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Analytical ultracentrifugation for the study of protein association and assembly

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Analytical ultracentrifugation remains pre-eminent among the methods used to study the interactions of macromolecules under physiological conditions. Recent developments in analytical procedures allow the high resolving power of sedimentation velocity methods to be coupled to sedimentation equilibrium approaches and applied to both static and dynamic associations. Improvements in global modeling based on numerical solutions of the Lamm equation have generated new sedimentation velocity applications with an emphasis on data interpretation using sedimentation coefficient or molar mass distributions. Procedures based on the use of multiple optical signals from absorption and interference optics for the analysis of the sedimentation velocity and equilibrium behavior of more complex interactions have now been developed. New applications of tracer sedimentation equilibrium experiments and the development of a fluorescence optical system for the analytical ultracentrifuge extend the accessible concentration range over several orders of magnitude and, coupled with the new analytical procedures, provide powerful new tools for studies of both weak and strong macromolecular interactions in solution.

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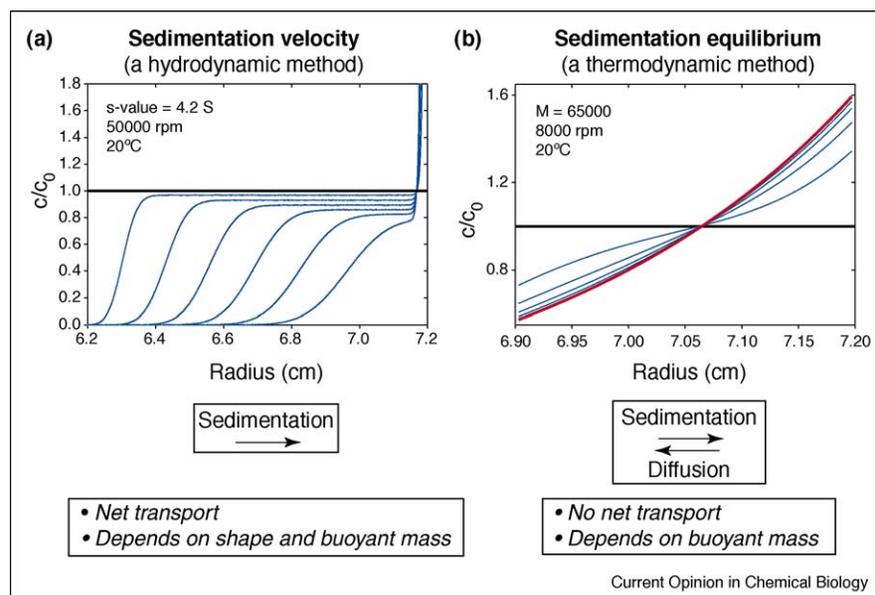
Introduction

Most biochemical reactions and processes are carried out by macromolecular complexes that interact in a reversible manner to form functionally active structures [1]. Therefore, the quantitative characterization of reversible macromolecular associations, in terms of stoichiometry and strength of the interactions, is essential to our

understanding of normal biological processes, pathological aggregation and degradation reactions, and the stability of biological macromolecules with respect to aggregation. For these reasons, there exists a large variety of methods for studying protein interactions, many of which have been recently reviewed [2,3]. However, in spite of the obvious biological relevance of multi-protein complexes and the large battery of tools available, it is noteworthy that the number of these complexes that are well characterized in terms of complex stoichiometry, affinity and reversibility is very low, especially in the case of transient protein interactions [3,4]. Here, we focus on analytical ultracentrifugation (AUC), which is one of the most powerful and versatile techniques for the quantitative characterization of macromolecular associations in solution. Two established techniques for measurement of solute gradients in AUC experiments are: (i) real-time data acquisition in analytical ultracentrifuges (XL-A and XL-I, Beckman-Coulter, Inc.) that allow an optical signal (UV-VIS, interference) to be measured as a function of radial distance; and (ii) post-centrifugation data acquisition using preparative ultracentrifuges followed by automated centrifuge tube microfractionation, a technique especially adapted to perform tracer sedimentation equilibrium (see below) experiments (for a comparative analysis, see [5]). The aim of this manuscript is to review recent advances in the use of AUC to study protein association and assembly since 2003, with special emphasis on novel techniques and applications. The reader should consult [6–8,9*,10] for more comprehensive reviews on AUC covering the field before 2004.

