Analysis of Protein Aggregation, Stability, and Lot Comparability by Sedimentation Velocity

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ABSTRACT: Sedimentation velocity is a powerful tool for detecting and quantifying aggregates in protein pharmaceuticals and proving comparability of conformation and aggregate content for different manufacturing processes or lots. Recent software techniques, including new approaches developed in our lab, extend the power of the method and make the data easier to interpret.

Several examples based on studies of antibodies for our clients will be presented. Comparing several lots of monoclonals from two different manufacturing processes demonstrated that the conformation of the majority species is indistinguishable, but different lots contained from 2.0% to 6.0% of dimers and higher aggregates. This aggregate content is significantly higher than detected by SEC, due to aggregate loss on the column matrix. One significant advantage of sedimentation velocity is the ability to run samples under a wide range of solvent conditions. By testing another antibody directly in two different formulation solvents we showed that a low ionic strength formulation gives markedly less dimer, a difference that could not be detected under the high ionic strength conditions necessary for SEC.

A common problem in protein products is the irreversible accumulation of aggregates with time or thermal stress. When these aggregates precipitate or form very large particles (“snow”) they are easy to detect, but smaller non-covalent soluble aggregates (which are often precursors to “snow”) can be difficult to detect and quantitate. Using sedimentation velocity on heat stressed samples we studied variations in aggregate amount and size distribution with various additives. These studies also detected a significant change in antibody conformation under conditions that minimize aggregate formation, but it is unclear whether this conformation change is directly a cause of improved reversibility of unfolding.

Objectives

1. Use sedimentation velocity to characterize the size and mass fraction of aggregates in bulk manufacturing or in-process samples to:
   a. detect lot-to-lot variations
   b. help identify steps where aggregates are formed
   c. demonstrate equivalence between different processes
2. Characterize aggregates in protein samples under formulation conditions and after thermal stress to help optimize the formulation
3. Measure aggregation induced by physical stresses during manufacturing or storage such as shear- or surface-induced denaturation
4. Develop improved data analysis methods to support these goals

Methods

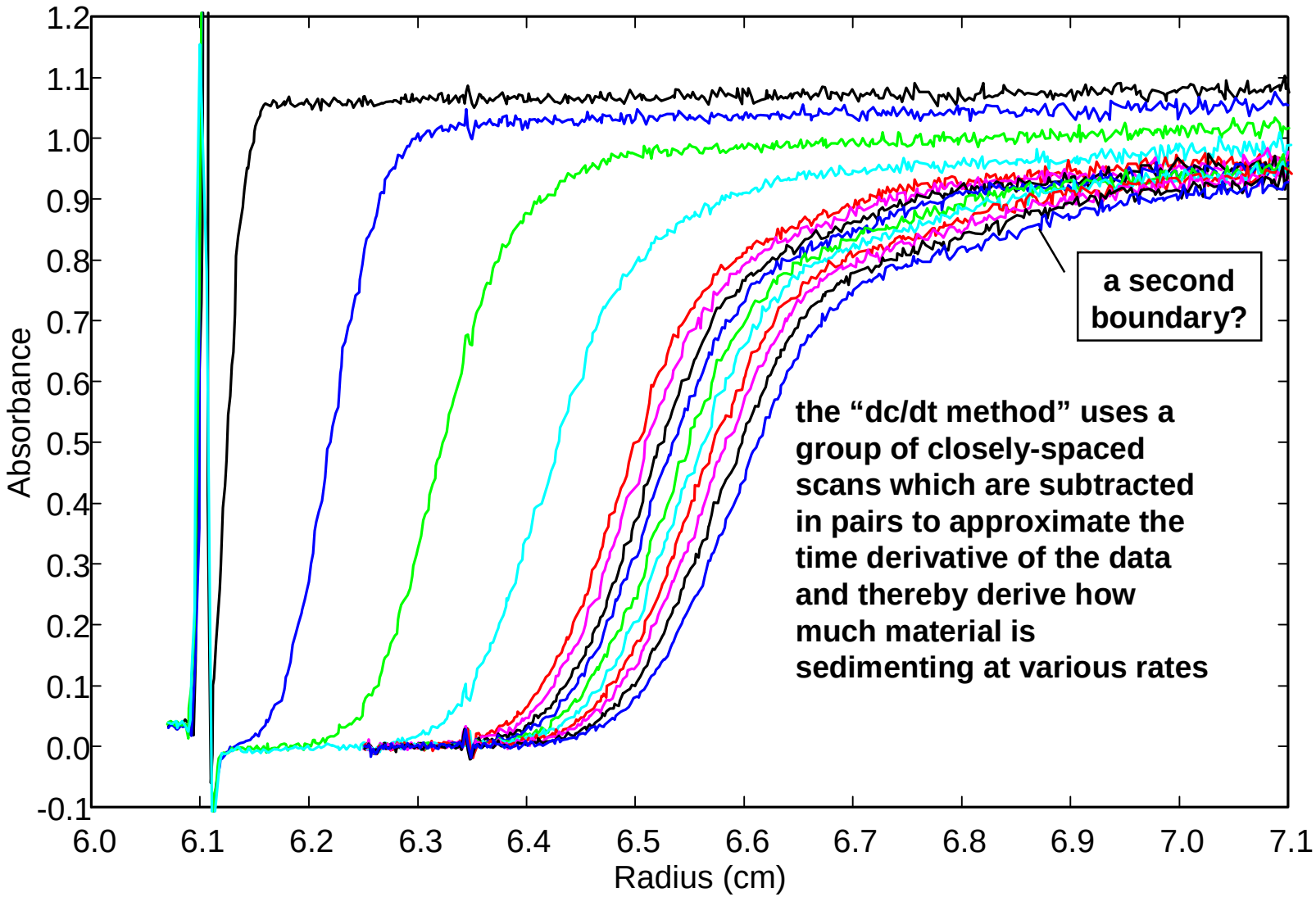
Sedimentation velocity studies were done in a Beckman Optima XL-A analytical ultracentrifuge using 2-channel charcoal epon centerpieces. For most of the studies here the rotor speed was 45,000 rpm, protein concentration was ~0.5 mg/ml, and absorbance scans were done at 280 nm.

