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MINIREVIEW

Analytical Ultracentrifugation in the Pharmaceutical Industry

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Introduction

Hydrodynamic and thermodynamic techniques have been used extensively to characterize and determine the size, shape, molecular weight, and interaction of macromolecules. One of the most widely used techniques is that of analytical ultracentrifugation whereby the concentration gradients of sedimenting molecules are measured during the application of a centrifugal force. The design of instrumentation to accomplish this was a significant challenge and through the classic work of Thé Svedberg[1,2] resulted in a centrifuge that laid the foundations for modern protein chemistry and molecular biology. In particular, the early experiments on hemoglobin demonstrated conclusively that proteins were actually homogeneous macromolecular entities.[3] Since those classic experiments, great strides have been made in instrumentation as well as in analysis of sedimentation data. The purpose of this review is not to recount the huge amount of work in sedimentation in biochemistry but rather to review and discuss how analytical centrifugation has been used in the pharmaceutical industry. For those interested in a historical perspective as well as some of the latest advances in the field, several excellent recent books are available.[4−6]

Instrumentation

An analytical ultracentrifuge is a centrifuge that maintains good temperature and rotational speed control and is equipped with optical systems that allow for the determination of concentration gradients during the sedimentation experiment. The original analytical ultracentrifuge designed by Svedberg was driven by an oil turbine,[1] and despite the improvements made in the design, Svedberg wished to design a centrifuge that could attain higher rotor speeds in order to obtain greater separation of macromolecules in a high sedimentation field. Considerable effort went into redesigning the centrifuge as well as designing new rotors that could be accelerated up to 400000*g*.[7] By the mid-1930s oil turbine centrifuges were used routinely in Svedberg's and a few other laboratories. The proliferation of this technology occurred when the electrically driven ultracentrifuge was developed by Pickels and colleagues, leading to the availability of a commercial instrument, the Spinco model E in 1947, which proved to be the workhorse for analytical centrifugation technology. This centrifuge was equipped with UV absorption, Rayleigh interference, and Schlieren optical detection systems. Although the basic design of the commercial instrument did not change over the next 40 years, a few major improvements were made. Notably the old gear-driven mechanical speed control was replaced with a superior electronic control unit.

The Schlieren and interference optical systems used photographic film or glass plates for acquisition of data. This necessitated the upkeep of good darkroom facilities, as well as the use of expensive optical comparators for magnification and analysis of the images. The UV optical system also used photographic technology and subsequent densitometric scanning to convert the images into concentration gradients. Arguably, one of the key improvements was the replacement of the UV absorption system with a split beam photoelectric UV absorption optical scanning detector. The invention of the UV photoelectric scanning system[8] eliminated the need for development and processing of film and made the investigation of UV-absorbing macromolecules a less time-consuming endeavor. Moreover, the development of the photoelectric scanner enabled many researchers to acquire the data via A/D converters for input

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into the newly emerging desktop computer, further decreasing the time for analysis. Additional improvements of the model E were also made by various laboratories in developing real-time Rayleigh interference optical systems that used video camera technology to acquire the data for input into computers. However, the majority of the biochemistry labs using the commercial instrument did not have the benefits of these additional improvements.

The difficulty in acquisition and analysis of centrifuge data from the model E may have resulted in a decrease in the use of analytical ultracentrifugation as more rapid, but less quantitative, techniques were developed and refined. In particular, a major use of the analytical ultracentrifuge was the determination of macromolecular mass. Techniques such as SDS polyacrylamide gel electrophoresis and gel sieving chromatography enabled researchers to obtain rough estimates of molecular weight more rapidly than with the model E.