When selecting a method to detect or characterize macromolecular interactions one must take into account whether the interaction is expected to be static or dynamic. Static associations are very slowly reversible or irreversible on the time scale of the experiment; it is possible to physically separate different states of association (e.g. monomer, individual oligomers) and characterize them individually. They are typically strong attractive self- and/or hetero-interactions leading to the formation of non-covalent complexes. In analytical ultracentrifugation, static interactions are most commonly studied by the technique of sedimentation velocity (SV). This is a hydrodynamic method where the rate of transport is measured and macromolecular complexes are fractionated at high centrifugal force on the basis of differences in mass, density and shape (Figure 1a). Analysis of the time-dependent gradients leads to estimates of the sedimentation coefficient and molar mass of each sedimenting species [11].

Figure 1



Basic concepts in analytical ultracentrifugation. Simulation of the sedimentation of a 65 kDa single species at 20 °C with s -value of 4.2 S. c/c_0 is the ratio of the protein concentration at a given radial position and time to the loading concentration before centrifugation (black). **(a)** An SV (high-speed) experiment. Successive gradients correspond to elapsed time intervals of 5 min. **(b)** An approach to equilibrium (low-speed) experiment. Successive gradients correspond to elapsed time intervals of 4 h. The final gradient (red) represents a close approximation to SE.

Dynamic associations are rapidly reversible on the time scale of the experiment; it is not possible to physically separate different states of association for the duration of the measurement. In this case, information about the underlying interactions is obtained from the analysis of the composition dependence of some total property of the interacting system. Moderately strong attractive self- and/or hetero-interactions leading to non-covalent complexes that exist in composition-dependent equilibrium with separated reactants belong to this class of interaction. Dynamic interactions have been most commonly studied by the method of sedimentation equilibrium (SE), which provides information about the composition dependence of signal-average buoyant molar mass. At sedimentation equilibrium there is no net transport and the equilibrium distributions (Figure 1b) can be analyzed using thermodynamic models for associations [12].

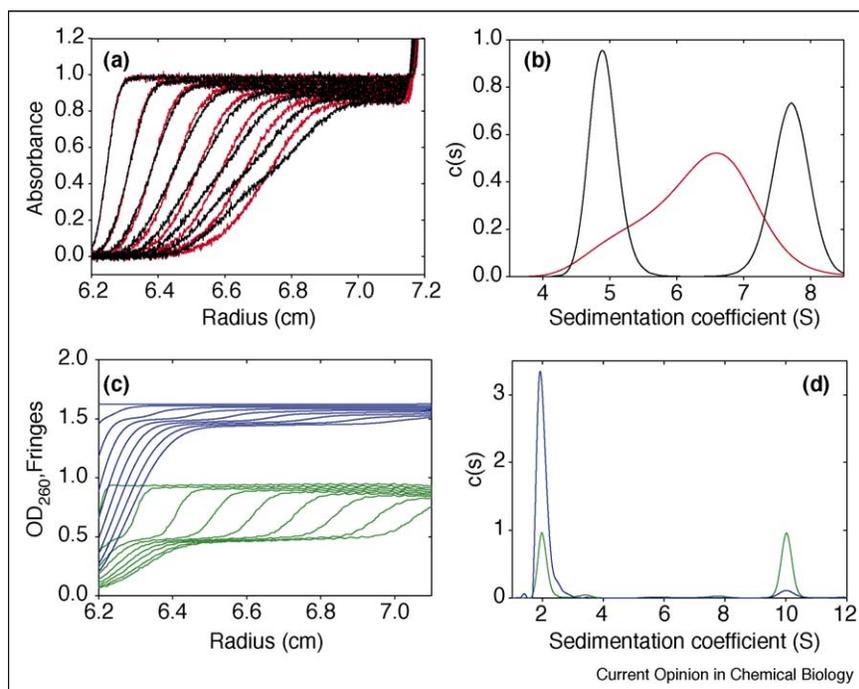
Advances in sedimentation velocity

The SV behaviors of static and dynamic macromolecular associations are quite different. This is illustrated by the simulations in Figure 2a. Radial distribution data for a static monomer–dimer system yield two discernable sedimenting boundaries that resolve into two peaks (Figure 2b), corresponding to the sedimentation coefficient of monomer and dimer, using continuous size distribution analysis [13]. For a dynamic monomer–dimer interaction, a single broad sedimenting boundary is observed, yielding a skewed sedimentation coefficient distribution (Figure 2b) with sedimentation coefficients

ranging between the values for monomer and dimer. Information regarding dynamic macromolecular associations may be obtained from SV experiments through analysis of the composition dependence of the weight-average sedimentation coefficient [14,15]. Schuck [16] demonstrated that when reactants are in rapid equilibrium, the weight-average sedimentation coefficient can be accurately evaluated by appropriate integration of the sedimentation coefficient distributions [$g(s^*)$, $ls_g^*(s)$, $c(s)$], provided that the distribution accurately describes boundary evolution with time. Thus, integration of the distributions in Figure 2b for the static and dynamic monomer–dimer interactions yields the same value for the weight-average sedimentation coefficient. These values are related to the molar masses and frictional coefficients of the individual species in solution and can be used, in principle, to select appropriate models for self-association for more detailed SE analysis.