Data analysis was done using the program DCDT+ written by John Philo.[1]

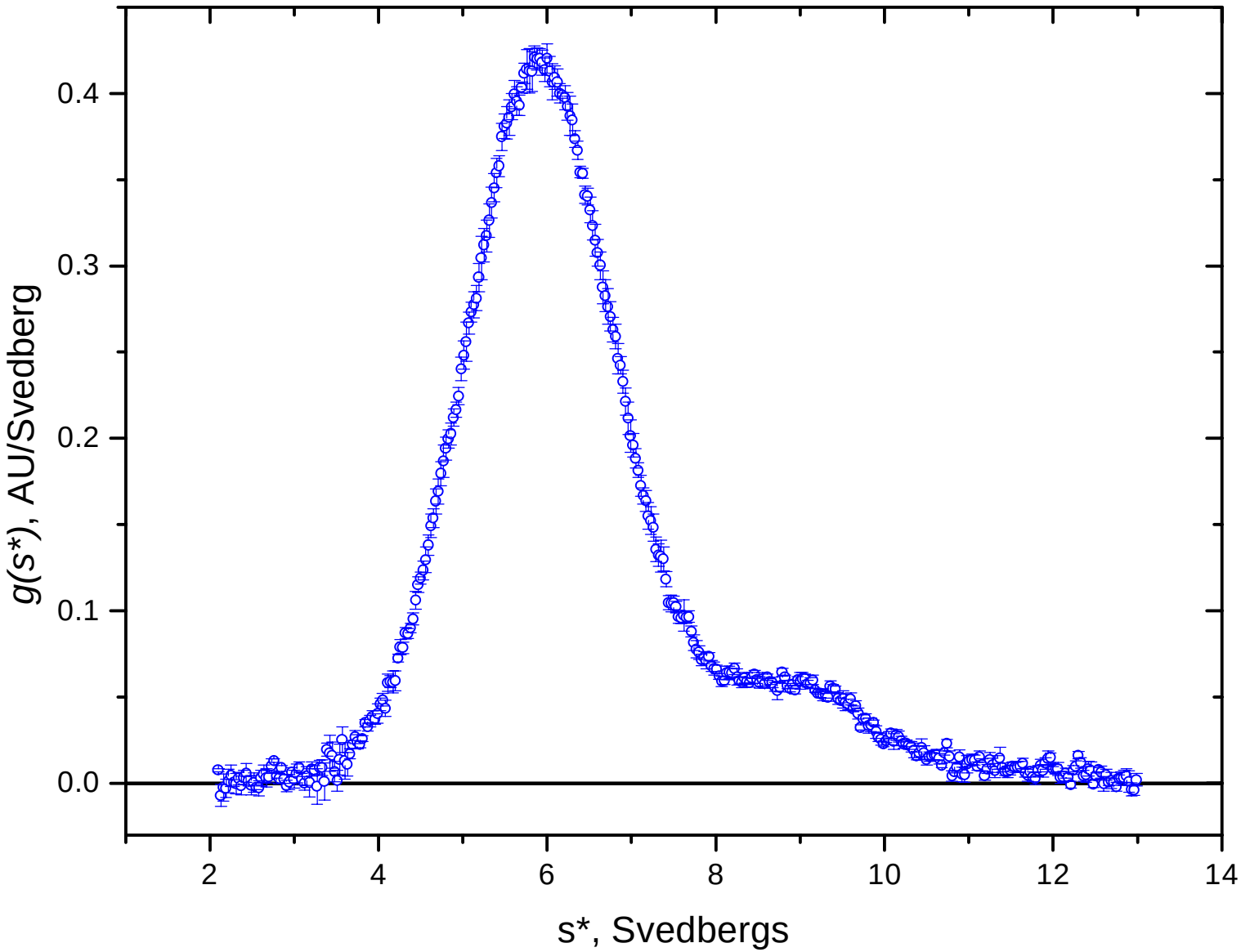
[1]Philo, J.S. (2000). A method for directly fitting the time derivative of sedimentation velocity data and an alternative algorithm for calculating sedimentation coefficient distribution functions. *Anal. Biochem.*, in press.

