Protein aggregation, particle formation, characterization & rheology

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A B S T R A C T
In this review, we attempt to give a concise overview of recent progress made in mechanistic understanding of protein aggregation, particulate formation and protein solution rheology. Recent advances in analytical techniques and methods for characterizing protein aggregation and the formed protein particles as well as advancements, technique limitations and controversies in the field of protein solution rheology are discussed. The focus of the review is primarily on biotherapeutics and proteins/antibodies that are relevant to that area. As per the remit of Current Opinion in Colloid and Interface Science, here we attempt to stimulate interest in areas of debate. While the field is certainly not mature enough that all problems may be considered resolved and accepted by consensus, we wish to highlight some areas of controversy and debate that need further attention from the scientific community.

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1. Introduction
The development of stable protein-based formulations with controlled rheological response is an area of high interest for the high-growth biotechnological industry, as well as for more traditional industrial sectors such as foods. Although the final applications in these two industrial sectors are very different, the complex self-assembly and particle formation processes under various formulation conditions (pH, ionic strength, buffer salts, temperature) must be well-understood, characterized, and controlled. This then allows the development of formulations which remains stable with long shelf life and that exhibits rheological properties that enhance/optimize the application performance — e.g. processing, delivery through injection in the case of therapeutic proteins, and texture/sensory features in the case of foods. The early detection and characterization of protein particles or aggregates — their size, structure, morphology, interactions and rheology in therapeutic protein formulations are critical to reduce safety issues (e.g. immunogenic response in biologics) and to ensure stability and optimized delivery etc. [1–4]. In food based systems, the food protein self-assembly, microstructure and resulting rheological properties must be characterized and controlled in order to ensure optimized textual/sensory experiences for the consumer and ensure issue-free processing [5, 6]. Due to the multiple length scales and time scales of interest in protein aggregate formation, the need arises for different techniques that span these wide ranging length and time scales. This article will review the progress made in the understanding of protein particle formation and advances made in analytical techniques and analysis methods that allow the development of new insights into the formation of protein particles and their corresponding properties-size, structure, microstructure, and rheology.

2. Native and non-native aggregation: reversible and (effectively) irreversible aggregates
Proteins can self-assemble in a number of ways. They can form highly specific, structured complexes such as receptors with ligands [7], multimeric native states with or without metal complexation [8,9], and multi-protein “machines” such as the ribosome [10]. Those types of protein complexes typically have sufficiently strong inter-protein interactions that one must work at extremely dilute conditions in order for the complex to not be the natural or “native” state. We do not review such systems explicitly here, as a majority of pharmaceutical proteins currently or recently in development do not associate so strongly unless it is via non-native conformers [11–13]. When self-association of native or folded proteins occurs in pharmaceutical products or model proteins that mimic pharmaceuticals, it primarily occurs via transient and relatively weak interactions that require one to work at high concentrations (on the order of 10⁻³ M or larger) [14–18]. In this case, one might consider an array of possible aggregate species (dimers, trimers, tetramers, etc.) that interchange with one another dynamically. These species are typically easily reversed simply by moving to lower protein concentrations and/or slightly shifting the solution pH or ionic strength to alter the charge–charge interactions between monomers [14,15,17,19]. As a result, one should anticipate that
aggregates of this type that are isolated (e.g., via purification) or characterized with ex situ methods that require dilution and/or a change in solvent conditions (cf. discussion below) will likely not be quantitative-ly, or possibly qualitatively, representative of the aggregate population(s) that exists in situ.

For purely reversible aggregates, one often can ignore the precise mechanism — i.e., the detailed steps and the order in which they occur in at a molecular level — if the time scales for equilibration of the aggregate population are short compared to that for production and storage of protein products such as pharmaceuticals. That is, one may only need the equilibrium aggregate size distribution, or equivalently the concentration of each species (monomer, dimer, trimer, tetramer, etc.) if the system equilibrates quickly [20]. For a simple diffusion-limited biomolecular reaction $M + M_j \rightarrow M_{j+1}$ ($M =$ monomer, $M_j =$ oligomer composed of $j$ monomers), the characteristic time scale for equilibration of such a “reaction” may be expected to be too small ($< 1$ s) to resolve with many experimental techniques that are in current practice (cf. discussion below). However, this is an important consideration when selecting techniques to monitor/detect/quantify aggregation, and when interpreting the results. Depending on the choice of experimental technique and analysis methods, one can reach quite different conclusions regarding the size and concentration of different oligomers or “clusters” [14,16,21]. In general, one requires systematic and detailed experimental characterization over a wide range of protein concentrations in order to refine even simple mass-action or multimer-equilibrium models with any quantitative certainty [17,20].

Not all aggregates are reversible. In some cases, what might be thought of as otherwise reversible aggregates can convert to stable species that are “bound” together so strongly that they are effectively irreversible on practical time scales and concentration ranges. In practice, this typically manifests as aggregates that do not dissociate appreciably upon multi-fold dilution or upon shifts in solution pH or ionic strength — although, the latter can cause aggregates to grow dramatically [22,23]. Furthermore, creation of such aggregates typically involves changes in the secondary and/or tertiary structures of the constituent monomers in a given aggregate species. These structural changes do not need to involve more than a (small) portion of the overall monomer chain(s) [24–26]. In the case of small proteins, there is often a marked increase beta-sheet content [27–30], but in general it remains unclear precisely what structural changes are required to create net-reversible aggregates. High concentrations of chemical denaturants (urea, guanidinium, ionic surfactants, etc.) or high pressures ($> 10^3$ bar) are sometimes able to dissociate such aggregates [31–33]. In such cases, small aggregates (dimers, etc.) may initially form as reversible species, but ultimately one often recovers or detects only the net irreversible species in most experimental techniques that resolve the different species from one another. In such cases, the mechanism(s) of aggregation become important because changes in the relative rates of different steps in the overall aggregation process can dramatically shift the population (concentration) of different sized aggregates, as well as potentially affecting the structure/morphology of the aggregates that are detected [34]. The next section provides additional details regarding illustrative aggregation mechanisms as a context for the discussion below regarding the importance of mechanism(s) and what controls them when one is considering how best to monitor and quantify protein aggregates.

### 3. Illustrative mechanisms of non-native aggregation

This section provides a brief overview of some the mechanisms by which non-native aggregates form. It is not realistic to exhaustively enumerate all conceivable aggregation mechanisms within the available space, nor is it necessary, as the examples below illustrate key conceptual approaches that aid when interpreting experimental results for aggregating systems. In what follows, the term non-native aggregate will be synonymous with net-reversible aggregate, although reversible intermediates can also be involved (see below). Net-reversible protein aggregation is described without explicit formation of new covalent bonds. While changes in covalent bonds can promote aggregation [35–37], the rate limiting step(s) in many cases involve formation of non-covalently linked aggregates prior to covalent linkages forming that further stabilize the initial aggregates [38]. That notwithstanding, aggregation mechanisms are conceivable in which non-covalent bond formation is rate-limiting, and therefore can be important from both the perspectives of kinetics and the resulting aggregate morphology [39,40].

