Analytical Biochemistry 437 (2013) 185–197

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Monitoring protein aggregation kinetics with simultaneous multiple sample light scattering

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ARTICLE INFO

Article history:
Received 29 October 2012
Received in revised form 15 February 2013
Accepted 19 February 2013
Available online 7 March 2013

Keywords:
Protein aggregation
Light scattering
Aggregation kinetics
High-throughput screening
High-concentration protein formulation
High-concentration monoclonal antibody

ABSTRACT

A simultaneous multiple sample light scattering (SMSLS) prototype instrument was built to simultaneously measure light scattering from many independent monoclonal antibody (mAb) solutions in order to monitor their time-dependent aggregation behavior and to characterize, via absolute Rayleigh scattering ratios, their molecular masses and second, third, and fourth virial coefficients under non-aggregating conditions at concentrations up to 190 mg/ml. One stable mAb and another prone to aggregation were studied. Early phase aggregation rates spanned six orders of magnitude over temperatures 30 to 83 °C for both mAbs and divided into "Arrhenius" and "Stochastic" regimes. The Arrhenius regimes comprise two thermal regimes whose breakpoint occurs near the first thermal unfolding temperature of the mAb domain structure. The Stochastic regime occurs for $T \leqslant 40$ °C. Rates yielded activation energies and temperature and concentration crossovers among rate-limiting regimes. Virial coefficients were closely related to aggregation kinetics. Hydrodynamic diameter relationship to virial coefficients provided further insight into stability. SMSLS detected as few as three dimerization events among 1000 monomeric proteins. Although early phase aggregation is linear in time and reproducible, aggregation becomes chaotic in later phases. SMSLS dramatically increases protein monitoring throughput, providing continuous monitoring for hours, weeks, and longer. New samples can be changed in and out without affecting other sample measurements in progress.

Aggregation is a common degradation process for proteins and often represents a major obstacle in the development of robust and stable injectable biologics for human use [1–4]. Concerns regarding the impact of aggregates on the safety and quality of protein therapeutics have been well documented and relate primarily to adverse immunological effects and loss of potency [5–7]. The unpredictable nature of protein aggregation arises because mechanisms are not fully understood and because of the tremendous diversity and complexity of protein structure itself. Consequently, the problem of monitoring, controlling, and predicting aggregation propensity is especially challenging but is central to improving the efficiency and success rate of the biotechnology pipeline. Increasing clinical "shots on goal" by accelerating molecule selection and reducing the subsequent time needed to lock down stable scalable formulations is, thus, an important goal. One underlying challenge is to efficiently measure physicochemical and structural properties that are expected to correlate with *pharmaceutical* stability—that is, stability relevant to the manufacturing stresses of large-scale processing operations, long-term storage, shipping, administration, and patient handling. As the biotechnology industry increasingly seeks to develop high-concentration protein formulations, the consequences of non-ideality related to protein–protein interactions becomes a significant obstacle [8]. Intrinsic physical properties that are expected to influence aggregation propensity include, but are not limited to, molecular size, shape, hydrophobicity, charge (total charge, charge distribution, and dipole moment), and thermal unfolding stability (defined by Gibb's energy change and unfolding temperature midpoint, T_m). Thus, it is unlikely that a single intrinsic parameter can be identified that will conveniently predict a protein's physical stability with respect to the diversity of stresses applicable to biotechnology drugs such as freeze–thaw, agitation, pH change, shear, air–liquid interface, and elevated temperatures. Consequently, identifying approaches that can provide more effective mechanistic insights into stability-limiting aggregation pathways is important. In this study, we have applied simultaneous multiple sample light scattering (SMSLS)[1] to characterize the

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[1] *Abbreviations used:* SMSLS, simultaneous multiple sample light scattering; mAb, monoclonal antibody; SEC, size exclusion chromatography; SLS, static light scattering; DLS, dynamic light scattering; IgG1, immunoglobulin 1; ND, neutral density; CCD, charge-coupled device; EDR, extended dynamic range; LS, light scattering; DSC, differential scanning calorimetry; QSSA, quasi-steady-state approximation; S/N, signal/noise.

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http://dx.doi.org/10.1016/j.ab.2013.02.014

respective aggregation kinetics of two structurally distinct monoclonal antibodies (mAbs) under conditions of thermal stress.

Currently, the most widely applied methods for assessing protein aggregation during pharmaceutical development are size exclusion chromatography (SEC), field-flow fractionation (FFF), sedimentation velocity (SV), and static and dynamic light scattering. These techniques have been compared directly, and each was shown to have specific strengths and drawbacks [9–11]. Nevertheless, SEC remains the primary technique for quality control due to its adequate sensitivity, high throughput, method simplicity/robustness, and cost effectiveness. In its simplest form, it is assumed that monomeric protein elutes within the separation regime of the SEC column(s) used and that large aggregates elute either in a resolvably different portion of the separation region or in the exclusion limit of the column(s). A refractive index detector usually provides a convenient means of providing a signal proportional to concentration at each elution point, so the ratio of the integrated presumed aggregate elution peak(s) to the integrated peak of the protein monomer is presumed to give the mass fraction of aggregate in the sample. SEC can be particularly valuable for following the disappearance of the protein monomer. When aggregate mass is plotted versus fraction of remaining aggregate, it is possible to distinguish aggregate growth mechanisms such as stepwise monomer addition and aggregate–aggregate coalescence.