The field of protein biotechnology that evolved, allowing researchers to express proteins in bacterial and mammalian cell culture systems, focused on many qualitative issues, such as expression and activity levels. As recombinant DNA technology evolved and became more commercialized, techniques were developed to express and purify large quantities of human proteins from mammalian cell lines. As more of the expressed proteins have entered the stage for development into human pharmaceuticals, there has been a greater need to use more sophisticated and rigorous physicochemical techniques to determine the best conditions for purification and ultimately formulation of the protein drug. In addition, the development of "rational drug design" required the use of more quantitative techniques to understand in detail the interactions of macromolecules with their biological targets. As will be discussed later, analytical ultracentrifugation remains one of the best techniques for investigation of molecular interactions. Thus, there was a need for a modern commercial ultracentrifuge that was "user" friendly and relatively maintenance free.

A new analytical ultracentrifuge, the XLA/I, has been designed that includes complete computer interface, and novel precision absorption scanning and integrated Rayleigh interference optical systems. The instrument is equipped with a highly precise and stable temperature control system and has a maximum speed of 60 000 rpm. The absorption optics uses a highly stable, UV-enhanced xenon flashlamp as the light source and a toroidal diffraction grating monochromator to provide the wavelength range from 190 to 800 nm.[9] The Rayleigh optical system uses a 30 mW, 675 nm laser as the light source.[10] It is extremely useful for analyzing the sedimentation of nonabsorbing macromolecules in solution.

Theory

The purpose of this section is to acquaint the reader with the essential and general facets of analytical ultracentrifugation theory. There are several excellent references that should be consulted for a greater in-depth treatment of the theoretical background of this field.[11–16]

The optical systems of an analytical ultracentrifuge can be used independently or in conjunction with each other to provide complementary information about the hydrodynamic and thermodynamic properties of macromolecules in solution. The analytical ultracentrifuge can be operated in two basic modes: sedimentation velocity and sedimentation equilibrium, and the type of information that can be obtained is dependent on which of the two basic modes is used.

Sedimentation Velocity—Sedimentation velocity is often conducted at relatively high rotor speeds for, at most, a few hours. The larger sedimenting species under a strong centrifugal field forms a unique sedimenting boundary in solution. The velocity and shape of the moving boundary can be used to estimate the sedimentation coefficient (s), diffusion coefficient (D) of macromolecular species, and in some case the stoichiometries and equilibrium constants of interacting systems.[17]

During a sedimentation velocity experiment, macromolecules are separated on the basis of not only the size but also the shape. The sedimentation rate of macromolecular species is directly related to the molecular mass M and translational diffusion coefficient D by the Svedberg equation,

$$s = \frac{v}{\omega^2 r} = \frac{M(1 - \bar{v}\rho)}{Rf} = \frac{M(1 - \bar{v}\rho)D}{RT} \qquad (1)$$

where v is the rate of sedimentation, ω the angular velocity, r the radial distance from the center of rotation, M the molecular weight, $\bar{v}$ the partial specific volume of solute, ρ the density of solvent, f the frictional coefficient, R the gas constant, and D the diffusion coefficient. In theory, the sedimentation coefficient of each species can be determined by analyzing the rate of movement of a midpoint in the boundaries, or more rigorously the migration of the second moment of the boundary. However, such analysis only yields an average s value for a complicated system composed of multiple sedimenting species. The diffusional spreading of the boundaries can obscure the presence of other species. These problems can be addressed at least in part by several more sophisticated sedimentation velocity methods, including the Van Holde and Weischet method,[18,19] the time derivative method,[17,20] and the whole boundary curve fitting method.[21] The sedimentation coefficient and diffusion coefficient determined from these methods can be used to study more about the shape and size of the sedimenting species.[22,23]

