NEW SOFTWARE METHODS ENHANCE SEDIMENTATION VELOCITY ANALYSIS OF PROTEIN AGGREGATION AND CONFORMATION

John S. Philo, Alliance Protein Laboratories, Thousand Oaks, CA www.ap-lab.com

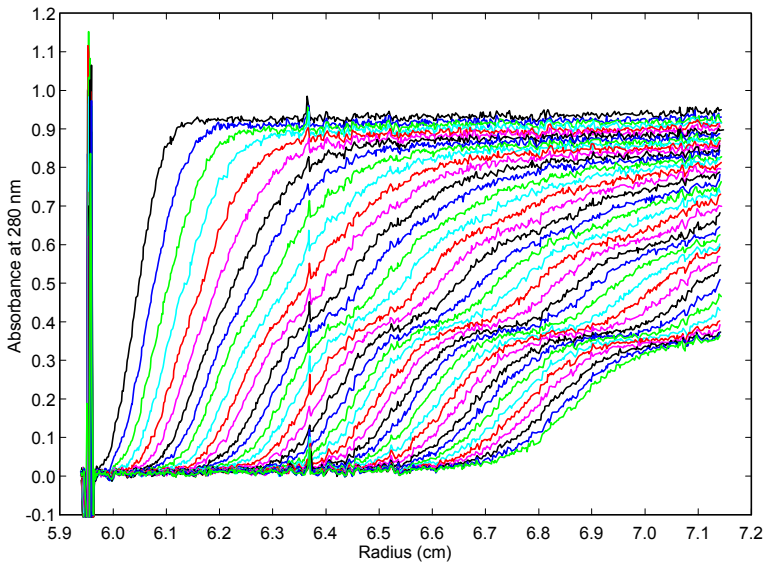
Some data analysis methods developed over the last year or two have significantly improved the resolution and sensitivity of sedimentation velocity analysis, making it an even more useful tool in comparability protocols, formulation, process development, and QA/QC. A method developed by Peter Schuck at N.I.H. greatly enhances the effective resolution of multiple components and also gives high signal/noise. With this approach the ability to resolve small oligomers appears to exceed that of the highest-resolution SEC columns, with the additional advantages of being able to analyze directly in formulation buffers and without potential loss of aggregates to a column matrix. The high signal/noise also enables detection of very minor components (well below 1%). Several examples of applications to comparability testing and formulation will be presented.

The principal drawback of Peter Schuck's method is that it discards information about the diffusion coefficient (and hence the mass) of each component. However, another new method developed in our laboratory extends the sensitivity of the more standard *dc/dt* analysis, allowing both conformation and mass information to be obtained at extremely low concentrations or for minor components. An example of analysis of antibody samples at concentrations of 1-2 microgram/ml (<1 microgram total protein) will be shown.

