Beckman Coulter Webinar Series
Analytical ultracentrifugation as a complementary technique for structural analysis of proteins and macromolecular complexes
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Presented by: Andrew Herr, PhD
Divisions of Immunobiology & Infectious Diseases
Cincinnati Children's Hospital Medical Center
August 13th, 2015

Q&A Session

Univ of Guelph, Canada
Will a recording of the webinar be made available? It would be an excellent resource for the undergraduate course on Analytical BIochemistry that I am teaching next semester!
Yes, the webinar will be available on-demand for several months following this live session. It will also be available to view on the LabRoots website.

EPFL Switzerland
Dear Dr. Herr, Thanks a lot for the webinar, We are a laboratory working on aggregation of a protein involved neurodegeneration, and we would like to catch the composition of the sample like Htt protein during the early time point of the aggregation to determine the aggregation determinant (tetramer, octamer ,,,) do you think that AUC would be useful for our desired assay?
Sedimentation velocity AUC can be very helpful to analyze aggregation in vitro, but you have to carefully consider several details. I'm assuming you have a source of purified recombinant Htt, and that nucleation of aggregation happens without a separate initiator protein. If this is not the situation, you'll need to label Htt with a chromophore for selective detection. The second important parameter is the relative time frame under investigation—if nucleation is a slow process, or has a significant time lag, then an AUC study is quite feasible. If the early time points you're studying have an elapsed time of less than 1 hour, then you may run into problems with the time required to achieve full vacuum and thermal equilibration at the start of the AUC run. Based on other published work on Htt, I think neither of the above issues would be a problem. Sedimentation velocity AUC would help you to resolve oligomeric species at different time frames (as long as they're not exchanging under rapid equilibrium, which would create reaction boundaries that appear as "moving" peaks in the c(s) distribution). To really figure out the critical nucleating species, you probably will need to do some additional higher-level analysis—see for example, Ron Wetzel's paper in *Nature Structural & Molecular Biology* 18:328, 2011.

NCI
How can one know/determine the best speed for our protein to be analysed by AUC?
There are different answers depending on the experimental approach. For sedimentation velocity, modern software such as Sedfit removes a lot of the challenges we used to face, since the deconvolution procedure explicitly accounts for diffusion. In most cases, you can simply spin at close to the maximum rotor speed (e.g., 48000 for typical charcoal-epon centerpieces). If you have really large species, you

may see that the samples completely sediments in just a few scans—if the protein completely sediments in less than 50 scans, you probably should redo the experiment at a somewhat lower rotor speed in order to increase the number of scans for analysis.

For sedimentation equilibrium, it is a bit more complicated. You ideally want at least three equilibrium curves per sample (i.e., data from at least 3 speeds) with a good range of curvature—one rather shallow, one intermediate, and one showing a good amount of curvature. In addition, you'll load 3 concentrations per sample (typically covering a 10-fold range in the loading concentration) and then do a global analysis of all the curves. The rule of thumb for choosing rotor speeds is that the value of □, the reduced buoyant MW, should range from 2 up to 15, although a range of 2 to 10 is probably easier to deal with. Keep in mind as well that if you have an unknown system or a protein undergoing assembly, you often will need to either do the experiment more than once, or add one or two additional speeds to account for larger species (e.g., adding a lower speed) or unknown MW of your sample. By the way, I highly recommend the program Sednterp as a wonderful utility for figuring out rotor speeds (and a lot of other relevant parameters) as you start almost any AUC experiment (online at http://sednterp.unh.edu; also available as a downloadable executable).

John Anders, Nanotherapeutics
Upon separation in SV experiments are the areas of the resultant peaks of deconvoluted sedimentation boundaries quantitative in nature? Or in other words can we assess % relative abundance to each peak in the sample.
Yes—the peak areas are proportional to relative abundance. This is a big advantage of SV experiments, since with a single experiment you can use the c(s) analysis or similar approaches to generate a sedimentation coefficient distribution and see how many species are in your sample, along with their relative abundance. More specifically, it's very straightforward to determine relative % contribution of each peak to the total signal. If you want to determine molar concentrations, especially with a mixture of different proteins, it can be a bit more complicated, since you may be dealing with different v-bar values (especially for a mixture of glycosylated and non-glycosylated proteins) or widely varying shapes, both of which will impact determination of MW from the sedimentation data.