Many of the recent studies with pharmaceutical proteins that form larger aggregates do not require covalent bonds to form between proteins, although some examples for mimics of food systems show a mix of behaviors [39,40]. The discussion below does translate, in qualitative terms, to aggregates that form by covalent linkages, although the detailed kinetics and time scales involved can be quite different [34]. To try to maintain as much generality is possible, most of the discussion below is cast in terms of relative rates of different steps, as it is only the relative rates that ultimately determine which competing pathway(s) are ultimately observed for a given protein and a given solution condition or storage environment.

Fig. 1 shows a schematic representation of a number of the key steps involved in competing pathways of protein aggregation that have been shown or speculated in the recent literature (see also, Figure caption), and adapted from [41,42]. Alternative representations are also possible, and many of the published mechanisms that have been validated in detail are similar to or essentially the same as in Fig. 1 [34]. Double arrows for any steps in the diagram indicate net irreversible steps. Single line arrows represent net irreversible steps, with ellipsis indicating a series of similar or analogous steps. Block arrows indicate steps that may be poorly or only qualitatively defined to date, and may involve multiple steps that are lumped into one block arrow.

Starting with folded monomer protein (blue), the monomers could conceivably form weak, easily reversible folded dimers or small oligomers (Fig. 1). Alternatively, a folded monomer is able unfold or partially unfold (red) and refold dynamically while in solution. The partly unfolded monomers expose more hydrophobic amino acid sequences that can help to drive initially reversible dimer or oligomerization (Fig. 1), and ultimately if the different protein chains can find ways to form both strong hydrophobic contacts and satisfy their hydrogen bonding needs (e.g., with inter-protein beta sheets) then they can "lock" into net irreversible, non-native oligomers that can stay as just dimers/oligomers or can grow through different mechanisms. If one considers sufficiently high concentrations then it may be feasible that otherwise weakly bound native oligomers will become sufficiently populated to be the faster pathway for transitioning from reversible oligomers to irreversible ones (Fig. 1) [43], although that would require a rather complex process of a native oligomer sufficiently unfolding and then misfolding as a cluster to form the non-native oligomer(s) that remain stable or grow to much larger sizes.

In qualitative terms, growth can first be categorized as dominated by monomer addition or by aggregate–aggregate coalescence (cf. labels in Fig. 1). In the former case, electrostatic repulsions between aggregates are sufficiently large that aggregates do not aggregate with one another except if one exhausts the available monomer pool [30,41,44,45]. In the latter case, aggregates are sufficiently attracted to one another that monomers are only consumed by the creation of new dimers/small oligomers, and those small aggregates rapidly coalesce with one another to form larger aggregates that propagate the aggregate coalescence process [44–48]. In the extreme, interactions between aggregates can become so favorable that the aggregates undergo bulk phase separation to form macroscopic and microscopic/subvisible particles [22,23]. Of course, these mechanisms can also occur simultaneously and so the behavior can change over the course of time as a sample is stored [44,45].

If one also considers aggregate formation via bulk interfaces, then the following qualitative features summarize key findings from a
number of recent studies: (1) proteins readily adsorb to bulk interfaces between water and solids (e.g., glass, plastic, metal, ice), liquids (e.g., silicone oil), and vapor (e.g., air or N₂ headspace) [49–55]; (2) formation of large aggregates/particles can be accelerated by turnover of proteins at the interface via convective mass transfer [56,57], by compressing/dilating the interface [51], and/or by creation/destruction of the interface (e.g., by bubbles forming/bursting) [57,58]; (3) to a first approximation, the rate at which the concentration of large aggregates increases over time in such “stressed” samples is proportional to the amount bulk interfacial area between the protein solution and whatever solid/vapor/liquid it is in contact with; (4) one does not typically observe large increases in much smaller aggregates (e.g., dimers, oligomers) during these types of experiments. The mechanistic details of how protein gets to/from the interface(s), whether it is folded or unfolded en route to the interface and at the interface, the structure of the protein layer(s) at the interface, and how large particles that are detected in the bulk liquid are formed from the protein molecules at the interface are all questions that have not been generally answered to date. As such, Fig. 1 (bottom) does not attempt to capture those in mechanistic detail, and only indicates that protein interactions with bulk interfaces have been implicated in many studies to date (an illustrative selection are cited above).

What follows from Fig. 1 is that different mechanisms compete with one another, and it is not clear a priori which mechanism or mechanisms will be most relevant for a given protein in a given solution or sample environment. The next subsection addresses a question that appears to have been overlooked, or at least not highlighted in the literature, to the best of our knowledge. Specifically, a given mechanism dictates how aggregation proceeds from monomer to small aggregates to larger ones, and so on. The resulting material balances or population balances must be adhered to, and therefore one cannot simply obtain an arbitrary distribution of aggregate sizes (characteristic dimension $R$, average molecular weight $M_w$, or average mass-per-particle $M_p$). This restricts the practical “operating space” for
where/when a given mechanism will be viable to monitor/quantify with existing experimental techniques.

4. Aggregate concentrations and sizes are not independent — mechanisms matter

As noted above, the underlying aggregation mechanism restricts the possible combinations of aggregate population sizes and concentrations. As an illustration we consider aggregation pathways that can be described either as bulk-mediated aggregation, which occurs in solution, or as surface-mediated aggregation, which occurs at an interface (e.g. glass–liquid interface). If a solution is not seeded with aggregates, a mass balance or population balance of the aggregate growth process in bulk solution results in the concentration and average mass-per-aggregate for all possible sizes of aggregates in solutions [48,59]. For surface-mediated aggregation, a simple mass balance on the control volume (the glass syringe or vial) can be used to relate the average aggregate mass and concentration (i.e., particle counts per mL) in a semi-quantitative manner. In both the surface-mediated and bulk-mediated pathways, a mass balance results in fundamental coupling of aggregate mass and concentration. In the interest of brevity and space limitations, the mathematical description of these balance equations and their solutions is provided in Supplementary material, as well as the original published reports for the bulk-mediated case [41,44,48,59].

In bulk-mediated aggregation, the full mass balance model for aggregation was previously distilled into moment equations in which the entire aggregation mechanism is explained in terms of competing processes: nucleation, growth by monomer addition or chain polymerization (CP), and growth by condensation or aggregate-association polymerization (AP) [48]. The moment equations relate the monomer concentration, the overall concentration of aggregates, and aggregate weight-average molecular weight to the aggregation rate coefficients or characteristic time scales for nucleation, and those for growth by CP and AP. A nucleation event creates a new aggregate while consuming monomeric protein. Growth by CP consumes monomers and increases the aggregate mass, but leaves the overall aggregate concentration unchanged. A condensation or AP event takes two existing aggregates to create a single larger aggregate (e.g., a dimer and a decamer create a dodecamer). Each condensation event necessarily decreases the net aggregate concentration. During aggregation for pharmaceutical proteins, all three mechanisms can occur simultaneously, but at different rates [45]. By varying the ratio of the rate coefficients or time scales for each process, one can determine realistic ranges of aggregate concentration and molecular weight that bound the expected behavior for real systems. Finally, by choosing a realistic fractal dimension (ν) for the aggregates [30,44,46], one can bound the “space” of realistic ranges for average aggregate or particle size (e.g., radius of gyration, R_g) and concentration. Previous scattering results for a number of protein systems have reported aggregate morphologies that range from insulin amyloid fibrils [60] (ν ~0.65) to acgm or IgG amyloid aggregates that resemble short or long flexible chain-like polymers [30,44] (ν ~0.75) and IgG aggregate clusters [46,61] (ν ~0.4).