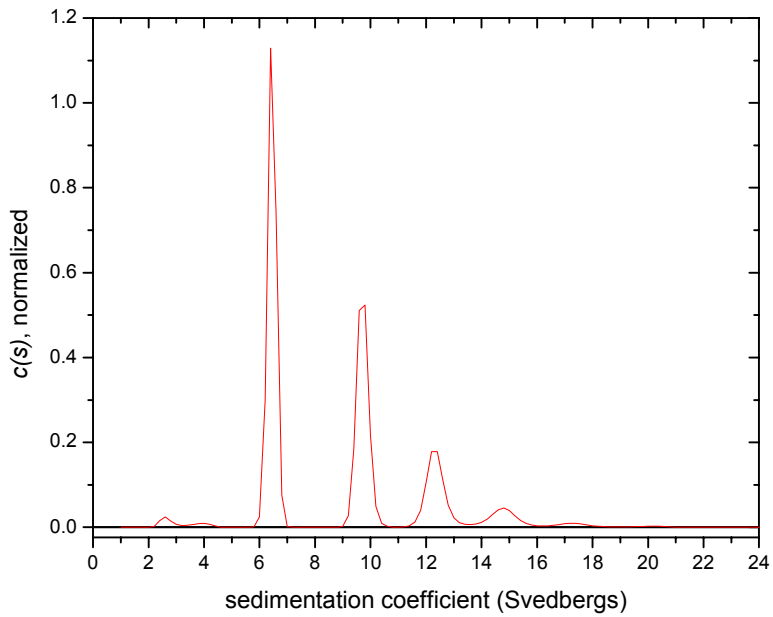
A. The high-resolution *c(s)* method

Peter Schuck at N.I.H. has developed a new software method[1] that in effect removes the influence of diffusion and thus greatly increases the resolution of different species. The resultant "*c(s)*" distributions also have very high signal/noise so very minor components can be detected.