Sedimentation Equilibrium—Sedimentation equilibrium is performed at lower rotor speeds. Under these conditions the rate of sedimentation under the centrifugal field is opposed by the rate of diffusion, and eventually when they reach the equilibrium, a time invariant exponential concentration gradient of solute is established throughout the centrifuge cell. The concentration distribution at equilibrium can be rigorously described with thermodynamic theory and has been widely used to determine the molecular weight, stoichiometry, binding affinity, and virial coefficient. For an ideal, non-interacting multiple-component system, the concentration distribution at equilibrium can be described as

$$C(r) = \delta + \sum_{i=1}^{n} c_i(r_0) e^{M_i(1-\bar{v}\rho)\omega^2(r^2 - r_0^2)/(2RT)} \qquad (2)$$

where $C(r)$ is the concentration at radial position, r, $c_i(r_0)$ the concentration of the ith species at an arbitrary radial reference position, r_0, δ the baseline offset, M_i the molecular weight of ith species, T the temperature in kelvin, ω the angular velocity, $\bar{v}$ the partial specific volume of solute, ρ the density of solvent, and R the gas constant. For a system containing only a single sedimenting ideal species, the molecular weight measurement can be made by simply fitting the data of concentration as a function of radius position. For more complicated systems, in principle eq 2 can be further modified to allow the sedimentation equilibrium data to be fit to models that incorporate self-association or heteroassociation systems as well as correc-

tions for thermodynamic nonideality by including virial coefficients as fitting parameters.[24]

Applications

Structure-Based Drug Research and Design—Often the interactions and biological activities of proteins are regulated and impacted by their quaternary structure. Analytical ultracentrifugation has proven to be one of the most important and widely used methods for the characterization of self-association and heteroassociation of macromolecules in solution. Investigation of these interactions enables researchers to design molecules that can modulate these important functional interactions.

Examples of this approach have been discussed by Holzman and Snyder.[25] In one of the examples discussed, sedimentation equilibrium analysis was used to characterize the interaction of the ligand CTP with an enzyme, CKS (CTP:CMP-3-deoxy-D-mannooctulosonate cytidylyl transferase), responsible for growth in Gram-negative bacteria. This enzyme activates an eight-carbon sugar, KDO, for incorporation into the outer bacterial cell-wall lipopolysaccharide. These studies conclusively demonstrated that CTP modulates enzyme oligomerization and that an analogue of KDO that inhibits the enzyme activity modulates the oligomerization in a similar fashion as does CTP. In particular, the analytical ultracentrifuge studies strongly suggested that the active enzyme is a homodimer. This was further supported by the determination of the X-ray crystal structure of CKS that shows that the enzyme crystallizes as an asymmetric unit comprising two monomers.[25]

Another recent example shows how both sedimentation velocity and equilibrium techniques can be used to investigate in detail the self-association of insulin analogues under formulation conditions.[26] The implications for the pharmacokinetic and dynamic responses of these insulin analogues were discussed, and it was suggested that sedimentation analysis can help in developing improved rapid-acting insulin therapies. In addition, as already pointed out by Varley et al.,[27] the analytical ultracentrifuge is one of the few techniques whereby analysis can be performed directly on the formulation and result in quantification of the protein interactions under those conditions.

Molecular biology has provided a clearer understanding of the structure and function of target molecules on cell surfaces that are often involved in regulating biological responses. This knowledge has allowed pharmaceutical scientists involved with the research and discovery of new pharmaceutical entities to undertake a rational drug design approach. By studying the detailed interactions of receptors or cell surface molecules with their target molecules, researchers can design pharmaceuticals that can modulate these interactions. An excellent example of the application of sedimentation equilibrium analysis to characterize protein ligand and receptor interactions is the recent work by Philo and his colleagues. The heteroassociation of erythropoietin (EPO) and the extracellular domain of the EPO receptor, sEPOR (soluble form of erythropoietin receptor), was analyzed by sedimentation equilibrium[28] at several rotor speeds. Global analysis of several data sets clearly demonstrated that two sEPOR can bind to a single EPO monomer with very different binding affinity. These data suggest that dimerization of receptors by erythropoietin may be important for signal transduction and activity. Thus, it may be possible to increase the potency of the molecule be engineering a second binding site with greater affinity. Other examples of the characterization of receptor protein ligand interactions are the studies on TNF-α and -β (tumor necrosis factor) with the soluble forms of their target receptors.[29,30] Those studies demonstrated that the TNF oligomer unit is capable of binding more than one receptor.