A surface-mediated aggregation pathway can be driven, for example, by the favorable interaction of the hydrophobic interface (e.g., air-water) and the hydrophobic patches in the protein, which may become exposed during adsorption. Aggregates or particles are speculated to form either on the bulk interface, or as unfolded/misfolded proteins desorb from the interface, although the precise mechanism remains debatable. The impact of air–liquid and solid–liquid interfaces on rheology is described in detail in Section 7. In this section, the discussion is limited to aggregation kinetics as it relates to bulk interfaces.

Fig. 1 (bottom panel) illustrates a simple thought experiment: (i) proteins adsorb at the interface and some or all unfold and interdigitate to some degree, forming a “film” over time; (ii) the film may or may not be flexible, but upon sufficient “stress” such as due to deformation of the surface or rigorous agitation to aid desorption, portions of the film will “shed” or break off from the surface; (iii) these “patches” of the film that have been shed from the bulk interface will not stay extended as sheets once back in solution, but instead will “crumple” into higher fractal dimension objects, or may “bundle” in extended fibril-like objects; (iv) available detection techniques (see below) typically monitor only the particles that find their way back into the bulk solution. As a worst-case scenario — i.e., highest particle counts for a given particle size — a simple mass balance states that if the entire film breaks into equally sized “patches”, then the number of particles (N) is equal to the area of the initial film divided by the average area of a patch. The concentration of particles or aggregates is N divided by the liquid volume (~1 mL or ~10 mL for a pre-filled syringe or a small vial, respectively). The average mass per particle (M_p) is equal to the patch area divided by the area-per-protein (roughly πD^2/4, with D = effective protein diameter), and multiplied by the protein molecular weight. The characteristic size of the particles then follows from the choice of fractal dimension for the “crumpled” or “bundled” patches that shed from the protein film(s).

Fig. 2 shows the range of average R_g and total (molar) concentration of aggregates for bulk- and surface-mediated aggregation mechanisms, calculated based on the discussion above, and assuming only a small amount of monomer loss (e.g., 1% for bulk-mediated aggregation) so as to be in keeping with pharmaceutically acceptable levels. For surface-mediated aggregation, the amount of monomer loss is much less than 1%, as the calculations are based on a monolayer of protein adsorbing and the breaking off as “patches” from a film.

Bulk-mediated aggregation has a family of curves, with each curve representing a different set of ratios of the rate coefficients for nucleation, CP and AP. The red family of curves follows from nucleation + CP, while the blue set of curves follows from nucleation + AP. The curves calculated for surface-mediated aggregation are based on the estimated solid–water interfacial area for the residual bubble in a 1 mL cylindrical pre-filled syringe with 0.23 cm i.d. (black curve) and for the stagnant air–water interface a 10 mL cylindrical vial with an i.d. of 1.1 cm (green curve). These provide upper estimates for the concentration of aggregates (y-axis in Fig. 2) compared to, e.g., the amount of air-water interface in a pre-filled syringe or stagnant vial. Changing the
fractal dimension rescales the set of curves slightly on the scale of the axes in the Figure, but the general size and concentration ranges remain essentially unchanged. The results in Fig. 2 use a representative value of (ν = 0.66), and illustrate that it is natural to expect “gaps” in the accessible size-concentration space for aggregates/particles, based on different aggregation mechanisms. The sections below include discussion of how this concept ties into the practical and fundamental aspects of detecting, quantifying, and characterizing protein aggregates/particles, as well as means to control their formation.

It is worth noting that in the case of surface mediated aggregate or particle formation, one could easily conceive of additional features in the model or mechanism — e.g., renewal of the protein film after shading; only a fraction of protein molecules at the surface creating aggregates; the film not shading as equally sized particles; etc. Only a simple model was applied above, as currently there is no experimentally validated mechanism available that addresses these issues. Therefore, the results in Fig. 2 should be considered illustrative, and are provided as simple graphical representation of the fact that the size and the concentration of aggregates are not completely independent once one considers the question of “how” the aggregates formed.

5. Characterization techniques: protein aggregation: thermodynamics, kinetics & aggregation mechanisms

One of the key requirements in formulation screening in the biotherapeutic area has been to understand the aggregation propensity and phase behavior of protein formulations, through exploring various thermodynamic properties that are accessible experimentally on practical time scales. There has been a significant amount of recent work in this area by a number of different groups focusing on developing and applying different data analysis methods to light scattering data in order to obtain thermodynamic parameters that are indicative of the strength of protein–protein interactions. Thermodynamic parameters of interest that have been obtained through such analysis and applied to quantifying protein–protein interactions include a set of related quantities: the osmotic second virial coefficient (B2) and reduced osmotic second virial coefficient (B2/R02); Kirkwood Buff integrals (G2); and the so-called interaction parameter (Kd) [62,63]. Although the analysis method varies in order to obtain these different parameters, they are all primarily obtained through light scattering techniques. There are two modalities of light scattering — dynamic light scattering (DLS) and static light scattering or (SLS). In DLS, the intensity fluctuations of light scattered from particles moving due to Brownian motion is measured, while in SLS the time averaged intensity of scattered light at a certain angle is measured. Measurement of either the protein osmotic second virial coefficient or Kirkwood–Buff integral can be obtained through static light scattering measurements by varying the protein concentration, while Kd can be obtained through DLS to measure the collective diffusion coefficient as a function of protein concentration. The thermodynamic parameters obtained from these light scattering techniques can provide indicative trends in terms of relative stability of different protein formulations. In the case of virial coefficients, equilibrium analytical centrifugation can also be employed.

Understanding and controlling aggregation kinetics is another key aspect to gaining insights into the aggregation mechanism and the resulting final aggregate microstructure[6,41]. Protein denaturation and aggregation brought about by isothermal incubation is often times the desired method in order to probe the aggregation kinetics. DLS and size exclusion chromatography (SEC) with multi-angle laser light scattering (MALLS) are increasingly being utilized in order to perform such measurements, as well as complementary techniques such as analytical centrifugation [64]. SEC is a robust analytical technique in which proteins are separated, in principle, by their hydrodynamic volume. It is a commonly used technique that is utilized in the pharmaceutical industry to quantify monomer loss. MALLS is a static light scattering technique in which scattered light is measured at multiple angles. The angle-dependent scattered light information provides the radius of gyration (Rg) and weight average molecular weight of the scattering species. MALLS is especially relevant for high molecular weight species where the scattering depends on the scattering angle.