raw data



sedimentation coefficient distribution

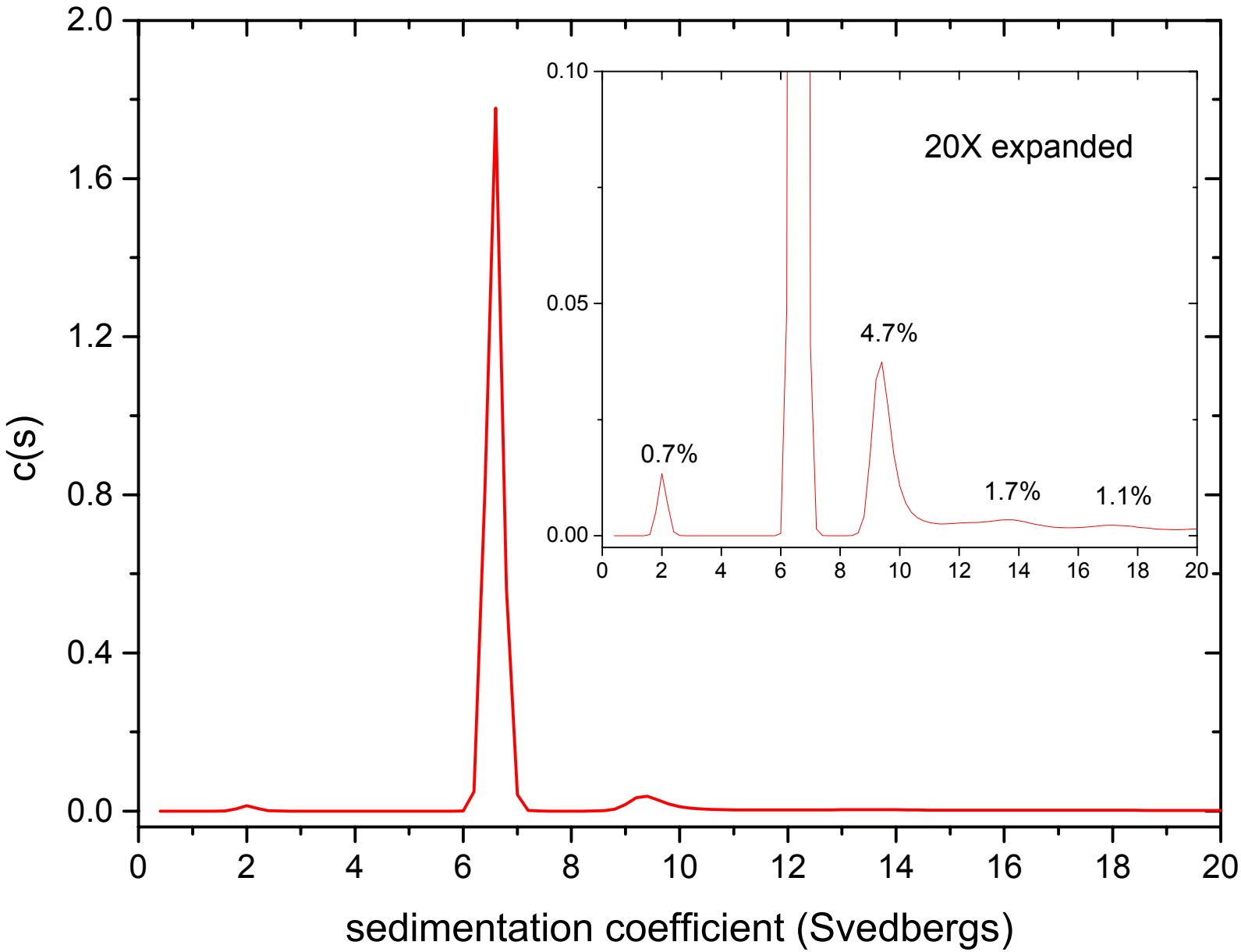


[1]Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. ***Biophys. J.*** 78, **1606-1619.**

