Characterizing the Solution Properties of Supramolecular Systems by Analytical Ultracentrifugation

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Dedicated to Professor Jean-Marie Lehn on the occasion of his 60th birthday

Abstract: Analytical ultracentrifugation is the study of the movement or the local distribution of dissolved supra- or macromolecular particles under the influence of centrifugal force, by measuring and evaluating concentration-versus-radius distributions in the sample. This paper describes application of the method to the determination of the state of association of supramolecular compounds in solution. Both principles and experimental techniques are considered and applied to a special metal coordination array. Those methods analyzing the "transient" sedimentation patterns, namely, sedimentation velocity experiments and the "approach to equilibrium", can yield information on aggregate size within less than approximately 3 h. In particular, by the approach to equilibrium method (Archibald method) the average molar mass of the dissolved compound can be determined within about 30 min. Sedimentation equilibrium analysis can yield the percentage of the different aggregates present and, for reversibly associating systems, association constants.

Keywords: analytical methods · analytical ultracentrifugation · association in solution · supramolecular chemistry

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Introduction

Designing, synthesizing, and characterizing supramolecular systems is one of the current principal topics in modern chemistry, as demonstrated by the large number of publications and conferences in the field during the last few years.[1] The general concept applied is to arrange special molecules into highly ordered architectures by self-assembly through noncovalent interactions. It is anticipated that, by this "chemistry beyond the molecule",[1a, 2] structures possessing new and interesting mechanical, thermal, electrochemical, photochemical, or magnetic properties can be constructed,[3] with potential applications in, for example, nanotechnology.[1]

With respect to the physical characterization of supramolecular architectures, powerful methods are available for systems present as single crystals or as ordered surface layers.[1c, 4] For supramolecular complexes in solution, however, the situation is much less favorable, in particular for metallo-supramolecular systems. This even holds for studies on rather basic problems, like that of the molar mass or the related problem of the state of association of a supramolecular structure in a particular solvent. It is this latter type of study which is the concern of the present paper.

Information on the state of association of supramolecular systems has been sought previously mainly from vapor pressure osmometry and from electrospray mass spectroscopy[1c, 5]. It is obvious, however, that the results of these methods do not necessarily reflect the state of the compound studied in solution (in addition, the former technique is barely applicable to charged systems[1d]). Recently, we have suggested the application of an alternative, true solution method, analytical ultracentrifugation.[6] This classical method of biochemistry and macromolecular chemistry for determining the molar mass or the state of association of macromolecules[7] has long been considered as obsolete, mainly due to instrumental difficulties. However, during the last few years, following the introduction of a new commercial analytical ultracentrifuge, it has seen a remarkable renaissance. Application of this technique to supramolecular chemistry does, in fact, lead to a number of problems concerning both technical aspects (resistance of the cell components towards the organic solvents required for most studies) and more fundamental

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problems (avoidance of nonideal sedimentation behavior, determination of the compound's partial specific volume). We have shown in a feasibility study, however, that these problems can be overcome,[6] thus opening the way towards a general acceptance of this method for studies on supramolecular compounds.

A major obstacle in establishing analytical ultracentrifugation as a standard tool in supramolecular chemistry is that, due to two decades of virtually complete neglect of this classical technique in physical chemistry of large molecules, most researchers in the field are unaware of its potential and advantages. It is the aim of the present paper to contribute to removing this obstacle, by 1) briefly describing the foundations and methods of analytical ultracentrifugation, and 2) demonstrating its potential by describing its application to a model system, a metal coordination array (Figure 1). This compound consists of four molecules of 4,6-bis(5″-methyl-2″,2′-bipyrid-6′-yl)-2-phenylpyrimidine and four cobalt(II) ions plus appropriate counterions, held together by noncovalent interactions.[8, 9] M_r of the compound (including 8 PF_6 counterions) is 3366. A very similar compound was used in the feasibility study referred to above.[6] The association behavior of several other compounds of this class will be described elsewhere.[10]