The combination of these two techniques provides a powerful tool to separate and characterize the high molecular weight aggregates formed during the protein aggregation process and help provide insights into the aggregation mechanism. In addition to simply using MALLS to assign a molecular weight to separable peaks in SEC, this approach can also be used to characterize HMW particles or aggregates that co-elute in SEC [65]. Li et al. illustrated that the combination of SEC-MALLS and extraction of weight average molecular weight, radius of gyration, apparent polydispersity and mass fraction of monomer provide necessary signatures to distinguish between different aggregation mechanisms (chain polymerization vs cluster–cluster aggregation) responsible for the formation of the HMW aggregates. Measurement of the aggregate characteristics utilizing SEC-MALLS was all carried out on quenched samples, where the sample was heated to a specific temperature and quenched in ice-water at different time points to capture the aggregate characteristics at that time point. Although this allows determination of aggregate size at a specific time point in the aggregation process, it is also desirable to measure the size evolution as the sample is held in situ at the incubation temperature, or as the sample is heated up to the incubation temperature. This can be achieved in the measurement through DLS measurements in a closed cell Peltier. A similar approach of utilizing both SEC-MALLS and DLS to characterize size of HMW aggregates was lucrative for food protein systems — e.g., whey protein, in addition [β-lactoglobulin [66].

Although a detailed discussion is beyond the scope of this review, it should be mentioned that understanding the secondary and tertiary structural changes associated with aggregation is essential in order to further obtain mechanistic insights into the aggregation process and establish the underlying ‘cause’ for aggregation. These are often times utilized in addition to DLS and SEC-MALLS, serving as complimentary techniques to both follow size/microstructural changes and secondary/ternary structural changes [62] Common techniques for elucidating structural changes in aggregating proteins include, Fourier Transform Infrared Spectroscopy (FTIR), Circular Dichroism (CD), Intrinsic Fluorescence (FL), and Raman Spectroscopy. FTIR provides information primarily on secondary structure, while CD and Raman can provide information on both secondary and tertiary structures, provided one can obtain sufficiently high-quality data. FL spectra provide information regarding primarily local tertiary structure in the vicinity of tryptophan and tyrosine side chains. CD measurements to obtain secondary structural information require data in far-UV region, while those for tertiary structure information require data in near-UV region. Raman and FTIR spectroscopy are both based on the vibrational spectra of proteins in solution. With current commercially available equipment, CD and FL measurements require orders of magnitude lower protein concentrations than those for Raman or infrared spectroscopy. Raman spectroscopy has advantages over FTIR for protein systems, as the relative background signal contribution of water is stronger in FTIR then in Raman. Raman spectroscopy can be carried out for solutions, gels and solids and this has clear advantages in studying aggregation for proteins systems that enter into a gel phase as aggregation progresses.

The DLS approach discussed above yields size and polydispersity information at the earlier stages of aggregation. However, the data analysis at later stages becomes highly challenging due to the presence of multiple scattering as the samples become turbid as high concentrations of HMW aggregates can occur. This issue has been addressed to a certain extent through two relatively new developments in DLS—backscattering and 3D cross correlation. In the backscattering approach, the scattered light is detected at a higher angle (e.g. 173°) and the scattered light is measured close to the cuvette wall, thereby allowing more turbid samples to be probed. This approach allows DLS measurements to be carried out on moderately turbid systems during early
stages of aggregation and additionally allows DLS to be utilized for tracer micro rheology measurements[67]. In 3D Cross Correlation,[68] single scattering data is obtained from turbid samples through simultaneously carrying out two light scattering experiments at the same q vector (scattering vector) and same sample volume and cross correlating the measured scattered intensities from both experiments with each other. This ensures that only single scattering contributes. This measurement has been extended to multiple angles [68] that allowed time resolved studies on aggregating systems to be carried out. The authors illustrated the utility of the technique in providing insights into the temporal evolution of aggregation in acidified skim milk for yogurt production. This technique has not yet been extended to investigating the temporal evolution of aggregating therapeutic protein formulations, but is clearly an area where new insights into the aggregation kinetics could be envisioned as many of the formulations exhibit significant increase in turbidity as aggregation progresses under stressed conditions.

More detailed understanding of the interaction potentials, microstructure and morphology of the formed protein particles however requires techniques that expand the q-vector (q = (4πn / λ)sin (θ / 2), where n is the refractive index, λ is the wavelength, and θ is the scattering angle) range of the scattering techniques, as the length scales of the formed structures, the time scales of relaxation mechanisms, and the distances over which interactions occur can vary significantly through the aggregation process; this can eventually lead to arrested dynamics, network formation, etc. Experimentally following these processes therefore requires the utilization of techniques such as small angle neutron scattering (SANS), small angle X-ray scattering (SAXS), Ultra Small Angle Light Scattering (USALS), and electron microscopy. The length scales usually probed by SANS is in the range of 10–1000 Å for and that usually probed by SANS is 10–200 Å. Light scattering usually probes length scales in the 2000 Å to 100 µm range. These techniques are increasingly being utilized in both the food and biotechnological area to provide additional insights into the phase behavior and microstructure evolution of aggregating protein systems.

Small Angle Neutron Scattering (SANS) allows an understanding of the interactions in protein systems through an analysis of the structure factor. The structure factor S(q) obtained from a SANS experiment is determined by the ensemble-averaged interparticle distance and interparticle interactions. An analysis of the structure factor therefore allows a more detailed understanding of the extent of the specific type and range of interaction present in the specific protein system and under specific formulation condition. The location of the structure factor peak and its subsequent fitting to a relevant interaction potential can in turn lead to generation of a hypothesis regarding the microstructure state of protein aggregate. One of the most prevalent recent hypotheses in the protein self-assembly field has been the interpretation of structure factor peaks based on the formation of equilibrium clusters in concentrated protein solutions. This experimental observation and data interpretation were first done by Stradner et al. [21], where the peak at high q was attributed to lysozyme monomer interactions within a single cluster and the peak at low q was attributed to cluster–cluster interactions. However as discussed in the review by Mezzenga [5], although there have been multiple subsequent studies utilizing other techniques such as light and X-ray scattering that support the cluster hypothesis, there also have been alternate non-cluster based interpretations of SANS data as well. One of the earlier studies is based on SANS work done by Shukla [69], where they observed a concentration-dependent shift in the SANS scattering peak and postulated that the peak can be attributed to lysozyme monomer interaction interacting via short range attraction and long range screened electrostatic repulsion.