GSK
Can one quantitate amount of components that appear? How would hetero-glycosylated protein appear?

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Yes, as described above, you can quantitate the relative abundance of the species you observe. Glycosylated proteins will still show up as individual peaks (generally the micro-heterogeneity due to glycosylation will not be apparent) and can be analyzed with a little more effort. The main issue with glycoproteins is that the v-bar (partial specific volume) for protein is typically around 0.73, whereas it's closer to 0.55 for glycans. Thus, the actual v-bar of a glycoprotein is a weight-average of the contributions from protein and glycan. Since there is often some uncertainty in the % mass contributed by glycans, the error on MW determination is a bit higher for glycoproteins, but you can get reasonable values if you know the number of glycan chains and the type of glycosylation (e.g., complex versus high-mannose).

Pakistan
What is the accuracy of our structural analysis through this technique of analyzing protein and how it is verified?
With proper analysis and good experimental technique, the results from AUC will be accurate and precise. You could use other techniques to verify the results if you wanted to—if the conditions are comparable—but in general, AUC is often considered the gold standard by which other techniques are measured. Keep in mind that the important issue here is that AUC allows analysis of proteins in solution with physiologically relevant buffers. Mass spectrometry, for example, can allow higher precision in terms of mass determination, but not under the same type of well-controlled, physiologically relevant conditions in solution.

Altheacmo
What are the units on the x and y axes in your first sedimentation equilibrium slide?
The y-axis is always concentration, whether detected by absorbance or interference optics. The x-axis is $r^2/2$, where *r* is the radial position (i.e., distance from the center of the rotor) in cm^2. These data have been transformed from the original units of radial position (cm) into the $r^2/2$ units needed for determination of □, the reduced buoyant molecular weight.

BMS
What concentrations of excipients such as sucrose and polysorbates cause significant interference with AUC analyses?
If not accounted for, these excipients could cause aggregated protein species to be underestimated. For example, Arthur and Gabrielson have shown that c(s) analysis of samples with 5-10% sorbitol can lead to underreporting of aggregate species by 17-77%. However, the good news is that one can model the

excipient cosedimentation explicitly in Sedfit to get accurate results. The numbers I mention here come from a chapter by Arthur, Kendrick and Gabrielson soon to be published in the Analytical Ultracentrifugation issue of *Methods in Enzymology* (the accepted proofs are available online via ScienceDirect).

NCI
Can we obtain the sample of interest based on the separation in AUC?
AUC is an analytical approach, not preparative. Thus, you can recover sample after the experiment, but it is not intended for purification of distinct species. However, it is perfectly complementary to other separation techniques—you could separate out species by gel filtration or field-flow fractionation for example, and determine which species are present in each fraction at high resolution using AUC.

Nanotherapeutics
Can one determine the partial specific volume of a protein for use in AUC by Dynamic Light scatter with determination of the hydrodynamic volume and mean radius? If not how do you determine this factor?
Typically, we calculate the v-bar (partial specific volume) based on amino acid composition using the helpful program Sednterp (now online at http://sednterp.unh.edu or available as a downloadable executable). For standard non-glycosylated proteins, the accuracy is very good. For glycosylated proteins or protein:nucleic acid complexes, you have to calculate the % mass contributions from the protein vs glycan or nucleic acid components, as they have quite different v-bar values. In these latter situations, it might be worth determining the v-bar experimentally; this can be done using a density meter to measure density as a function of concentration and fitting the results to determine v-bar. There are also published methods to determine v-bar by conducting parallel sedimentation experiments in different density buffers (e.g., H_2O and D_2O).