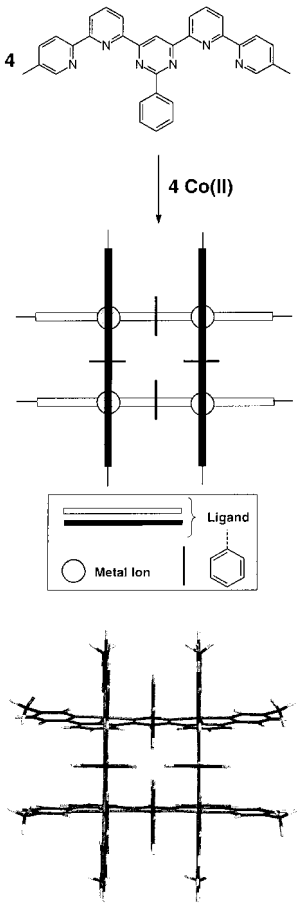

Figure 1. Building blocks, reaction scheme, and structure of the cobalt coordination array (wireframe model, MacSpartan, level MM2; for an X-ray structural analysis of a similar grid, see ref. [8]).

Background

General: The purpose of analytical ultracentrifugation is the study of the movement or the local distribution of molecules (in solution) under the influence of a centrifugal force. This is usually done in an instrument based on a preparative ultracentrifuge (as routinely used in biochemistry) to which are added three different accessories: a special rotor, special cells equipped with transparent windows, and an optical system allowing the measurement of the local absorbance or the local refractive index of the sample at any radial position in the ultracentrifuge cell and at any time after starting the ultracentrifuge (Figure 2).[11] The concentration-versus-radius

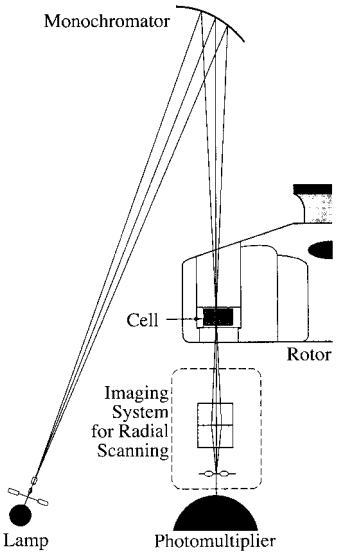

Figure 2. The optical system of the Beckman Optima XL-A analytical ultracentrifuge.[11]

Abstract in German: *Analytische Ultrazentrifugation beschäftigt sich mit dem Studium der Bewegung oder der lokalen Verteilung von gelösten supramolekularen oder makromolekularen Systemen unter dem Einfluß der Zentrifugalkraft. Hierzu wird die Konzentrationsverteilung der Moleküle als Funktion des Radius gemessen und ausgewertet. Dieser Artikel beschreibt die Anwendung der Methode auf die Untersuchung des Assoziationszustandes von supramolekularen Systemen in Lösung. Sowohl das Meßprinzip als auch die experimentellen Techniken werden behandelt und auf ein spezielles metallosupramolekulares Metallgitter angewendet. Die Methoden zur Untersuchung der „transienten" Sedimentationsverteilungen, nämlich Sedimentationsgeschwindigkeitslauf und Annäherung an das Gleichgewicht (Archibald-Methode), können Informationen über die Größe der Aggregate in weniger als 3 h liefern. Insbesondere die Archibald-Methode liefert eine Aussage über die mittlere Molekülmasse der gelösten Verbindungen in weniger als 30 min. Die Gleichgewichtsläufe geben detaillierte Auskünfte über den Anteil der unterschiedlichen vorhandenen Aggregate; im Falle von reversibel assoziierenden Systemen können sie auch Gleichgewichtskonstanten liefern.*

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curves obtained from the measurements contain information on the molar masses of the molecules under study and, additionally, on parameters related to the shape of the molecules (sedimentation, diffusion, or frictional coefficient).[7] When applied to associating molecules, the data can also yield association constants.[7] Although the method has been used for molecules as small as sucrose ($M_r = 342$), it is more useful for molecules with molar masses between ≈ 1000 and several million g mol^{-1}. This range includes the sizes characteristic for systems of supramolecular chemistry.

