Journal of Pharmaceutical Sciences 105 (2016) 2310-2318

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

Characterizing Reversible Protein Association at Moderately High Concentration Via Composition-Gradient Static Light Scattering

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ARTICLE INFO

Article history:
Received 13 December 2015
Revised 6 May 2016
Accepted 9 May 2016
Available online 28 June 2016

Keywords:
biopharmaceuticals characterization
biotechnology
protein aggregation
protein formulation
stability
light-scattering

ABSTRACT

Analysis of weakly self-associating macromolecules at concentrations beyond a few g/L is challenging on account of the confounding effect of thermodynamic nonideality on the association signal. When the reversible association comprises only 1 or 2 oligomeric species in equilibrium with the monomer, the nonideality may be accounted for in a relatively rigorous manner, but if more association states are involved, the analysis becomes quite complex. We show that under reasonable assumptions, the nonideality in a composition-gradient static light scattering measurement may be accounted for in a simple fashion. The correction is applied to determining the stoichiometry and binding affinity of a protein previously characterized via sedimentation equilibrium and dynamic light scattering. The results of the new analysis are remarkably self-consistent and in line with the expectations for the form of self-association predicted previously from analysis of the surface residuals, establishing composition-gradient multi-angle static light scattering with nonideality corrections as a critical technology for characterizing associative interactions in concentrated solutions.

Introduction

Characterization of protein self-association at high concentrations is important for many biomolecular applications, including drug product and process development. One of the few techniques capable of measuring and analyzing protein–protein interactions at high concentrations is composition-gradient multi-angle static light scattering (CG-MALS). Automated CG-MALS as applied to characterization of protein–protein interactions was first described by Attri and Minton.[1] This technique involves automatically mixing and delivering a series of single or dual-component compositions to 2 detectors, a static light scattering detector and a concentration detector. The light scattering and concentration signals are fit to appropriate association models to determine monomer molar masses, stoichiometry of association, binding affinities in terms of equilibrium association constants, and nonspecific interactions in terms of virial coefficients. CG-MALS does not require any tagging or immobilization of the molecules of interest, and formulation buffer can typically be used as diluent solvent, and hence provides a true in-solution characterization of unmodified formulations.

Several publications[2,3] have since shown that CG-MALS produces essentially the same results as sedimentation equilibrium (SE), the historic "gold standard" for determining stoichiometry and association constants of reversibly self- and hetero-associating proteins. In addition, CG-MALS offers a few attractive features: (1) the short time required to complete a measurement—typically 30 min to a few hours, (2) the relative simplicity of data interpretation, (3) an extended range to characterize stronger interactions, and (4) the low cost of the instrumentation. The method has also been compared with surface plasmon resonance and found to provide very similar results for antibody–antigen binding constants in the range of K_d = 100 pM to μM, while also determining the stoichiometry of these reversibly bound complexes.[3]

CG-MALS Theory

Interpretation of CG-MALS data in terms of protein self-association at concentrations in the range where thermodynamic nonideality may be ignored—that is, typically below about 1 mg/mL in a solution of reasonable ionic strength—is a relatively straightforward analysis. A set of equations describing the system are fit to the light scattering data acquired at a series of concentrations. Following Attri and Minton,[1] these are the equations for

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http://dx.doi.org/10.1016/j.xphs.2016.05.018
0022-3549/

conservation of mass, mass action, and ideal light scattering (Eqs. 1-3):

$$c_{tot} = c_1 + \sum_{i>1} i c_i \tag{1}$$

$$c_i = K_i (c_1)^i \tag{2}$$

$$R(0) = \tilde{K}\left(\frac{dn}{dw}\right)^2 \left[Mw_1 + \sum_{i>1} M_i w_i\right] = \tilde{K}\left(\frac{dn}{dw}\right)^2 \left[M^2 c_1 + \sum_{i>1} (iM)^2 c_i\right]. \tag{3}$$

Here, c represents protein molar concentrations; i is the degree of self-association of each oligomeric (i-mer) state, where $i > 1$; c_{tot} is the total concentration of free and bound monomers in solution; c_1 and c_i are the partial concentrations of free monomers and i-mers, respectively; K_i is the equilibrium association constant for monomer—i-mer association; $R(0)$ is the Rayleigh ratio determined from the intensity of scattered light over multiple scattering angles, extrapolated to zero angle and assuming incident light linearly polarized in the direction perpendicular to the scattering plane; $\tilde{K} = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}$ is a function of the free-space scattering wavelength λ_0 and solvent refractive index n_0; $\left(\frac{dn}{dw}\right)$ is the refractive index increment of the protein in solution with respect to weight concentration w; and M is the monomer molar mass. The equations are readily generalized to account for heteroassociations and even simultaneous self- and heteroassociation. A cursory inspection shows that, for a fixed c_{tot}, R increases with increasing aggregation and is in fact proportional to the weight-averaged molar mass of species in dynamic equilibrium.