There have been reported a wide range of applications of the use of sedimentation equilibrium to characterize protein oligomerization including the dimerization of human growth hormone by zinc,[31] the trimeric structure of TNF-α,[32] self-association of biglycan,[33] dimerization of human relaxin,[34] and self-association of human interferon alpha.[35] However, for more complicated self-assembly systems, sedimentation velocity analysis has been found to be very useful due to its sensitivity to both size and shape. This approach has been extensively applied to characterize many complicated systems, including the assembly and disassembly of cpn60,[36] salt-dependence of folding of chromatin,[37] amyloid protein aggregation,[25] and assembly of viruses.[38–40] In addition, sedimentation velocity can be used to characterize complex size distributions because of the separation power of the technique coupled with recent advances in analysis of heterogeneity by determination of the differential sedimentation coefficient distribution function.[20] Recently, this method was used to characterize a number of immune complex distributions formed by several monoclonal antibodies.[41] Not only was the size of the complexes determined, but also the potential structure of the complex was investigated by hydrodynamic modeling. This detailed structural information about protein interactions has provided an important basis for the further effort to develop and improve this drug therapy.

Stability and Homogeneity—For biopharmaceutical products, it is important to know whether they are stable and homogeneous under certain process and storage conditions. The degradation products of biopharmaceuticals often include aggregates and fragments. One possible way to identify these degradation products is to determine their average molecular weights by using sedimentation equilibrium analysis.[27,42] For highly purified and homogeneous molecules, the average molecular weights for the pure and non-self-associated macromolecules under ideal conditions should be very similar to the monomer molecular weights. On the other hand, for macromolecules that have undergone aggregation or fragmentation, the apparent average molecular weights under ideal conditions will diverge from the monomer molecular weights.

The purity of biopharmaceuticals can also be determined by sedimentation velocity analysis. Several sedimentation velocity methods have been developed to determine the distribution of protein species. Among these methods, the van Holde and Weischet analysis[18,19] has been well documented to provide a rigorous test of sample homogeneity.[43] This method has been extensively utilized by several groups to study the homogeneity of macromolecules. These include the characterization of oligonucleosomes,[44] transcription factors, sigma 70,[45] and NusA and transcription termination factor rho.[46] A different extrapolation method based on the time derivative of concentration distribution has also been found very useful to identify impurities from macromolecules.[17,20] This method has been used in combination with other techniques, such as SDS polyacrylamide gel electrophoresis and gel sieving chromatography, to help us to determine the purity and homogeneity of macromolecules in solution.[47]

Activity of Biopharmaceuticals—The binding affinity of protein interaction is an important thermodynamic parameter that will affect the activity of many biological molecules. The sedimentation equilibrium experiment is one of the most rigorous thermodynamic methods to measure binding affinity.[43] This technique generates large quantities of high-quality data with broad concentration ranges from a single experiment. Therefore, it can greatly increase the confidence of model determination with non-

linear least-squares fitting. The sedimentation equilibrium method has been successfully and extensively used by a number of investigators to determine the association constants of an interacting system.[28,48,49]