NCI
Can you please explain about the relativeness of the molecular weight and dispersity of a protein? Do you think single molecular weight protein is always monodisperse?
It depends on the particular sample you're working with. You could have a purified protein that shows a single band on an SDS-PAGE gel, but in solution it forms dimers, tetramers and octamers, so you need to assess polydispersity in solution, and SV-AUC is the best way to initially assess this. You could have a single-MW protein that is not monodisperse, for example you might have have two populations of

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monomeric protein, one that is properly folded and one that is misfolded. If the folded and misfolded monomers have different-enough shapes, that might show up as two species by SV-AUC. This can also show up in assembling systems as a sub-population of "incompetent monomer" during sedimentation equilibrium analysis.

Altheacmo
Why not use the faster technique of FFF-MALS for antibody aggregates?
FFF-MALS is a useful technique, but the resolution is much lower than AUC. If you have multiple species co-eluting in a single peak, you have to keep in mind that the MALS output is a weight-average MW, so you could get misleading results. It would probably be best to verify the FFF-MALS results by AUC before using it in your primary workflow.

U Brasilia
The nM concentration is possible even in absorbance only AUC?
You can get down to nanomolar concentrations using absorbance optics, but it depends a lot on the buffer used. A lot of buffer components start to absorb dramatically at wavelengths below 250 nm, particularly reducing agents. If your protein is happy in a simple phosphate/NaCl buffer system, you can then go down to much lower wavelengths. We've gone down as low as 203 nm, which lets you follow the very strong absorbance from the peptide backbone.

GSK
Is absorbance enough, or interference also needed?
I think it really just depends on your needs. We have done a huge amount of work with absorbance alone, and there are some advantages, especially if you are analyzing samples from outside users that may or may not have perfect buffer blanks. The interference optical system gives you higher-precision raw data, and interference is necessary if you are analyzing samples that don't absorb. Interference optics also let you analyze higher concentrations that might be beyond the linear range of the absorbance optics (but even with absorbance optics, you can analyze high-concentration samples by measuring at a different wavelength or using thinner centerpieces with a shorter path length).

IIS
Can we have a class sometime for anaysis using Sedfit software?
Peter Schuck (NIH) and his colleagues give detailed (and really good) workshops on a regular basis that teach the use of both Sedfit and Sedphat software packages.

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Emergent Biosolutions
Whether AUC can be used to analyze very complex mixtures such as Amyloid beta oligomer preparations which contain mixtures range from monomer (4.2KDa) to dimer, trimer and up to 100KDa aggregates?
Absolutely—SV-AUC would be a fantastic way to analyze a system like this. The main issues will be whether you have co-sedimenting species of different shapes (but this can be resolved using the 2D size-and-shape analysis) or whether you have species in rapid equilibrium. If the latter were occurring, you'd expect to see reaction boundaries with apparent MW values intermediate between the actual species. You would want to do several experiments at different protein concentrations to help resolve whether the c(s) peaks represent well-resolved stable oligomers (e.g., in slow exchange) or reaction boundaries that will move as the species distribution shifts at higher concentrations.

Xenetic
For a protein-DNA complex, how accurate is the v bar estimation?
It can be very accurate, but it will depend on how much information you have going in. If the protein:DNA complex has unknown stoichiometry, then you'll need to calculate the v-bar separately for each potential complex stoichiometry since the v-bar of the complex is the weight-average value based on the relative % mass contribution from protein and DNA. We use Sednterp for the protein v-bar and the NucProt server (http://www.molmovdb.org/cgi-bin/psv.cgi) for the nucleic acid v-bar and then calculate the weight-average v-bar for different stoichiometries. For similar examples from my lab (with protein-RNA complexes) see Li et al., JBC 284:13881 (2009) or Lu et al., Structure 18:1032 (2010).

UCL
How accurate can you determine the molecular weight of a small protein?
You can get very accurate MW even for small proteins with modern analysis approaches. Originally this was very challenging to determine from SV-AUC experiments, but since Sedfit explitly accounts for diffusion in the c(s) analysis approach, you can get very good results even for small proteins.