A limitation in the use of average sedimentation coefficient analysis for studies of complex interactions is the difficulty in obtaining enough accuracy to distinguish different models of association. Significant progress has been made in the direct fitting of SV data using numerical solutions of the Lamm equation. The programs SEDANAL [17] and SEDFIT [13] allow both static and dynamic interactions for both multi-species and multi-component systems. These programs can accommodate very complex models with a range of forward and reverse rate constants for association. A requirement in the fitting

Figure 2



SV of interacting systems. **(a)** The individual simulations were calculated for 10 min intervals at 50 000 rpm. The red lines are for a rapidly reversible monomer–dimer system assuming spherical shapes, a monomer molar mass of 50 kDa and an equilibrium constant (in optical density units) of 2. The black lines are simulations for an identical monomer–dimer system where interconversion of the species is irreversible over the time course of the experiment. **(b)** Continuous sedimentation coefficient distribution analysis of the simulated data in (a). **(c)** Experimental SV data (50 000 rpm, 20 °C) using interference optics (blue) and absorption optics (green) for a protein–DNA mixture containing a nucleoprotein complex with an *s*-value of 10 S and an excess of unbound protein that sediments with an *s*-value of 2 S. **(d)** Multi-optical signal analysis of the data in C to obtain component distributions shown by the blue and green lines.

process is to deconvolute the effects of diffusion from boundary spreading due to species heterogeneity. One approach is to use a weight-average frictional coefficient as a fitting parameter to relate the molecular weight and sedimentation coefficient of individual species [18]. An alternative approach, applied to studies on the self-association of tubulin [15], is to use a hydrodynamic model [19] to estimate the shape factor for individual species. An advantage of the SV approach is the ability to obtain information on both the size and shape of the complexes. However, this can also be a disadvantage in that the number of fitting parameters becomes large for complex interactions. A powerful new approach is to combine the analysis of SV and SE data. The program SEDPHAT [16] has been specifically designed for this purpose. An application of this approach is provided in a recent study of the self-association of different isoforms of apolipoprotein E [20*].

A recent publication [21*] describes the integration of multiple optical signals into the sedimentation coefficient distribution analysis of components, which combines size-dependent hydrodynamic separation with the discrimination of the sedimenting species according to their extinction properties. An illustration of this approach is

given in Figure 2c,d. The data shown were obtained using either interference optics or absorbance optics for a protein–DNA mixture. The difference in the relative sizes of the sedimenting boundaries reflects the separate contributions of protein and DNA to the optical signals. Using the multi-optical signal approach these boundaries can be resolved into sedimentation coefficient distributions where the separate contributions of the two components to the distributions can be identified. This method permits determination of the stoichiometry of each sedimenting species without prior knowledge of the assembly reaction.

SV has been recently used to detect and characterize unusual features of the oligomerization of the bacterial cell division protein FtsZ [22]. In this study, diffusion-deconvoluted sedimentation coefficient distributions were calculated from SV experiments and found to exhibit a concentration-dependent bimodal distribution. These results, together with results obtained from precipitation assays and images from electron and atomic force microscopy, could be explained by a mechanism in which intrinsic curvature of protein fibrils leads to formation of closed circular fibers with a narrow size distribution.

Advances in sedimentation equilibrium

Novel sedimentation equilibrium methods and applications

The modeling of concentration profiles of solutions containing multiple interacting solute species at sedimentation equilibrium may be rendered mathematically ill-conditioned by the required evaluation of the meniscus concentration of each solute species [23]. This requirement can be partially or completely eliminated through the use of mass conservation constraints and prior knowledge of component loading concentrations. However, valid employment of these constraints requires precise knowledge of the radial position of the base of the sample column, not directly obtainable experimentally, and actual conservation of mass, not always achieved in real systems of biomolecules. Schuck and collaborators [24•] have introduced a model for implicit mass conservation, in which the column base position and effective loading concentrations of solute components are taken as fitting parameters, subject only to the assumption that these remain constant among several datasets obtained at multiple rotor speeds. The implicit mass conservation conditions, which may be applied with variable stringency, are found to greatly simplify the task of defining a unique model for reaction equilibria that can simultaneously account for all experimentally observed equilibrium gradients. In addition to the previously cited work by Chou and co-workers on apolipoprotein associations [20•], the strategy of using this SE approach in conjunction with multi-signal analysis has also been applied, for example, to study the structural organization and self-association properties of the merozoite surface protein 3, an effective protector against parasite infection in primates [25], and the self-association and hetero-association reactions of an iron-regulated protein [26].