The utility of SANS as a tool to understand the interaction potentials and microstructure in high-concentration protein systems has recently been extended to therapeutic proteins such as monoclonal antibodies, primarily with a view to understanding viscosity increases in these systems as a function of protein concentration and formulation conditions. Yearly et al. [19] carried out SANS measurements on two monoclonal antibodies (named as MAb1 and MAb2 respectively) which differed by small sequence alterations but exhibited very different patterns of how viscosity increased with increasing concentration (MAb1 exhibited significantly higher viscosity increases with concentration than did MAb2). A detailed structure factor analysis allowed the authors to gain insights into the different protein–protein interactions (PPI) present in these two different proteins and how that was impacted by concentration. It was shown that the MAb1 PPI changed from strongly attractive net potential at small volume fractions to a PPI with negligible attraction at high concentration. This then led to the postulation of the formation of dynamic clusters which in turn gave rise to higher viscosity than MAb2, as the PPI for MAb2 were dominated by charge repulsion between monomers. In addition to a detailed structure factor analysis, the authors developed an analytical three-arm form factor for monoclonal antibodies which addresses the problem of deconvoluting the form factor and structure factor in a numerically practical manner. To further support SANS analysis of protein data, Clark et al. [70] also carried out molecular Monte-Carlo simulations together with molecular dynamics simulations to gain further insights into intermolecular and intramolecular interactions that impact functional performance of these therapeutic proteins. SANS is a potentially powerful tool that, together with theoretical developments and atomistic molecular simulations and free energy analysis, is expected to provide new insights into the interactions and microstructural evolution in concentrated protein formulations.

Similarly, small angle X-ray scattering (SAXS) is a potentially powerful technique for advancing the understanding of the therapeutic protein formulation stability. Although the technique has previously provided information mostly on static solution structures or bead model representations of proteins, a recent study [71] has extended the technique to infer the effects of solution conditions (ion type, pH) on antibody protein dynamics in solution and on stability. As SAXS represents the average scattering pattern from all molecular conformations (as does SANS), data interpretation can be complicated. The authors employed an ensemble-optimized method (EOM) to determine the conformational space of an IgG protein under changing formulation conditions. The EOM allows optimization of the fit of the scattering data by comparing and optimizing the averaged individual scattering patterns from different conformers with real SAXS data from the protein solution. This analysis allowed the molecular flexibility around hinge region of monoclonal antibodies to be determined under the influence of different kosmotropes and chaotropes in solution and led to an understanding of conformational dynamics and stability for the specific IgG protein.

The self-assembly and aggregation processes seen in both therapeutic and food protein systems due to thermal or chemical treatments eventually can lead to a liquid–solid transition and to the formation of a gel. The microstructural and morphological changes associated with these processes require utilization of techniques which allow the visualization/probing of the large length-scale structures associated with these processes. The key techniques which have been utilized extensively in the food protein area have been based on microscopy. The key microscopy techniques of interest have been scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy. The techniques have illustrated that for globular protein gels formed from food protein systems, the morphology tends to be primarily particulate or fine stranded. The morphology that will form is strongly dependent on the ionic strength/charge conditions of the formulation. At conditions close to the isolectric point or at high ionic strength, the microstructure consists of associated spheres forming relatively compact clusters. At conditions of high electrostatic repulsion the gels that are formed are more filamentous or worm-like networks. [6]. Although the mentioned microscopy techniques have limitations e.g. viscosity issues in utilizing cryo-TEM, they provide a relatively direct route for probing the morphological changes associated with
aggregation, and thereby offer insight into the underlying aggregation mechanism(s) and observed rheological properties.

Moving from molecular–scale aggregation to bulk phase separation, an improved understanding of the liquid–solid transition process has also been progressed based on utilization of novel light scattering techniques such as Ultra Small Angle Light Scattering (USALS) [72]. USALS allows static light scattering to be carried out over a scattering vector range corresponding to 0.1 μm⁻¹ < q < 2 μm⁻¹. The scattered intensity behavior over this q range allows the following of a spinodal decomposition process and capturing of the changes in a characteristic microstructural lengthscale, ξ, associated with this process. Gibaud has utilized this technique together with video microscopy to follow liquid–solid transition in lysozyme solutions. The combination of these two techniques allowed them to demonstrate the formation of an arrested bicontinuous network when the solution was quenched. They were also able to show that the correlation length exhibited a temperature dependence that closely followed the critical scaling expected for density fluctuations during early stages of spinodal decompositions. This approach can be a useful tool for interrogating liquid–solid transitions that are seen in therapeutic protein formulations, but to the best of our knowledge has yet to be employed in that context.

6. Characterization techniques: protein particle detection and size

Protein particles formed as a result of aggregation can span many orders of magnitude from oligomers spanning tens of nanometers all the way to visible aggregates spanning several hundred micrometers [3,4]. It is clear that one single analytical instrument cannot be utilized to capture this wide range of length scales. Even more challenging is the fact that within a certain size range, the actual distribution of particle sizes and concentrations of particles of a particular size are difficult to obtain due to limitations of some of the techniques being utilized [4].

In the monomer-to-oligomer size range which can span from several nanometers to tens of nanometers, sizing and a quantification of the polydispersity in the particle sizes have traditionally been carried out by light scattering techniques either independently or in combination with a separation technique such as SEC and Field Flow Fractionation (FFF)[65,73]. The following sections highlight the relevant techniques for the various size ranges of particles or aggregates that are encountered.

6.1. Protein particle sizing: 1 nm–2 μm

a) Dynamic Light Scattering (DLS)

Light scattering has been discussed in the context of following the protein aggregation mechanism. Once aggregates are formed or in systems which do not undergo aggregation DLS can provide useful size and polydispersity information for monomers/oligomers and particles spanning between 1 nm and 1 μm. As already discussed cuvette based DLS measurements can allow the following in-situ of size changes when a protein undergoes aggregation. There are however limitations in DLS in the sense that size distribution obtained from DLS is biased towards larger particles due to dependence of the intensity to the sixth power of the diameter. This can however be advantageous if the objective is the detection of small quantities of large particles. In SLS one obtains a z-average molar mass, which can make it difficult to understand quantitatively the relative contribution of different species present in the formulation. Combination of these techniques with a separation technique such as SEC enhances the resolution and allows better separation of the contribution of different size particle populations.

b) Nanoparticle Tracking Analysis (NTA)

Some of the issues highlighted with dynamic light scattering based particle sizing, such as bias to larger particles can be overcome through complimenting the measurements with novel developments in particle tracking [4,74]. Nanoparticle Tracking Analysis or NTA utilizes laser illumination to track the Brownian motion of deeply submicron nanoparticles in liquids. The laser illumination can be through using of a 405, 532 or 638 nm light source and the particle movement is detected through a CCD camera. A modified Stokes–Einstein equation is employed in order to obtain the particle size from the mean squared displacement of the particle. This particle–by–particle approach provides both a high resolution particle distribution and measures concentration. The size range covered by NTA is between 30 nm and 1 μm. The lower size limitation is influenced by the refractive index of the particles. For low refractive index particles, such as protein particles, the lower limit is usually in the 40–50 nm size range. The clear advantage that NTA presents is in the ability to picking up the differences in particle tracks in different parts of the sample. This is especially important in heterogeneous samples such as protein formulations.