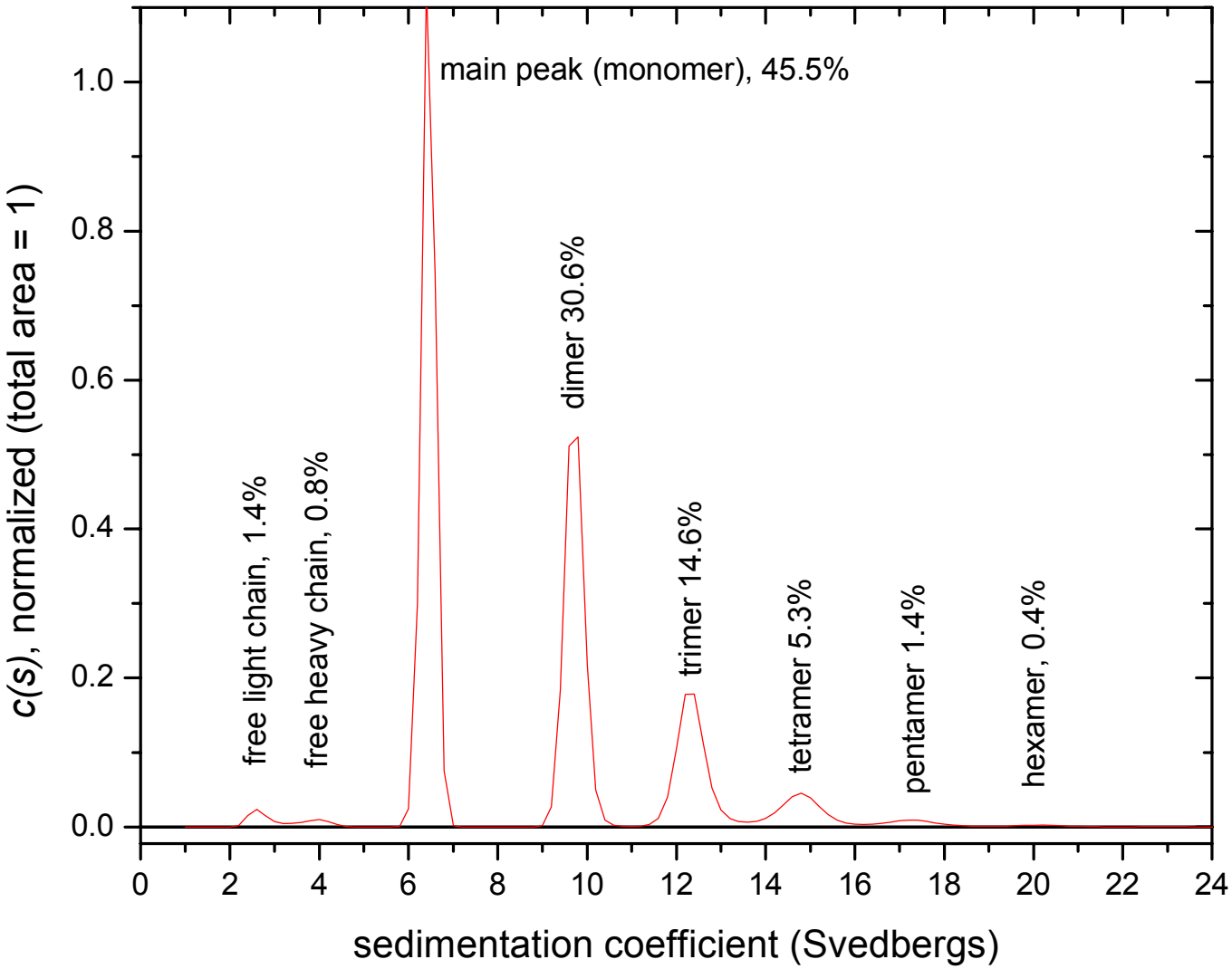
Examples of the *c(s)* method

1. When applied to a monoclonal antibody sample, this method resolves at least 4 minor components in addition to the main peak at 6.5 S: a fully baseline-resolved dimer peak at ~9.4 S, small peaks at ~13.7 S and 17.5 S (probably trimer and tetramer), and a low mass contaminant at 2 S (probably free light chain).
2. A heavily stressed sample of another antibody shows a whole series of small oligomers, as well as free heavy and light chains, for a total of 8 species.
3. Another useful feature is the ability to cover an enormous range of sizes in a single analysis. For example, in formulation studies of a ~20 kDa recombinant protein, marked increases in aggregate formation were seen upon addition of salt, particularly very large aggregates (16-40 S, ~500-2000 kDa) that were not resolved by SEC.