For more complicated and high-affinity bivalent antibody and antigen interactions, the sedimentation equilibrium methods are often limited, due to the sensitivity of the current analytical ultracentrifuge detectors and complication of complex distribution. Conventional methods, such as ELISA and biosensor, have often been used to determine such interactions. Analysis by ELISA is highly dependent on having the appropriate antibody for detection that does not interfere with the binding reaction that is being studied. Apparent affinity constants are often determined by using an ELISA to measure the amount of free ligand in solution. This requires extensive control experiments to ensure that the dilutions and process used in the ELISA do not perturb the interaction equilibrium.[50] In addition the immobilization of complexes onto the plate surfaces may also perturb the association equilibrium. Biosensor measurements also require immobilization of one of the reactants, and the manner in which the macromolecule is immobilized may alter the thermodynamics of interaction because of complications from molecule orientation on the surface and interference with the polymer matrix used for immobilization. The latter may alter the kinetic measurements because of mass transport through the matrix support. These complications have been discussed along with criteria needed to assess the reliability of biosensor data.[51] An alternative approach based on the sedimentation velocity method has recently been suggested by us to study the interaction of human IgE with an anti-IgE antibody.[52] In this method, a soluble form of the IgE high-affinity receptor (sFcϵRI) is used as a binding competitor to probe the IgE and anti-IgE monoclonal antibody (rhuMAb E25) interaction. Since the sFcϵRI is much smaller in size than rhuMAbE25 ($\sim$42 vs 150 kDa) and binds at or near the same site on IgE as rhuMAb E25, it is possible to distinguish the complexes formed by IgE$-$sFcϵRI and IgE$-$rhuMAb E25 by using sedimentation velocity experiments. By studying the shift of the complex distribution, we are able to distinguish the binding activity of rhuMAb E25 from that of other similar monoclonal antibodies.

Molecular Crowding Effect on Protein Drug Interaction and Formulation—Physiological media, such as human serum or cytoplasm, are very crowded environments that contain many large macromolecules. The volume exclusion arising from these high concentrations of macromolecules can have a large qualitative effect upon the equilibria and kinetics of protein drug interaction.[53] Sedimentation equilibrium is an ideal method to study protein drug interaction in a crowded environment. Although the current design of analytical ultracentrifuge optical detection systems lacks the sensitivity and specificity to distinguish a specific protein from the crowded background molecules, an alternative method that combines the sensitivity and specificity of radioisotope labeling and ELISA with preparative centrifugation techniques has been suggested.[54,55] This technology can increase the concentration range of traditional sedimentation equilibrium experiments. It has been used successfully to characterize a number of macromolecules, including ovalbumin, albumin, and γ globulin.[55,56]

The excluded volume effect resulting from small inert crowding molecules can also play an important role in protein drug formulation. Many protein drugs such as monoclonal antibodies requiring high dosing and sc administration will need to be formulated at a very high protein concentration. In addition, sugars have often been used to increase protein stability in solution[57] due to their exclusion from the surface of protein molecules. This has been attributed to a lowering of the protein free energy by minimization of exposed surface area.[58] Stabilization of the more compact folded form of the protein will obviously result in a surface area reduction. However, the surface area can also be reduced by intermolecular association, and thus, the excluded volume effect of sugars in liquid formulations can increase the propensity of proteins to self-associate. Recently it was shown, by sedimentation equilibrium analysis together with scale particle theory, that the high concentration of sugar and protein can dramatically increase the weak self-association of protein that may not be detectable at low and ideal conditions.[59] Such weak association can potentially decrease protein stability and affect other physical chemical properties.

Summary

The analytical ultracentrifuge has been cited as "still the tool of choice" to characterize associating systems.[60] The original development of the analytical ultracentrifuge helped provide the key data for the cornerstone of modern molecular biology and protein chemistry. At the height of its popularity few major labs in the country were without an analytical model E. Unfortunately, despite some of the improvements in the model E, the technology rapidly became a specialized area for researchers interested in improving the instrumentation for rapid data access and analysis. As cheaper and easier techniques were developed to measure rough molecular weights and obtain qualitative information on macromolecular association, the use of the centrifuge rapidly declined. Recently, with the advent of newer instrumentation, proliferation of user groups, workshops, and establishment of the National Analytical Ultracentrifugation Center at Storrs Connecticut, the technology has reemerged and is being used to a greater extent in academia as well as the pharmaceutical industry. This minireview, hopefully, has captured many of the recent advances and problems being investigated in the pharmaceutical and biotechnology arena.

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