The applicability of the NTA in carrying out size characterization of protein particulates is not only limited to spherical shape aggregates, but has been extended to look at fibrillar aggregates in a recent study carried out by Yang et al. [75]. In that study, NTA based particle sizing was carried out for DNA and Transthyretin a 56 kDa homotetrameric protein. For the DNA sample, which has a large aspect ratio and can be modeled as a semiflexible (wormlike) chain, the peak in the size distributions from NTA was at 178 nm and 32 nm. The authors considered this as excellent agreement with the size calculated from the semiflexible chain model. The concentration of fibrils obtained from NTA was however quite significantly underestimated. According to the authors, the extended dimensions of DNA lead to interference effects. This leads to a decrease of the scattered intensity of many DNA particles below the threshold of detection, leading to significant undercounting. As however illustrated by the authors, combining NTA with DLS data does allow the extraction of meaningful concentration information. Overall the technique together with DLS shows good promise as a technique to measure protein particulate size distributions allowing further insights into protein aggregation kinetics and mechanisms.

c) Resonant mass measurement (RMM)

Resonant Mass Measurement (RMM) is a technique which adds further quantification of protein particles in the size range between 50 nm and 2 μm. The technique is based on a microchannel resonator[76]. When a particle moves through the microchannel it causes a change in the resonance frequency of the microcantilever. The net frequency shift is proportional to the buoyant mass of the particle from which the size can be extracted. The frequency shift in the resonance frequency is measured by a laser which is focused on the tip of the microcantilever and the signal passed onto a photodiode detector. The technique provides accurate measurement on a particle by particle basis in the size range between 50 nm and 2 μm extending accurate sizing to a range just below that of flow microscopy. This starts to then provide a good overlap and transition into sizing techniques in the subvisible range.

6.2. Protein particle sizing: subvisible range >2 μm

The characterization of protein particles in the size range between 2 and 10 μm is increasingly growing in importance due to potential immunogenicity of particles in that size range. These include both proteinaceous and non-proteinaceous particles. Techniques which are being utilized to characterize particles in this size range include light obscuration, counter counter and flow imaging or flow microscopy.

Light obscuration is based on extracting the area/size of a particle from the loss in intensity as a particle passes through the path of a light beam. In a counter counter the particle sizing is obtained from voltage impulse due to the resistance it induces as it passes through an orifice with two electrodes. In flow microscopy the particle count and particle size distribution are obtained as a sample flows in front of the
objective of a microscope. All three techniques are utilized quite extensively in the subvisible size range and do provide useful size information. However there are certain limitations regarding all of these techniques [77]. The optical techniques-light obscuration and flow imaging have limitations when characterizing protein particles at high concentrations. These techniques tend to underestimate the particle numbers due to the low refractive index of the protein particles in a high concentration protein solution background. Additionally these techniques in many cases do require dilution and that can result in artifacts especially for reversible aggregates that only form at high concentrations. Flow imaging or flow microscopy additionally has some further limitations. As this is a flow based measurement there are limitations on the lower size that can be imaged/sized accurately. This limits sizing to 2–3 μm at the lower size end. Additionally resolution and picture quality are not high due to limited depth of field. Coulter counter can provide size information reliably up to about 150 mg/mL however it does require the use of an electrolyte with sufficient conductivity and sometimes underestimation of size is obtained especially for particles in the smaller size end of the size range probed. Overall, the limitations of these techniques under relevant buffer and high concentration conditions still make sizing and characterization in this subvisible size range highly challenging.

6.3. Characterize/distinguish proteinaceous and non-proteinaceous particles

In addition to correct estimate of the protein particulate sizing aspect, it is important for protein therapeutic formulation safety and stability perspective to be able to distinguish between proteinaceous and nonproteinaceous particles. One of the recent advances in this area has been through the application of a resonant mass measurement (RMM) technique based on a vibrating microcantilever which has already been discussed in an earlier section. The presence of a particle on the resonating microcantilever causes a shift in the frequency. Positively buoyant particles such as silicone oil droplets can be distinguished from negatively buoyant particles such as protein particles as they would cause either an increase or decrease in the cantilever frequency. Recent study by Weinbuch [78] has illustrated the utility of the RMM technique compared with the more standard micro-flow imaging (MFI) technique for detection and analysis of protein particulates and silicone oil droplets. It should be mentioned that the size determination and discrimination between proteinaceous and non-proteinaceous particles in MFI is based on very different principles then RMM. In MFI, 2D particle images are captured and size determination is carried out based on spatial dimension of the images defined by the outer boundaries. The discrimination between proteinaceous and non-proteinaceous particles is based on particle shape and transparency. Based upon the comparative studies carried out [78] it was concluded that RMM differentiation was more appropriate for particles below 2 μm, while MFI differentiation was more appropriate for particles above 2 μm. As the size range of protein particles that can be encountered in therapeutic formulations is very wide, complimentary use of both techniques was recommended.

7. Rheology of therapeutic protein solutions

7.1. Scope

We focus exclusively on the bulk shear rheology of protein solutions here while emphasizing the fact that amphiphiles like proteins adsorb spontaneously at the air (A)/water and oil (O)/water (W) interfaces (Fig. 1). Surfactants are commonly added to colloidalaly stabilize therapeutic proteins in solution by preferential adsorption, i.e. orogenic displacement. Since surface adsorption can apparently influence the measurement of the bulk shear rheology of surfactant-free protein solutions, we shall briefly address it later, given its importance to the formulation of bio-therapeutic proteins and peptides. We forgo discussions of food protein solution rheology in this review and focus mainly on therapeutic proteins.

We focus mainly on the shear viscosity, η, though other rheological material functions in steady and oscillatory shear as well as creep deformation also provide useful insights, but are more challenging for the layman to comprehend. Moreover, while, the debate on whether protein molecules unfold in shear flow still ensues and overlaps partially with the intended scope of this review, space limitations preclude its discussion. We refer interested readers to a recent review in this subject area by Bekard et al. [79].

7.2. Soluble clusters and their effects on viscosity: reversible self-association

We first scrutinize the effects of reversible self-association (RSA), which often occurs in formulations of therapeutic proteins, and is commonly mitigated by addition of excipients or by varying pH and/or ionic strength. RSA increases the viscosity of IgG solutions. Liu et al. verified carefully the effects of RSA on solution viscosity thereby extending the original results of Hall & Abraham [16,80], who focused on hydrodynamics.

The molecular underpinnings of increased viscosity, poorly elucidated hitherto in the literature, lie in the attractive inter–molecular interactions that drive RSA. These attractive interactions effectively suppress the mean squared displacement, \( <r^2(t)> \), that molecules undergo during time (\( t \))–dependent Brownian diffusion; angular brackets denote ensemble average. The reduced \( <r^2(t)> \), is reflected in the diffusion coefficient, \( D \approx <r^2(t)> / \tau \), where \( \tau \) denotes the relaxation time. Since the Stokes–Einstein relationship predicts that \( D \times \tau \) should be constant, we can therefore understand how reduced \( D \) leads to increased \( \eta \). Conversely, when repulsive interactions prevail in a protein solution, they facilitate diffusion and increase \( <r^2(t)> \). Thus, \( D \) increases and \( \eta \) decreases in repulsive systems. Repulsive interactions are therefore desirable from both stability and low viscosity, especially from the biopharmaceutical formulation perspective. Though the Stokes–Einstein equation was derived for dilute systems, a generalized Stokes–Einstein expression has been proposed for concentrated systems, wherein the hydrodynamic size is replaced by a correlation lengthscale [81]. The molecular arguments proposed here therefore hold for both dilute and concentrated protein solutions.