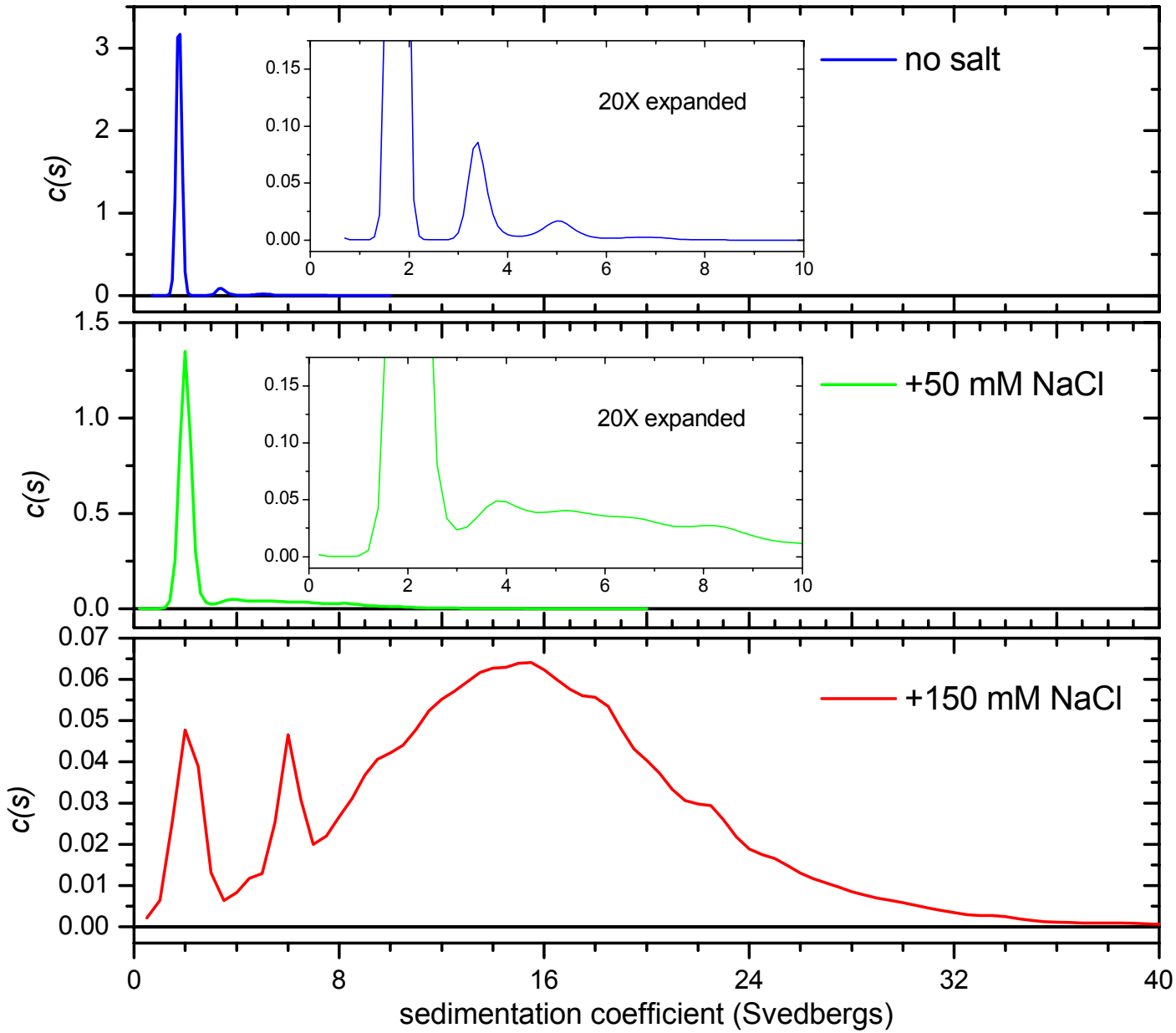
Minor components in an antibody sample



A highly stressed antibody sample shows a well-resolved series of oligomers up to hexamer, as well as free chains. Clearly the resolution and range are significantly better than any SEC method.