Applications of analytical ultracentrifugation which aim at determining basic properties of the molecules under study are usually conducted under conditions of "ideal" sedimentation behavior, which can be realized by using low enough solute concentrations and performing the measurements in the presence of at least 10–20 mM salt[7] (for situations where this ideal behavior cannot be achieved, the reader is referred to refs. [7a, 7c]). Under these conditions, the sedimentation of the molecules in a sector-shaped solution column is described by the Lamm equation [Eq. (1)],[7] where $c_k(r,t)$ denotes the

$$\frac{\partial c_k}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r}\left[r\left(D_k\frac{\partial c_k}{\partial r} - s_k\omega^2 r c_k\right)\right] + Q_k \qquad (1)$$

concentration of the molecular species k ($k = 1,\ldots n$) at radius r and time t, s_k and D_k denote the sedimentation and diffusion coefficient of that species, respectively, Q_k denotes the local chemical reaction rates (which are zero for noninteracting components), and ω denotes the angular velocity of the rotor. It can be shown that the term in parenthesis represents the local transport flux in the centrifugal field, and that s_k and D_k are related to each other and to the molar mass M_k of species k by the Svedberg equation [Eq. (2)],[7] where $\overline{\nu_k}$ denotes the partial specific volume (approximately the reciprocal density) of species k and ρ is the solvent density.

$$s_k = \frac{D_k M_k (1 - \overline{\nu}_k\rho)}{RT} \qquad (2)$$

Analytical ultracentrifugation experiments can be performed for the analysis of the transient states, that is, the evolution of $c_k(r,t)$ in time, which are referred to as sedimentation velocity methods, or for the analysis of the equilibrium distribution $c_k(r,\infty)$ attained after sufficiently large sedimentation times, referred to as sedimentation equilibrium experiments.[7]

Transient state analysis: The time-course of sedimentation $c_k(r,t)$ contains information on the molar mass and the sedimentation coefficient as well as the diffusion coefficient and hydrodynamic radius of the species k. Its measurement does not permit the same detailed analysis of the state of association of the sample molecules as do sedimentation equilibrium experiments. On the other hand, it allows a relatively quick assessment of the aggregation state of the sample, which may be very useful as a quality control preceding, for example, its use for preparing surface films[12] or similar applications.

Sedimentation velocity experiments are traditionally performed with special experimental configurations to allow the application of robust analytical solutions to Equation (1). In most cases, relatively high centrifugal fields are applied to an initially uniform solution ($c_k(r,0) = c_{k,0}$), in order to produce clear sedimentation boundaries of the macromolecular components. The movement of the sedimentation boundary allows the precise determination of the sedimentation coefficient and hydrodynamic properties of the macromolecules from a simple solution of Equation (1) for a single macromolecular component in the absence of diffusion.[7] Recently, however, methods have become available that allow the direct modeling of sedimentation data $c_k(r,t)$ with the Lamm equation [Eq. (1)].[13] They can also be used for the analysis of the sedimentation profiles produced by small molecules, where no clear sedimentation boundary can be achieved. It is exactly this latter behavior which is exhibited by many, if not most, systems of supramolecular chemistry, making this approach the method of choice (see below).

A special solution to the Lamm equation is available at the end of the solution column: As shown by Archibald,[14] the measurement of the concentration and its derivative at the meniscus, at any time in the centrifugation process, allows the estimation of the average molar mass of the molecules under study. While the original Archibald method suffers from optical artifacts which prevent precise concentration measurements at the meniscus position, more recently described variants[15] do not. In these alternative Archibald methods, the average molar mass of the sample molecules is extracted by applying the Lamm equation [Eq. (1)] from the time-course of the absorbance profiles in the close vicinity of the meniscus (measured in rapid succession after the start of the ultracentrifuge). This group of methods is the least precise but the most rapid among those described in this paper. It can be combined with a sedimentation equilibrium experiment, representing the initial phase of the latter one.[15b]

Equilibrium analysis: Although sedimentation equilibrium could simply be considered as the special case $t \to \infty$ of the general sedimentation process described by Equation (1), it is much more powerful, in that sedimentation equilibrium allows the direct application of equilibrium thermodynamic principles.[7] In addition, by virtue of the absence of hydrodynamic parameters, sedimentation equilibrium experiments are much more robust in their interpretation and are clearly the method of choice for more complex solutes.