Although the SEC methodology is considered to be the workhorse for quality control purposes, it has several disadvantages for probing aggregation kinetics and mechanisms. First, SEC is an equilibrium characterization method that assumes the protein population is not changing in time. If aggregation is slow compared with the time scale of an SEC measurement (typically 0.5 h, i.e., 30 min), then a "quasi-equilibrium" condition is met and the measurement can be meaningful. Second, there is an assumption that the entire aggregate population survives passage through the column and arrives at the detector to be measured. In fact, adsorption on and blockage in the column packing material, as well as blockage by any inline frits, pre-filters, or guard columns, may retain a fraction of the aggregate population, leading to an underestimate of aggregate content. Third, interaction of protein aggregates under high pressure with the column packing and the aforementioned frits and filters may lead to breaking up of aggregates, leading to an underestimate. Also of concern is the possibility that shear effects through these same SEC elements can lead to denaturation and aggregation of proteins; that is, the SEC method itself may create some aggregation or accelerate aggregation that is under way. Finally, SEC does not conveniently furnish aggregation kinetics, rather, it furnishes "snapshots" of aggregating proteins.

Spectroscopic techniques such as circular dichroism (CD) and fluorescence/Fourier transform infrared (FTIR) have also been applied, but their utility for quantitative analysis is hampered by the mass average nature of the signal and/or low sensitivity. Particle counting techniques such as nanoparticle tracking analysis [12,13] and microflow digital imaging [14,15] are emerging technologies that can monitor the formation of sub-micron- and micron-sized particles, respectively.

SMSLS was developed several years ago as a quantitative high-throughput instrument [16] particularly suited to both measuring the absolute molecular weight of many polymeric or colloidal solutions simultaneously and monitoring even the smallest changes in molecular weight of a polymeric or colloidal sample [17]. Such changes in molecular weight can occur from degradation, polymerization [18], microcrystallization, microgelation, micellization, and (in the case of a protein solution) aggregation. SMSLS is based on total intensity light scattering, often referred to as static light scattering (SLS). As summarized below, this allows absolute determination of molecular weight of solutes without recourse to molecular weight standards for calibration.

SLS is distinct from dynamic light scattering (DLS). Whereas SLS directly measures molecular weight, DLS measures a particle's mutual diffusion coefficient, which is less directly linked to particle molecular weight. In DLS, the fluctuations in scattered intensity are autocorrelated and related to the mutual diffusion coefficient of the scattering particles [19]. In the limit of zero scattering angle and polymer concentration, this yields the particle's translational diffusion coefficient, D, which in turn is reciprocally related to the particle's hydrodynamic radius, R_H, via the Stokes–Einstein equation. When the scatterers are polydisperse, D computed from the intensity autocorrelation function yields the z-average of the reciprocal of R_H, that is, $1/\langle 1/R_H \rangle_z$.

The relationship between diffusion coefficients, conveniently obtained by DLS, and molecular weight of macromolecules is not straightforward. For a given type of molecule, there is usually a relationship of the form $D = AM^{\alpha}$, where A is a proportionality constant. For rigid rod molecules, $\alpha = 1$, for random coils with excluded volume, $\alpha = 0.6$, for ideal coils, $\alpha = 0.5$, and for globular proteins, $\alpha = 0.333$; that is, D is rather insensitive to molecular weight. When aggregates form, this latter power law may continue, but in some types of aggregation processes, such as when a random coil begins to accrete other random coils (e.g., denatured proteins) without significantly changing hydrodynamic volume (i.e., a microgel of increasing density builds up), $\alpha = 0$, in which case there is no relationship between D and M. DLS can nonetheless be useful if $\alpha > 0$. Caution must be used when interpreting various algorithms that attempt to extract a particle size distribution from DLS autocorrelation functions by Laplace transform, smoothing, histogram, and other methods because these can introduce considerable artifacts. The average diffusion coefficient and first moment of the diffusion coefficient distribution are usually the most reliable characteristics.

SLS has been used to monitor the time dependence of protein aggregation in earlier work [20–23], but the current work is the first report, to the authors' knowledge, of a detailed kinetic and thermodynamic study resulting from high-throughput SLS measurements. Hence, the goal of this work is to introduce the SMSLS prototype and its capabilities, including issues of sensitivity, high throughput, and other performance aspects. It is then used for initial equilibrium characterization of two mAbs, one highly stable the other highly unstable, and subsequently to measure aggregation kinetics of the latter under a wide variety of temperature and concentration conditions. From these results, a detailed picture of the aggregation process for these particular proteins emerges and a hypothesis connecting measured equilibrium and non-equilibrium properties results. Conclusions about reproducibility, and the non-applicability of accelerated testing via elevated temperature stress, are also made.

Materials and methods

The monoclonal antibodies mAb1 and mAb2 were produced at Biogen Idec (Cambridge, MA, USA) via overexpression in mammalian cells using recombinant technologies. The proteins were purified, diafiltered, and concentrated into their respective formulation solutions and were stored frozen. Protein solutions for analysis were prepared by dilution and/or dialysis using the respective formulation buffer solutions when needed. mAb1 is an immunoglobulin 1 (IgG1) monoclonal antibody, and mAb2 is a bispecific antibody. mAb1 demonstrated good storage stability for 36 months at 5 °C. In contrast, mAb2 demonstrated significantly poorer stability under these conditions.

Dynamic light scattering

A Brookhaven Instruments BI-90 Plus (Holtsville, NY, USA) was used for DLS measurements of the antibodies. It used a 660-nm