The determination of the mass of detergent-solubilized membrane proteins via SE has been accomplished in the past by matching the density of buffer with that of detergent, thus eliminating the contribution of detergent to the buoyant mass of the protein [27]. Noy and collaborators [28] have eliminated the requirement for buffer density matching by assuming that the composition of a protein–detergent complex is independent of buffer density, and obtaining that composition via global modeling of multiple equilibrium gradients from solutions with different buffer densities.

A thermodynamically rigorous formulation of SE in a solution containing an arbitrary number of solute species at arbitrary concentration has been presented [29•]. This formulation permits the analysis of SE in the presence of both attractive and repulsive interactions between different solute species. Although the formalism can become computationally complex in the general case, a special case permits facile characterization of the effect of a

single concentrated solute upon interactions between dilute solutes [30].

Protein–nucleic acid interactions are most commonly characterized via gel-shift or filter binding assays. However, these assays are not conducted under equilibrium conditions, so interpretation of the results is questionable when protein–nucleic acid associations are nonspecific, hence of lower affinity and rapidly reversible. Ucci and Cole have employed SE to characterize the nonspecific binding of the RNA-binding domain of protein kinase R to two different oligoribonucleotides containing 20 bases each [31]. The analysis was facilitated by measurement of absorbance gradients at 230, 260 and 280 nm, permitting calculation of individual concentration gradients of protein and nucleic acid (see for example [32–34]) (Figure 3a,b). Equilibrium gradients measured in three solutions containing different ratios of protein to RNA were globally fit by combinatorial models describing either non-overlapping or overlapping binding of large ligands to a finite one-dimensional lattice, yielding best-fit values of an intrinsic binding constant.

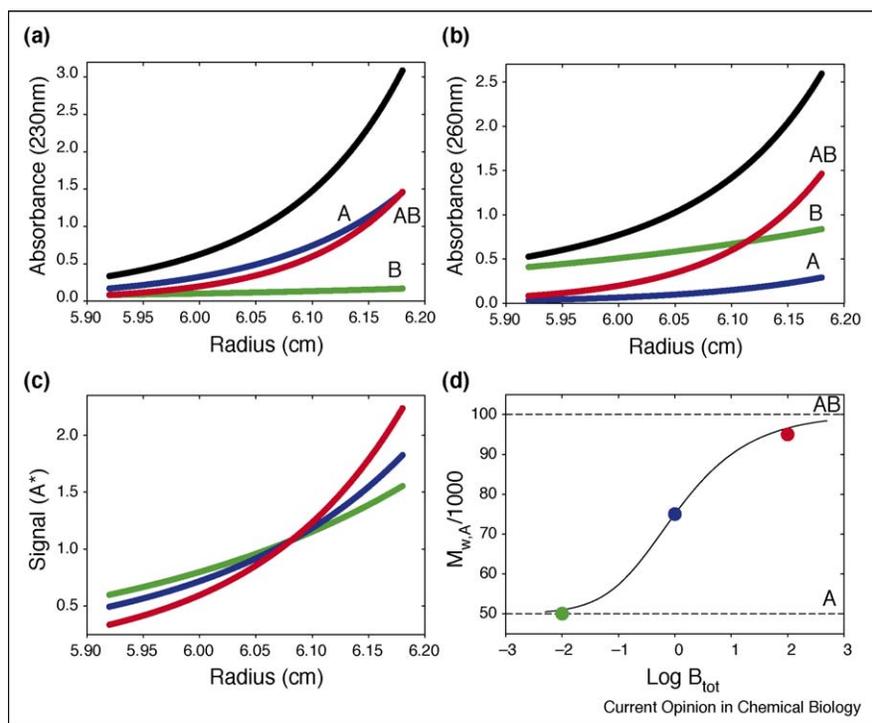
The self-association of transmembrane helices within detergent micelles has been characterized via SE by Fleming and collaborators [35]. The equilibrium constants for self-association are corrected to reflect the concentration of protein within the micelle rather than the total solution (aqueous buffer + micelles). Such a correction is based upon the assumption that the helices behave as if they were in an ideal solution within the micelle. Fleming previously proposed and implemented a simple experimental test of the validity of this assumption [36].