In most sedimentation equilibrium experiments, a much lower centrifugal field is used than in traditional sedimentation velocity experiments. This reduces the sedimentation fluxes to a magnitude comparable to the diffusional fluxes, such that after sufficient time (from several hours up to a few days) an equilibrium can be attained in which the concentration of the solute at the bottom of the solution column is only a few times higher than the concentration at the meniscus. It can be shown that in sedimentation equilibrium a single component k assumes a Boltzmann distribution [Eq. (3)],[7] where r_0 denotes an arbitrary reference radius. For

$$c_k(r) = c_k(r_0)\exp\left[\frac{M_k(1 - \overline{\nu}_k\rho)\,\omega^2}{2RT}(r^2 - r_0^2)\right] \qquad (3)$$

a mixture of n species, the resulting concentration distribution is the sum of the contributions of the different components [Eq. (4)]. Importantly, for dilute solutions, this equation holds

$$c(r) = \sum_{k=1}^{n} c_k(r_0) \exp\left[\frac{M_k(1-\bar{\nu}_k\rho)\,\omega^2}{2RT}(r^2 - r_0^2)\right] \quad (4)$$

true regardless of whether the components are stable or are only reversibly formed through noncovalent interactions. If different components are in reversible association equilibrium, and if there are no pressure effects on the equilibrium constant that governs the association, then the preexponential factors are simply coupled by the law of mass action. This permits expression of $c_k(r_0)$ in terms of $c_1(r_0)$ and the association constants K_{a1k} $(k>1)$. As a consequence, the measurement of the equilibrium populations of interacting components can be performed without their separation. This makes sedimentation equilibrium particularly powerful for the characterization of weak reversible macromolecular or supramolecular interactions.

In practice, the measurement of the molar mass of a single component can be best performed by fitting Equation (3) to the experimental sedimentation equilibrium profiles, using M_k and $c_k(r_0)$ as fitting parameters. The same procedure can, in principle, be applied to the analysis of multicomponent systems with Equation (4). However, as with all techniques that require the analysis of experimental data in terms of sums of exponentials, the resolution for multiple unknown exponents (here the molar masses) is very limited. Therefore, in practice, the study of multicomponent systems requires that the molar masses of the components be determined in separate experiments, which leaves the relative population $c_k(r_0)$ of all stable components and reversibly formed aggregates, respectively, as the parameters to be determined. This allows the resolution of up to 3–4 different species (provided that their respective buoyant molar masses are significantly different from each other). Reliable association constants can, however, be obtained only with systems of 2–3 components.

Materials and Methods

The cobalt coordination array used for demonstration was synthesized and purified as described elsewhere.[8, 9] It was dissolved in acetone (under stirring) at a concentration of approx. 700 μg mL^{-1}. Afterwards, the solution was brought to 25 mM ammonium hexafluorophosphate by the addition of a 150 mM stock solution of the salt in acetone.

Analytical ultracentrifugation experiments were conducted in a Beckman Optima XL-A ultracentrifuge with an An-60Ti or an An-50Ti rotor, titanium double sector centerpieces with optical pathlength of 1.2 cm, and polyethylene gaskets (BASF).[6] The rotor speed was 40000 rpm and the rotor temperature 20 °C. The absorbance-versus-radius profiles $A(r)$ of the samples were recorded at a wavelength of 380 or 450 nm, depending on the maximum absorbance desired. The centrifuge data were evaluated with the computer programs SEDFIT (sedimentation velocity), ARCHIFIT (approach to equilibrium) and DISCREEQ (sedimentation equilibrium) by P. Schuck.[13c, 13d, 15b, 16] Copies of the software are available from this author. The partial specific volume of the cobalt coordination array, $\bar{\nu}$, was assumed to be 0.54 ± 0.02 mL g^{-1}.[10, 17]

Experiments and Results

The aim of the experiments performed was to establish the state of association of the cobalt coordination array dissolved in acetone.