As RSA creates soluble clusters, an obvious question lies in understanding the dependence of \( \eta \) on cluster size, \( N \), the number of monomers in a cluster. Liljestrom et al. [82] have demonstrated that \( \eta \propto N \) linearly in an IgG1 formulation with RSA (Fig. 3). For a formulation

![Fig. 3. Solution viscosity as a function of cluster size in systems of two different monoclonal antibodies that exhibit reversible self-association. The linear dependence of viscosity on cluster size, measured by light scattering, is beautifully demonstrated in these data by the authors, Liljestrom et al., J. Phys. Chem. B (2013) (Ref. [82]).](image-url)
comprising mostly hexamers \((N = 6)\), \(\eta \sim 300 \text{ mPa} \cdot \text{s}\), which is twice that of a formulation comprising trimers \((N = 3)\). Invoking a molecular interpretation of viscosity enables easy rationalization of linear scaling of \(\eta(N)\). Viscosity is an ensemble average measure of the friction coefficient \((\zeta)\) experienced by monomers and all soluble clusters during Brownian motion. The force per unit velocity experienced by the soluble species, as \(\zeta\) is defined, scales with \(N\). Thus, a soluble cluster comprising \(N\) monomers experiences total friction of \(N\zeta\), and since viscosity itself scales linearly with \(\zeta\), the linear \(\eta(N)\) reported by Liljeestrom et al. is easily understood. This elegant prediction of Einstein’s Brownian motion theory describes the scaling of \(\eta(N)\). Nevertheless, the exact shape, linear vs branched, and conformation of the soluble clusters formed due to RSA still need to be determined, since they are important determinants of solution viscosity. Moreover, since the sedimentation behavior of proteins and their clusters formed by RSA depend on both size and shape, advances in the understanding of cluster shape, size and charge/charge distribution will bridge the gap between experiment and theory.

### 7.3. Effects of sub-visible particles on viscosity

Until recently controversy seemed to prevail about the effects of irreversible and insoluble aggregates on viscosity, but evidence now exists that particles increase the solution viscosity. Simulation and experimental studies of stable and unstable (aggregated) colloidal suspensions have demonstrated that aggregation increases the viscosity of colloidal suspensions, particularly at low shear rates. Therefore, measurements of low-shear viscosity on conventional rheometers can be of great use in deducing particle effects on viscosity and has relevance to formulation stability studies, which represent a zero-shear condition, slow flow in large pipes, and shipping of material [83]. The effect of particles on viscosity has been shown in a surfactant-free IgG1 solution kept at storage conditions (2–8 °C) [1] and also in another surfactant-laden IgG1 solution incubated at 40 °C for approximately 41 days (Fig. 4) [84,85]. Both papers clearly document the presence of a yield stress in these aggregating IgG1 solutions, and the increased viscosity in these works agrees with the result originally reported by Patapoff and Esue [86] that the solution viscosity of highly concentrated mAb solutions increases due to the formation of insoluble aggregates. All these workers measured flow curves on their protein solutions, which has facilitated a more complete understanding of the effects of particulates on solution viscosity.

The rheology of the aggregating solution in Fig. 4 shows a transition from Newtonian response in the monomeric/stable state to non-Newtonian response as aggregation proceeds in a thermally incubated environment, and a yield stress develops, though its origins are not completely understood currently. The low shear rate upturn in the viscosity is removed by filtering the solution, and the low wavevector, i.e., large real space length scale upturn in the scattering intensity in SANS experiments also disappears upon filtration [84,85]. This key result confirms that fractal sub-micrometer particles, formed by Reaction Limited-Aggregation (RLA) mechanism, drive the increase in the low shear rate viscosity as well as the low wavevector upturn in the scattering intensity of that aggregating antibody solution. The fractal dimension of the aggregates, inferred from scattering data, serves to verify RLA as the mechanism of aggregate growth, as is seen in many other unstable colloidal suspensions. RLA has been verified by static light scattering experiments [46] on other aggregating IgG1 systems too, thus confirming the agreement between light scattering and neutron scattering. The low shear rate viscosity of antibody solutions is thus a sensitive indicator of particle formation and growth. The sensitivity of low shear viscosity to the presence of particles underscores the importance of measuring the flow curve, especially the low shear rate response, of unstable antibody solutions over a broad shear rate range to clearly discern the effects of aggregation on the solution viscosity. Aggregating protein solutions are clearly non-Newtonian, while stable, i.e., mostly monomeric protein solutions are Newtonian (Fig. 4). Neither ultrasonic rheometry at 10 MHz (10\(^{-1}\) s\(^{-1}\)) [87] nor Dynamic Light Scattering approaches that rely on scattering from Polystyrene bead tracers [26,88] would detect this aggregation-driven increase in viscosity at low shear rates. However, particle-tracking, Diffusing Wave Spectroscopy, and Dynamic Light Scattering-based micro-rheology techniques using the approach of Mason and Weitz can allow one to measure the zero-shear viscosity. Micro-rheology therefore has promise as an ancillary screening technique for stability in protein formulations, if one carefully checks the data for artifacts.

While single shear-rate/frequency measurements are advantageous and indeed necessary for high throughput formulation screening in the bio-pharma industry, full flow curves can provide rich information to supplement stability data and should be measured to understand stability effects on viscosity and syringeability.

### 7.4. The effects of A/W, O/W and solid (S)/W interfaces on protein viscosity and stability

We begin with a discussion of solid/water interfaces first, as their consequences are impactful and not generally appreciated by most workers who study protein solution rheology. Critical evidence for the effects of solid surfaces is abundant in the bio-process engineering literature. Proteins encounter shear flow kinematics during manufacturing unit operations, e.g. in mixing, and also during transfer/filling using a pump, shipping and unit operations such as mixing. Recent work has shed much-needed molecular light on the effects of the solid–liquid interface during impeller-driven mixing [89]. As seen in Fig. 5, shear flow at high strain rates generated by an impeller’s rotation leads to monomer loss with simultaneous increase in the turbidity/opalescence of a dilute surfactant-free IgG solution formulated in Phosphate Buffered Saline at pH 7.4. The dominant factors found to affect the antibody stability were pH, which controls net charge on the protein surface, and also the surface roughness associated with the solid–liquid interface. These results point to the role of charge driven adsorption of antibody on the solid–liquid interface in surfactant-free solutions. Moreover, the formation of sub-visible particles during filling with pumps is also marked with a rise in turbidity [90]. Antibodies can also be unstable in the presence of flow around surfaces made of common materials such as stainless steel and ceramics. These observations lead to the important

![Fig. 4. The effects of sub-visible particles formed by prolonged thermal incubation on the shear rate-dependent viscosity of a monoclonal antibody solution at 40 °C. Note that the control (unheated) solution at the zero time point shows Newtonian response, which undergoes a marked transition to non-Newtonian response, especially at low shear rates. Eventually, a yield stress develops, and the inset shows the growth of the yield stress with incubation time. These data highlight the important effects that SVPs have on solution viscosity, as well as the necessity of measuring viscosity vs. shear rate flow curves. Adapted from Castellanos et al., Soft Matter (2013) (Ref. [84]).](https://example.com/fig4.png)
inference that surfaces, including those used in rheometry, should not be assumed to be benign to proteins.