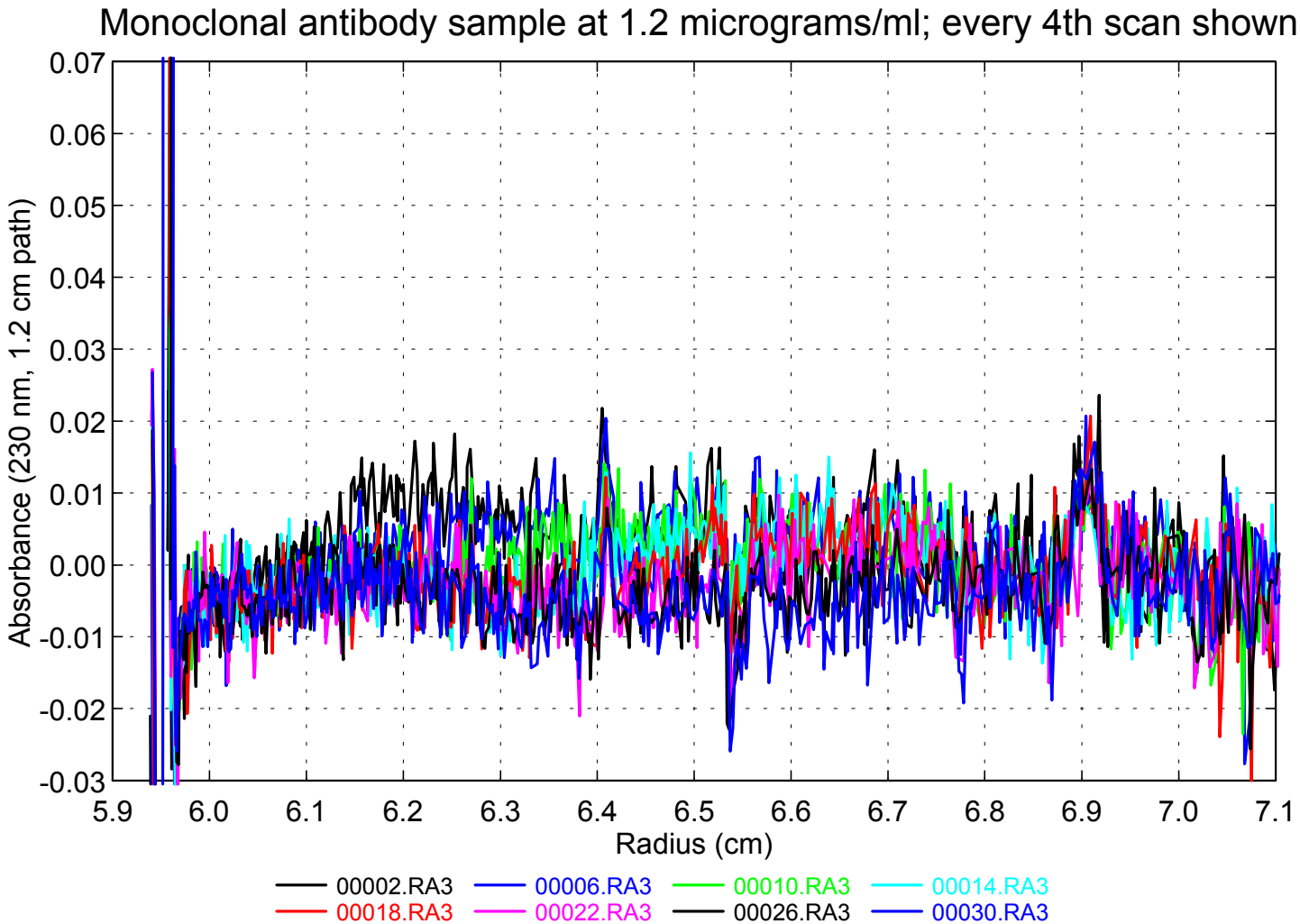


Formulation studies: effects of NaCl on aggregate content



B. Extending the sensitivity, range and accuracy of the *dc/dt* method

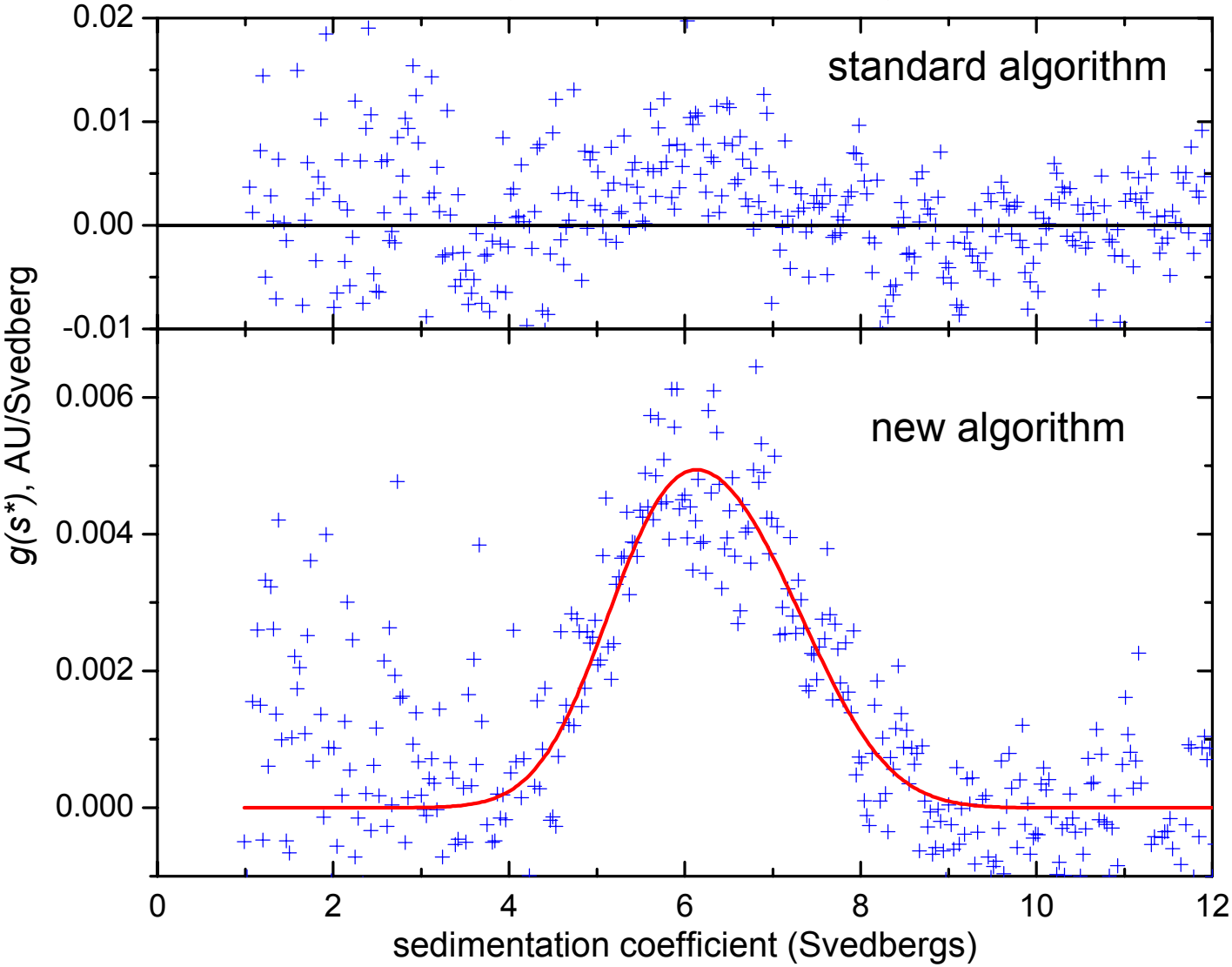
Can you see a boundary moving in these velocity data for a sample at 1.2 μg/ml (0.5 μg total protein)? A new algorithm we have developed can recover both the sedimentation coefficient and the solution mass from such data!