Sedimentation velocity experiments: The experimental sedimentation velocity pattern of the dissolved cobalt coordination arrays is shown in Figure 3. It consists of 25 consecutive

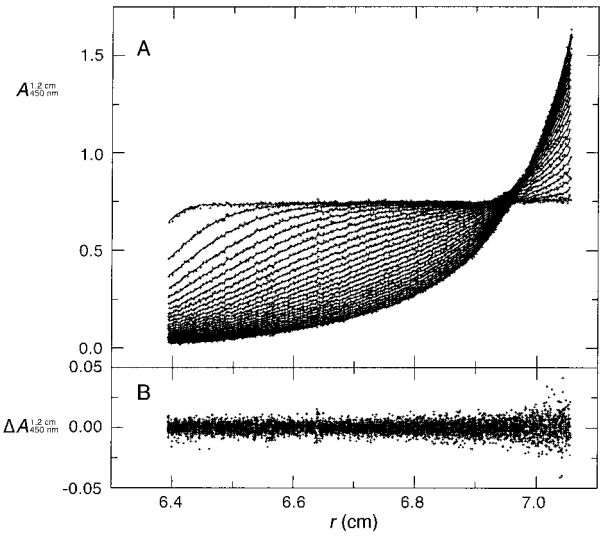

Figure 3. A sedimentation velocity experiment: (A) Experimental $A(r)$ data (collected at 450 nm) (+) and best-fit distributions according to Equation (1), assuming the presence of a single supramolecular aggregate, superimposed by the calculated time-invariant background signal (—). (B) Local differences between the experimental and the fitted data. The scans were taken at intervals of 420 seconds. Sample concentration was approx. 550 μg mL^{-1}, and sample volume was 300 μL.

scans taken at time intervals of 420 seconds. The $A(r)$ profiles, with their absence of a plateau region near the meniscus and the rapid disappearance of the plateau near the bottom of the cell, are typical of a relatively small and rapidly diffusing solute.[7, 15b] The figure also shows the curves fitted to the experimental data according to the Lamm equation [Eq. (1)], on the assumption that only one type of particles is present. It is obvious that the fit is of very good quality at all the time intervals considered. The best-fit parameter values for the apparent sedimentation and diffusion coefficients are $s = (4.6 \pm 0.2) \times 10^{-13}$ seconds and $D = (61 \pm 4) \times 10^{-7}$ cm^2 seconds^{-1}, respectively. From the Svedberg equation [Eq. (2)], with $\bar{\nu} = (0.54 \pm 0.02)$ mL g^{-1} and $\rho = 0.7935$ g mL^{-1}, a relative molecular mass of $M_r = 3200 \pm 300$ is obtained, which agrees, within the limits of error, with that of the intact monomeric compound (3366). Assuming the presence of two noninteracting molecular species did not improve the quality of the fits. This indicates that the sample molecules are essentially monomeric under the conditions used. However, the presence of a small percentages of aggregated material cannot be definitely excluded. From s and M_r, estimates on the dimensions of the sedimenting species can be obtained.[7, 10b]

In general, the application of this method requires sample volumes of 250–450 μL, at concentrations high enough to

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give 0.2–0.8 absorbance units at the wavelength selected for scanning the profiles. With the sample used in this study, taking advantage of the low solvent viscosity, the measurements could be performed within 3–4 h. Measuring times as low as 90 min are possible, but reduce the accuracy of the data.

Archibald method (approach to equilibrium): Experimental $A(r)$ patterns of an "Archibald experiment", extending from the meniscus to approximately 1 mm into the sample sector, are shown in Figure 4A. They were recorded during the first 30 min of a sedimentation equilibrium run (see below), at intervals of 240 seconds. The figure also shows fits to the data based on the Lamm equation [Eq. (1)] and the assumption that the sample is monodisperse.[15b] The fit yields M_r and s as a function of time; the results for M_r are shown in Figure 4C. The calculated M_r essentially does not vary with time and, again, agrees reasonably well with that of the monomeric cobalt coordination array. If the sample had contained significant amounts of oligomers, an average M_r would have been obtained which decreased with increasing time, because of preferential removal of the larger and thus more rapidly sedimenting species from the meniscus region.[15b]