Tracer sedimentation equilibrium in concentrated (non-ideal) solutions

A variation of SE that is particularly useful for the detection and characterization of complex interacting systems and repulsive interactions (i.e. in crowded solutions; see [37,38]) is tracer sedimentation equilibrium (TSE) [30,39] (Figure 3 c,d). A tracer is defined as a solute component present at low concentration relative to other solute components, the equilibrium concentration gradient of which may be reliably quantified independent of the gradients of other solute components [10,39]. A particular component can qualify as a tracer if it has a uniquely detectable signal (i.e. enzymatic or immunochemical activity) or if it can be provided with a unique signal via labeling.

By means of preparative ultracentrifugation coupled with microfractionation as described in [40], Zorilla *et al.* [29•] have measured the SE of RNase A at concentrations up to 200 g/l and analyzed their results in the context of the general theory presented in this paper. The data are shown to provide clear evidence of extremely weak

Figure 3



SE of interacting systems. **(a,b)** Multi-wavelength analysis of an $A + B = AB$ system (molar masses of A and B were 50 and 10 kDa, respectively; the extinction coefficient of A and B at 230 nm (and 260 nm) were 1 (0.2) and 0.2 (1.0) $(\text{mg/ml})^{-1} \text{cm}^{-1}$; the speed was 15 000 rpm. The solid lines represent the total and individual species gradients at 230 nm (a) and 260 nm (b). An experimental example of this kind of approach can be found in [32]. **(c,d)** Tracer SE analysis of an $A^* + B = A^*B$ system, where A^* denotes the tracer component A. Both the SE gradients (c) and the dependence of the apparent signal-average molecular weight of A^* as a function of the concentration of total B (d) are shown. The parameters used in the simulations are the following: the molecular weight of A^* and B was 50 kDa; the concentration of A^* was constant and equal to 1 mg/ml; the concentration of B was 0 (green), 1 (blue) and 100 (red) mg/ml; the association constant was 1 $(\text{mg/ml})^{-1}$; the speed was 10 000 rpm. An experimental example of this kind of approach can be found in [46].

self-association to form significant amounts of small, presumably nonspecific oligomers at concentrations exceeding ca. 50 g/l.

By means of preparative ultracentrifugation coupled with microfractionation, Liu *et al.* [41] measured the SE of three different monoclonal antibodies at concentrations of up to ca. 200 g/l. In high salt solution, the apparent weight-average molar mass of all three antibodies decreased strongly and identically with increasing protein concentration. Analysis of this dependence indicated that the primary source of this highly non-ideal behavior was volume exclusion. In contrast, in a low salt solution, one of the three antibodies exhibited significant self-association, and after approximate correction for non-ideal effects, the weight-average molar mass of this antibody was estimated to approach four times that of the monomer at a protein concentration of ca. 60 g/l.

Conclusions

In this review we have summarized the recent methodological advances in analytical ultracentrifugation and novel applications to study protein complexes in solution.

In the light of these advances, we anticipate the following developments:

1. Additional applications of the newly developed multi-signal SV technique [21[•]] to biologically relevant multi-protein complexes, which will complement and increase the resolution of previously developed multi-signal analyses of SE [32–34].
2. Increased use of global analysis of the results of multiple methods for the study of protein associations, such as the powerful combination of SV, a method well suited for characterization of solute heterogeneity, and SE, a method well suited for characterization of composition-dependent equilibrium average properties [16]. Although outside the scope of this review, it is clear that such studies would also benefit from additional information obtained from recently developed approaches to the measurement and analysis of static [42,43] and dynamic [44] light scattering.
3. Additional applications of TSE analysis, a powerful technique used only sparsely until now. This technique enjoys several significant advantages over conventional methods of characterizing equilibrium

associations in solution. First, the method can be implemented using non-optical probes of tracer solute behavior, hence the fluid medium may be opaque or translucent. Second, the concentrations of non-tracer components may be varied over an arbitrarily wide range without significantly varying the overall magnitude of the signal, permitting study of associations under the most advantageous experimental conditions, making strong, moderate, and weak interactions simultaneously accessible to study. Third, in dilute solution, the contribution of each postulated solute species to the overall measured signal may be precisely calculated *a priori*, given independently measurable values of individual component buoyant molar masses, total component concentrations, and assumed equilibrium relations and fractional abundances of species. In addition, a recent analysis [30] has shown that, under favorable conditions, the analysis of specific associations in highly non-ideal (crowded) solutions may be greatly simplified and may be implemented without ambiguities resulting from lack of information regarding weak nonspecific intermolecular interactions.

4. Finally, we anticipate that new instrumental developments, such as the recently introduced fluorescence detector for the analytical ultracentrifuge [45] will further increase the power and areas of application of AUC methods in protein science.

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