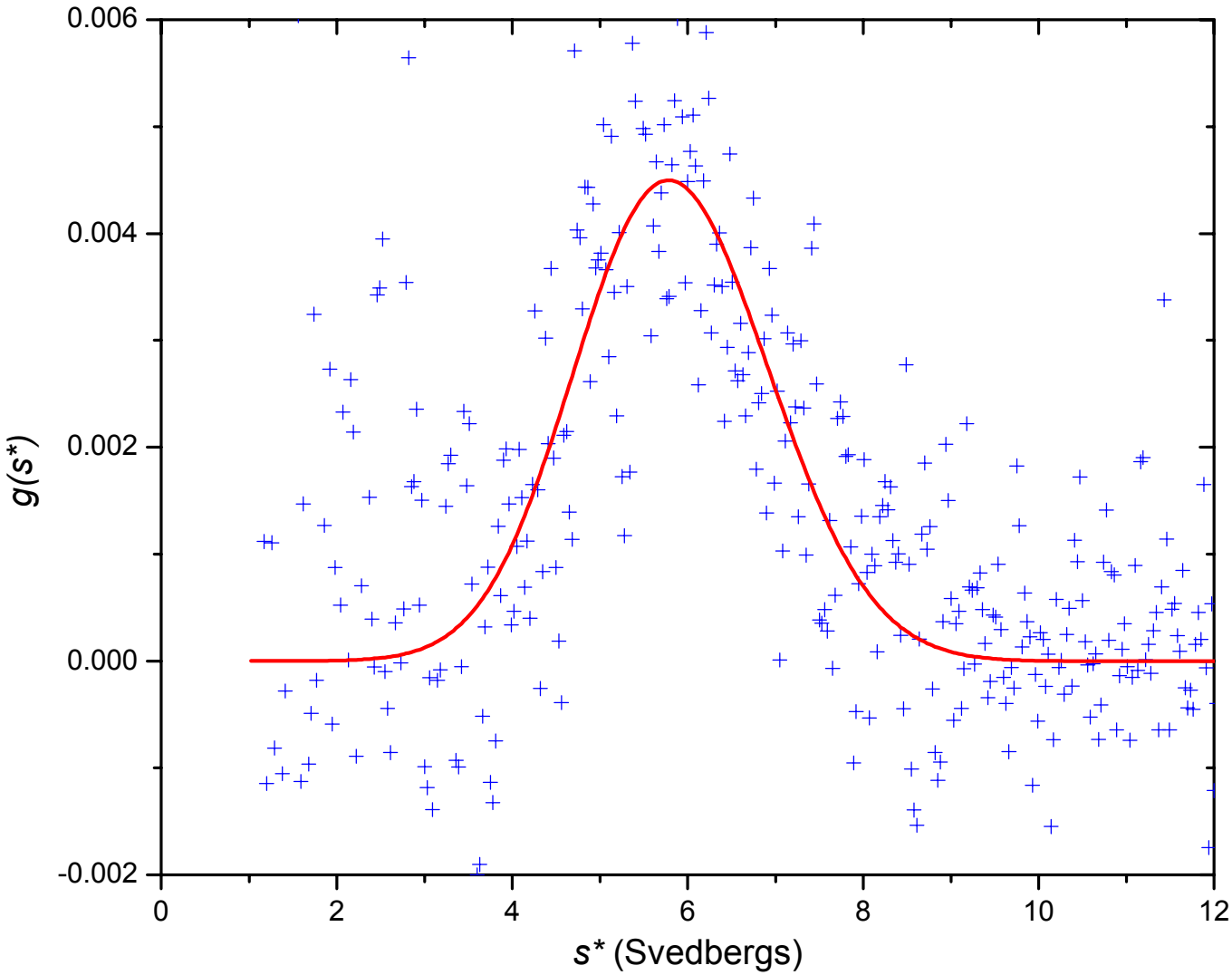
What are we trying to do?