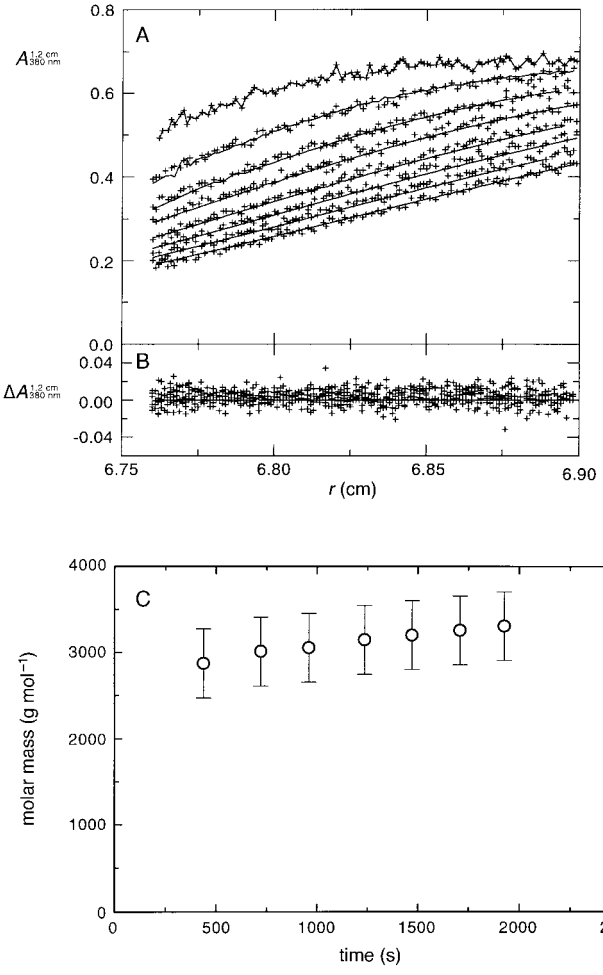

Figure 4. An approach to equilibrium experiment: (A) Experimental data sets of the depletion at the meniscus, recorded at 380 nm and at intervals of 240 seconds (+), and curves fitted to the data sets 2–8 according to Equation (1) (—). The first experimental scan was taken as initial condition for solving Equation (1). The experimental noise in this scan is rapidly suppressed in the calculated evolution, by virtue of the diffusion term.[13b] (B) Local differences between the experimental and the fitted data. (C) Time dependence of the apparent molar mass governing the depletion of the solute at the meniscus. Sample concentration: 30 μg mL^{-1}; sample volume: 150 μL.

Sedimentation equilibrium experiments: After sufficient running time of the ultracentrifuge, the absorbance-versus-radius profiles of the samples become time-invariant within the precision of the data acquisition, indicating that the samples have attained sedimentation equilibrium. For the sample shown during the approach to equilibrium in Figure 4, the corresponding sedimentation equilibrium distribution is plotted in Figure 5; it was reached after approx. 8 h. An

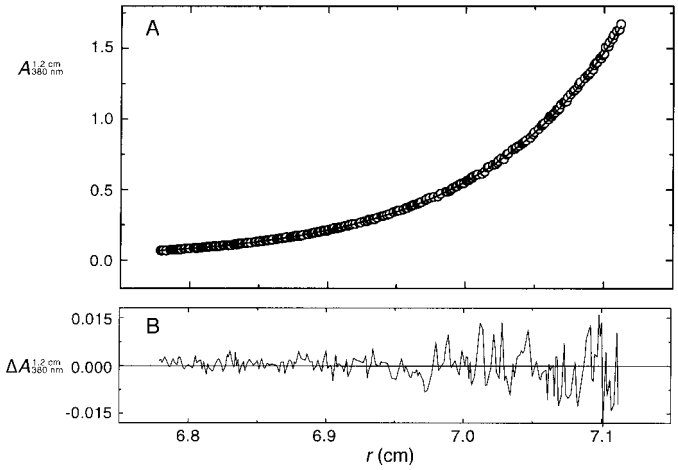

Figure 5. A sedimentation equilibrium experiment: (A) Experimental data $A(r)$ at 380 nm (○), and curve fitted to the data by assuming that the sample contains only the monomeric Co coordination array (—). (B) Local differences between the experimental and the fitted data. The experimental data are from the same centrifuge run as those of Figure 4.

