of the aggregates (possible a dimer) remains almost constant up to 6 M. Although the transition for HSA is not as smooth, the same trend is observed. Its size is increased by almost four-fold from 0 to 8 M urea. The size of the fully denatured protein agrees well with the value of 25.8 nm reported by Tanford et al. [30] for the end-to-end distance of albumin denatured in 6 M guanidine hydrochloride. Again, the size of the aggregates (possible a dimer) remains nearly constant for all urea concentrations. This may indicate that the aggregates are formed by already denatured protein.

Fig. 11 shows the partition coefficients versus urea concentration for both IgG and HSA. All aqueous two-phase

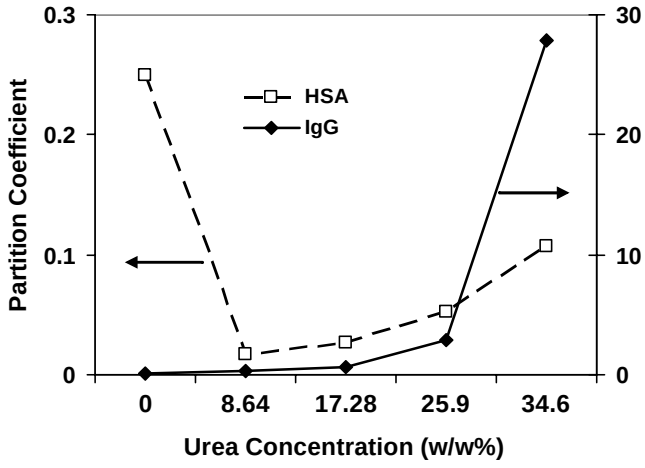

Fig. 11. Partition coefficients of IgG and HSA vs. urea concentration.

system were held at a constant pH of 8; therefore, the only three factors affecting the partitioning behavior of the proteins were: (1) the amount of urea present; (2) the subsequent exposure of the buried amino acids as denaturation proceeds, which results in an increase in the surface hydrophobicity of the proteins and, possibly, a change in their charge; and (3) possible changes in the phases' compositions caused by the addition of urea. The volumes of top and bottom phases remained unchanged upon the addition of urea, which indicates that the composition of the equilibrated phases does not appreciably change upon the addition of the denaturant. This is in agreement with the phase diagrams reported by Raemsch et al. [4]. Although the molecular weights of the polymers used by Raemsch et al. are not same as the ones used in this work, the relatively high polymer concentrations used in our work places our systems in a region in which changes in tie-line length with urea concentration are not significant. Therefore, the only two factors to take into account in the analysis of the results are items (1) and (2) above. The partition coefficient (K) is defined as the concentration of protein in the top phase divided by the concentration of protein in the bottom phase and is correlated with the charge of the protein (and therefore the pH of the solution) by:

$$\ln K = \ln K_0 + \Gamma z \quad (1)$$

where K_0 is the partition coefficient at zero charge, z is the protein's charge and Γ is a coefficient that depends on the type of protein, the type of phase system, and the type and concentration of salt used. The salt used in our investigations was sodium phosphate, which has a Γ value of -23 [31]. The partition coefficient of HSA remains small with its highest value of 0.25 at zero urea concentration. Once the concentration of urea reaches approximately 9% (w/w), the partition coefficient drops to nearly zero and only slightly increases to 0.1 as the concentration of urea is increased to ∼35% (w/w). This indicates that both native and denatured HSA remain in the bottom, Dx-rich, phase. However, because the partition coefficient is so small, no change in size or charge could be detected. IgG behaves rather differently. Its partition coefficient remains nearly zero until a urea concentration of 17% (w/w) is reached where it increases slightly to about 3 at approximately 26% (w/w) urea. There is a large increase in the partition coefficient from 25.9 to 34.6% (w/w) urea (K ∼25 at 34.6% (w/w) urea). We can conclude that the native antibody partitions preferentially into the Dx-rich phase but once it is fully denatured it prefers the PEG-rich phase. A well-known mechanism that is used to explain partitioning in aqueous two-phase system is exclusion volume. As the antibody is increasing in size, exclusion volume arguments would predict that as the denaturation proceeds K_0 should decrease—the presence of aggregates should have a similar effect. However, if upon denaturation, the net protein charge becomes more negative, the second term in Eq. (1) will increase. The combined effect is an increase in K, as we observed. This does not rule out a change in number and size of aggregates. It is a well-known fact that if aggregates

are of sufficient size (>200 nm) they have a tendency to accumulate at the liquid–liquid interface. Thus, their presence may go unnoticed.

The rationalization of our results is highly simplified when inputs from the three techniques are considered together. The partitioning experiments indicate that there is a change in the charge of the antibody upon denaturation and that the change occurs at approximately 4 M urea. This result is confirmed by the capillary electrophoresis experiments, which suggest an increase in charge of the protein when the urea concentration reaches 4 M. A straightforward connection between the observed changes in mobility and the structural changes of each protein that occur upon denaturation does not seem to exist. The partitioning results for HSA are less conclusive. The partition coefficients of this protein barely change with the addition of urea, at least at the conditions selected for this work. Although capillary electrophoresis in combination with dynamic light scattering confirms a change in the charge of the protein; this is clearly overwhelmed by a change in its size, which obscures the aqueous two-phase system results. Neither capillary electrophoresis nor aqueous two-phase systems are sensitive enough to univocally detect the presence of aggregates or even minor changes in the size of the proteins. This information is neatly supplied by dynamic light scattering. The dynamic light scattering experiments allow us to follow the size of the monomer of each protein through the denaturation process and to detect the presence of aggregates. Although aqueous two-phase system partitioning is much cheaper and straightforward to use than capillary electrophoresis, it seems to be able to detect large changes in charge/surface area ratio of the protein. An overall advantage to using these techniques is that not one of them requires extensive analysis times.

4. Conclusions

We have investigated the denaturation and possible aggregation of two commercially available proteins, IgG and HSA. The three techniques used by us to follow denaturation and aggregation offer complementary information. For example, capillary electrophoresis captured the changes in size-to-charge ratio of the protein that are likely to occur upon denaturation. However, the presence of aggregates was not easily detected by capillary electrophoresis. On the contrary, dynamic light scattering, that is insensitive to charge but extremely sensitive to the presence of aggregates, provided information about changes in the size of the proteins. Finally, aqueous two-phase system provided similar information to capillary electrophoresis. Aqueous two-phase systems are sensitive to protein size, and therefore, they should be sensitive to the presence of aggregates. However, aggregates may go unnoticed if they accumulate at the liquid–liquid interface.

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