- The *dc/dt* method developed by Walter Stafford has the useful property that the width of each peak in the distribution can be related to the mass of that species.
  - the peak width measures the diffusion coefficient, and the ratio of sedimentation coefficient to diffusion coefficient gives the mass
  - this mass/diffusion information is sacrificed in Peter Shuck's *c(s)* method in order to get higher resolution of different species
- Unfortunately with the *dc/dt* method one can use data from only a small portion of the entire run. This both limits the signal/noise and makes it difficult to measure samples containing a large range of sizes.
- The standard method can also give systematic errors of ~10% for masses.
- We have developed some new mathematical approaches that remove these limitations, as demonstrated below.

Analysis of the antibody data at 1.2 μg/ml (0.5 μg total). With the standard approach the peak at ~6 S is barely seen. With the new algorithm we get the sedimentation coefficient with a precision of better than 1% and a mass of 175 ± 35 kDa (*i.e.* it is a monomer).



In these data for a degraded antibody sample at 1.2 μg/ml, we can now see a shift to lower sedimentation coefficient, and we can be >99% confident that the width of the peak is too broad to be consistent with a single monomeric species. (Data at higher concentrations reveal additional species at ~4, 5, and 9 S.)



Significance of the improved algorithms

1. The improved sensitivity permits examining the conformation and solution mass of <1 μg of protein, which may be useful for examining side fractions collected from analytical columns or other materials available in limited amounts.
2. The higher accuracy for mass values is very helpful in assessing heterogeneity. If the apparent mass (derived from the peak widths) is too low, this implies unresolved heterogeneity in the peak.
3. With the new methods it is possible to analyze a broader range of sedimentation coefficients in one experiment.

Conclusions

- The resolution and size range of sedimentation velocity analysis is now significantly better than size-exclusion chromatography, adding to its other advantages:
  - wide range of solvent conditions, usually compatible with formulation buffers
  - no loss of components due to column interactions
  - absolute method (does not require standards)
- New methods also extend the sensitivity down below 1 microgram, and allow for more accurate determination of solution mass
- Low throughput is still an issue, but sedimentation velocity is nonetheless a powerful tool for comparability studies, validating SEC or other sizing methods, and/or providing supplementary data from an orthogonal approach