analysis of these data according to Equations (3) and (4) shows that a very good fit can be obtained just by use of a single exponential characterizing the monomeric cobalt coordination array (Figure 5). On the other hand, fits of virtually the same quality were obtained if the presence of a few percent of small oligomers of the compound (dimers, trimers) was assumed. Similarly, the fits yielded small percentages of these oligomers if $\bar{\nu}$ was assumed to be 0.56 mL g^{-1}, which is within the limits of error of the experimental value of 0.54 mL g^{-1}.[10] The uncertainties described are typical for any sedimentation equilibrium analysis.

The sample volume of 150 μL used in the experiment of Figures 4 and 5 is a good compromise between accuracy of the analysis and duration of the centrifuge run. Smaller sample volumes, as low as ≈80 μL, can be used for homogeneous samples, with a concomitant shortening of the time required to reach sedimentation equilibrium by up to approx. 75 %.[7] However, for heterogeneous samples the results of the analyses will become less reliable. The loss of reliability can be monitored by appropriate statistical analyses.[16] Initial sample absorbance (at the wavelength selected) should be around 0.4.

With macromolecular compounds, sedimentation velocity experiments are usually performed at distinctly higher rotor speeds than sedimentation equilibrium runs. For the much smaller compounds of supramolecular chemistry, the advantage obtained by maintaining this practice is small, in particular when the sedimentation velocity experiments are evaluated by fitting the data by the Lamm equation.

Discussion

During the initial characterization of newly synthesized supramolecular compounds, three questions arise which concern the compounds' solution properties:[6]

1) Do the compounds really exist in solution?
2) Do they show significant dissociation?
3) Do they show self-association and, if so, what type of self-association?

In a later phase of the studies, an additional question may arise:

4) Does a special sample (intended as, e.g., a starting material for the formation of surface films[12]) meet the requirements of uniformity with respect to its oligomeric state?

We hope to have made clear that analytical ultracentrifugation is ideally suited to answer the questions raised: for questions (1)–(3) by sedimentation equilibrium analysis, for question (4) either by the same method (if sufficient time is available) or by a sedimentation velocity or an Archibald experiment. We do not know of other experimental methods which could deal with the same problems with comparable rigor and versatility.

With respect to the Co coordination array studied in this paper, it is clear from our data that, under the experimental conditions applied, it neither shows dissociation nor self-association but is essentially "monomeric" (though the presence of a few percent of oligomeric material cannot be excluded). Thus, when asking the questions cited above, the answer is simple and unequivocal. This result is, however, not clear from the beginning: We have found that, during incubation at room temperature for several days, some of the sample molecules apparently degrade, leading to smaller as well as larger entities. This is supported by the findings on similar compounds.[10] In addition, under a variety of conditions most of the material settles to the bottom of the ultracentrifuge cell (despite being apparently well solubilized according to visual inspection) (see also ref. [6]). Thus, in order to know the state of association of the sample, a control by analytical ultracentrifugation is obligatory. With the sample of ref. [6], the use of monomeric Co coordination arrays was found to grant the formation of regular surface films of the compound.[12]

It must be kept in mind that, according to Equations (2)–(4), any evaluation of analytical ultracentrifugation data requires knowledge of the partial specific volume $\bar{\nu}$ of the compound under study. The value of $\bar{\nu}$ used in the present paper was determined by digital densimetry,[18] which requires the availability of at least 5 mg of the compound and a solubility of at least 5 mg mL^{-1}. If these requirements cannot be met, determining $\bar{\nu}$ may become the most difficult task in an ultracentrifuge study (for a more detailed discussion see refs. [6, 10a, 18]).

Acknowledgments

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