Characterization of reversible self-association at concentrations higher than ~1 g/L is challenging, not because of experimental difficulties, but due to the need to account quantitatively for thermodynamic nonideality. Nonideality arises from nonspecific interactions between molecules such as short-range, hard-core repulsion; medium-range, van der Waals, and dipole interactions; or long-range, electrostatic interactions. Intermolecular correlations induced by repulsive interactions affect the dependence of the light-scattering signal on concentration in ways that counteract the effects of association. The complete expression for multicomponent, static light scattering as expressed in fluctuation theory[4] is presented in Equation 4:

$$R(0) = \tilde{K} \sum_{j,k=1}^{n} \left\{ \frac{\Psi_{jk} w_k M_j}{|\psi|} \left(\frac{dn}{dw_j}\right)\left(\frac{dn}{dw_k}\right) \right\} \tag{4}$$

Here, n is the number of distinct species in solution; $\psi_{jk}=\delta_{jk}+w_j X_{jk}$, where $\delta_{jk} = 1$ if $k = j$, otherwise $\delta_{jk} = 0$; $X_{jk} = \frac{\partial \ln \gamma_k}{\partial w_j} = \frac{1}{RT}\frac{\partial \mu_k}{\partial w_j}$, γ_k is the thermodynamic activity coefficient and μ_k the chemical potential of species k; $|\psi|$ is the determinant of ψ; and Ψ_{jk} is the jk cofactor of ψ.

Thermodynamic nonideality is commonly expressed via the virial expansion, most familiar as the virial equation for osmotic pressure. For a single, nonassociating species, under the virial expansion, Equation 4 simplifies to Equation 5:

$$\frac{R(0)}{\tilde{K}} = \left(\frac{dn}{dw}\right)^2 \frac{Mw}{1 + 2A_2 Mw + 3A_3 Mw^2 + \ldots} \tag{5}$$

The molar mass and virial coefficients A_2, A_3, … may be determined by fitting the light scattering signals of a concentration series to a suitably truncated version of Equation 5. At high enough concentration, Equation 5 will generally require several virial coefficient orders, which may not be determined unambiguously by a simple unconstrained fit to the data. The virial correction becomes more complex when dealing with more than one species due to the myriad self- and cross-species interactions described by Equation 4 and expanded in terms of self- and cross-virial coefficients.

Minton and Edelhoch[5] applied a simplifying assumption to the analysis of protein—protein interactions via CG-MALS at high concentration, the Effective Hard Sphere Approximation (EHSA). In the context of EHSA, the molecule is assumed to undergo billiard-ball—like nonspecific interactions, albeit subject to an effective molecular radius that may differ from the actual physical or hydrodynamic radius of the molecule. For example, a net charge will add repulsion between molecules and hence increase the effective radius. Moreover, this assumption will generally only be valid if the "soft" interactions are of relatively short range[6]; for example, the net charge should be fairly well screened by counter-ions in the solvent for the overall potential to behave effectively like that of a hard sphere. As shown by Minton,[7] in such a model all of the self-virial coefficients may be expressed in terms of a single parameter, the effective radius r_{eff}, greatly simplifying the analysis (with some minor algebraic manipulation):

$$\frac{R(0)}{\tilde{K}} = \frac{Mw}{1+8\nu w+30(\nu w)^2+73.4(\nu w)^3+141.2(\nu w)^4 \Big/ (1-1.368\nu w)} \tag{6}$$

where $\nu = \frac{N_A}{M}\frac{4\pi}{3} r_{eff}^3$. Comparing Equations 5 and 6, we find the familiar result that the second virial coefficient of a solution of hard spheres may be calculated from the radius as:

$$A_2 = \frac{16\pi N_A r^3}{3M^2} = \frac{4\nu}{M} \tag{7}$$

A reasonable measure of the steric molecular interactions is the hydrodynamic radius,[8] measured versus concentration and extrapolated to the limit of zero concentration, which may be obtained from quasielastic light scattering. This is then the baseline value; an overall effective radius can be considered as a modification of the hydrodynamic radius value due to "soft" interactions.

Cross-virial coefficients may also be expressed correspondingly in terms of the effective radii of the interacting species using an appropriate model such as the scaled particle theory (SPT) or the modified SPT.[9,10] Attractive interactions are preferably folded into the association constants derived from fitting the data to an appropriate association model so that the nonideal terms incorporate primarily repulsive elements such as the steric interaction.[10]

Fernandez and Minton[11] further applied EHSA and SPT to CG-MALS analysis of chymotrypsin self-association up to 70 g/L, which was best described as equilibrium between 3 species—monomer, dimer, and either a pentamer or hexamer. To account for the nonspecific interactions of the oligomers, the authors made a further simplifying assumption, setting the effective specific volume of the oligomers equal to that of the monomer. The data were fit by Equation 4 under EHSA and SPT for monomer and the 2 oligomers.

The chymotrypsin system involved only 2 oligomeric association states. As more oligomers are added to the mix, just writing out Equation 4 becomes very cumbersome. A further simplification is in order for the high-concentration analysis of CG-MALS data to become more manageable and applicable. Such a simplification, described by Minton[12] as an approximate "universal correction," has been suggested by Chatelier and Minton[10] and shown to provide results quite close to those obtained with more rigorous, but