In addition to consequences for unit operations, interfacial adsorption poses non-negligible consequences for rheometric measurements, as adsorption results in partial unfolding and hydrophobically-driven aggregation of protein molecules that create a film. As an illustration, one can quantitatively connect the average aggregate size and concentration with a simple thought experiment for the case of particles forming via a “shedding” process from the air–water interface, as has been suggested based on experiments with antibody solutions [51]. Surface-adsorbed films are viscoelastic and respond accordingly to interfacial (two dimensional) shear and dilatation (changes in surface area). In a recent report, Sharma et al. [91] have traced the existence of a bulk shear yield stress in protein solutions solely to the viscoelastic film formed by adsorption at the A/W interface. They have also nicely shown that the yielding in dilute protein solutions, as measured by rotational rheometry, is due to the torque contribution of the adsorbed protein film at the A/W interface. A rheometer cannot identify the source from which the generated torque emanates. Both the bulk shear of the protein solution and the adsorbed protein layer at the A/W interface can contribute to the measured torque. The reader should note that carefully chosen measurement geometries can mitigate the surface contribution, and provide robust measurements for protein solutions, even surfactant-free ones. Moreover, it is now also known that particles can also cause non-Newtonian shear yielding behavior [84]. The rheology of concentrated protein solutions is manifestly non-Newtonian, and this non-linear response in the form of liquid-like shear thinning or solid-like shear yielding should be appropriately accounted for in the treatment of rheometry data.

7.5. Experimental data vs. theoretical models for protein solution viscosity

There are currently no protein-specific molecular theories for the composition dependence of viscosity of stable (monomeric) proteins in solution. Theoretical models for the viscoelasticity of unstable/aggregating protein solutions are understandably unavailable, given the complex challenge they pose in terms of concurrently modeling the hydrodynamic response of concentrated protein solutions comprising soluble monomers and aggregates as well as insoluble aggregates and SVPs. Monomer–monomer and monomer–aggregate interactions would also need to be accounted for in these concentrated systems. The rheology of these irreversibly aggregating systems is complicated also because they are inherently non-equilibrium systems, whose rheology reflects the time-dependent evolution of the morphology created by irreversible protein aggregation and the precipitation of SVPs.

While colloidal models such as the Russell–Saville–Schowalter model [92] and the Krieger–Dougherty model [93] and even entanglement scaling models [94] have been applied to model the composition dependent viscosity of protein and antibody solutions, they possess significant limitations. Application of colloidal models commonly assumes one-to-one equivalence between proteins and charged hard-sphere colloids, which have uniform surface charge distribution. This assumption fails for globular proteins like BSA [95] and also for multi-domain proteins such as IgGs [96]. The implications of such non-uniform charge distribution on RSA in IgGs are not negligible, and colloidal models cannot capture this complexity. Spherical models simply do not hold for the solution viscosity of globular proteins such as BSA and multi-domain proteins have even greater deviations from spheres assumed to have homogeneous surface charge distribution. All proteins in buffered solutions have a surface charge distribution that changes with pH. Even at its iso-electric point, pl, BSA possesses patches of negative and positive charges on its surface, while maintaining zero net charge. Charge–charge and charge-induced dipole interactions would still persist in protein solutions at the pl. Moreover, proteins also possess a hydration shell [97], which significantly alter their solution hydrodynamics, as reviewed by Halle in [98] and also their solution rheology, viz. the composition dependence of solution viscosity [95]. To test the applicability of colloidal rheology models to protein solutions, the volume fraction must be calculated based on protein composition. If the hydration shell is ignored in the calculation of the protein volume fraction, then one reaches the apparent, though erroneous, conclusion that colloidal models quantitatively predict/correlate the viscosity of protein solutions [91]. Charge and hydration are both fundamental to protein solution rheology.

In addition to differences in charge and hydration, analogies between proteins and colloids can be called into question due to a priori assumption of shape for protein molecules, regardless of whether they are in dilute or concentrated solutions. BSA, which has been assumed to be a hard sphere [97] for the treatment of small-angle X-ray scattering data from its crowded solutions, is neither a hard sphere in the hydrodynamic sense [95], nor in the thermodynamic sense [99]. Proteins are macromolecules, whose dynamic conformations are universally accepted. There is now evidence from near UV CD for changes in tertiary structure in crowded BSA solutions [99] and also in crowded IgG1 solutions from near UV CD, AUC and SANS. At high concentrations molecules can sometimes adopt less compact conformations, which experience larger hydrodynamic friction/drag and thus experience higher viscosity. While changes in friction coefficient of a hard sphere can be captured by changing the charge and charge distribution on its surface, these hard sphere models do not account for charge effects and focus only on excluded volume interactions. While excluded volume interactions are undoubtedly non-negligible in crowded protein solutions, they are by no means the sole determinant of protein solution viscosity. We therefore do not consider empirical quasi-spherical hard sphere models [100] for the protein concentration dependence of solution viscosity for discussion here. It is our assessment that a priori assumptions of protein shape and conformation in concentrated solutions have hampered progress in this field.

In contrast to quasi-spherical hard-sphere models, recent work has extended a scaling theory for the viscosity of semi-dilute polymer solutions [94] to the viscosity of high concentration antibody solutions. While the approach succeeds in fitting the data, the scaling approach assumes that antibody molecules form an entanglement network. To date, published literature reports have not found evidence of an entanglement network, which is typically manifested by a plateau in the shear storage modulus, G′. While this model succeeds in fitting the data, albeit with a significant number of adjustable parameters, this scaling approach assumes that antibody molecules form an entanglement network. However, no information is available in Ref. [94] that shows the existence of an entanglement network, as evidenced by a plateau.
in C'. The readers should note, however, that the assumption of an entanglement network can be considered to be simply a useful mathematical construct to facilitate comparison with experimental data, and it provides a simple means to relate the size of a transient network to the timescale for rearrangement of that network.

In summary, the field of protein solution rheology is wide open for new molecular theories to be proposed. Proteins are complex macro-molecules, whose solution viscosity depends of many factors, viz., molar mass (influenced by RSA), charge and charge distribution, hydration, conformation, protein–protein interactions etc. The authors hope that the coming years herald significant new advances in this field.

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Appendix A: Supplementary data

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