Some raw velocity data for an antibody---how can we interpret it?

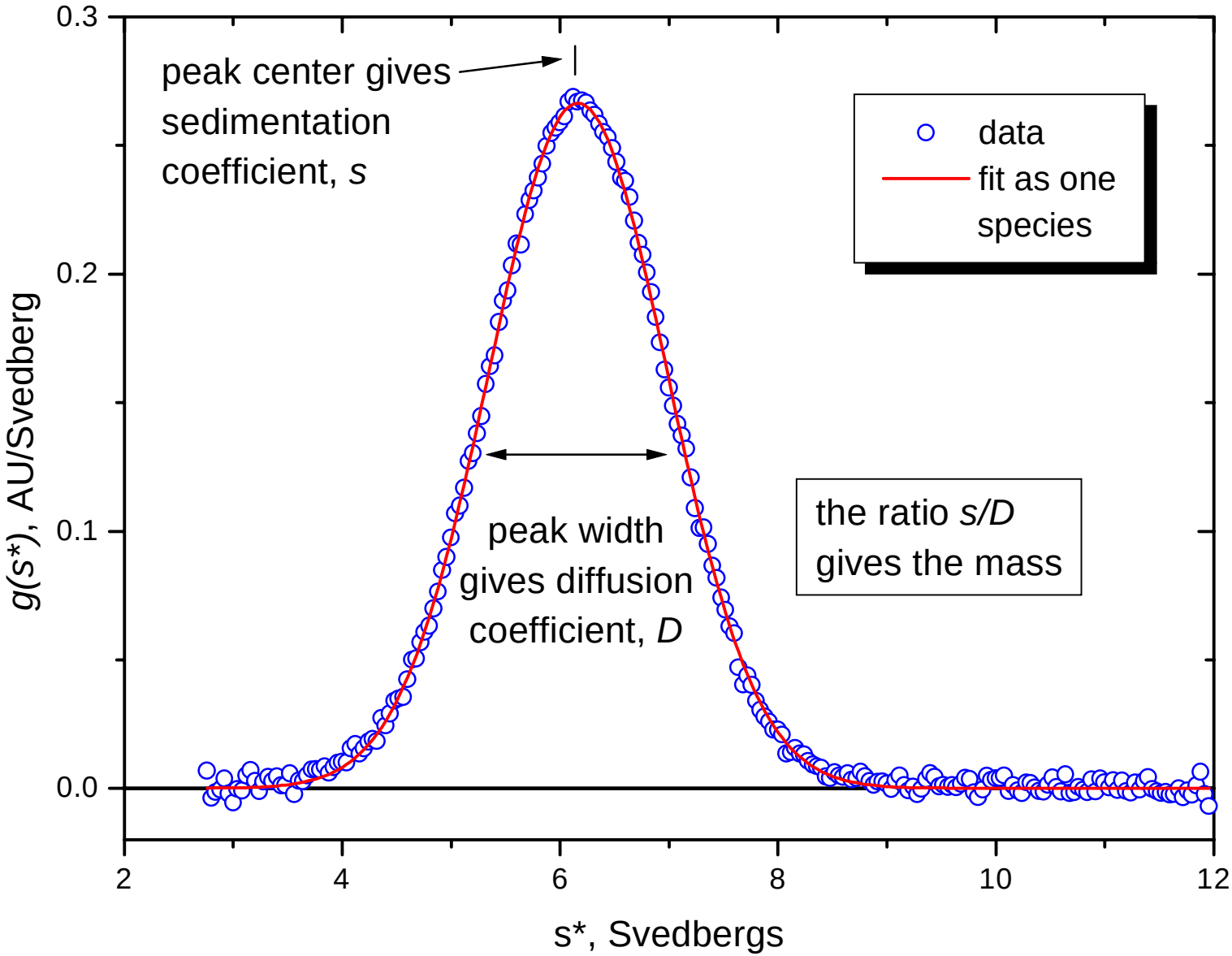
The rate of motion of the sedimentation boundary as the run proceeds is determined by the sedimentation coefficient, which depends on both mass and conformation. If the sample contains irreversible aggregates, they will form separate boundaries.



The sedimentation coefficient distribution function for this antibody sample shows it is heterogeneous and contains at least 3 species



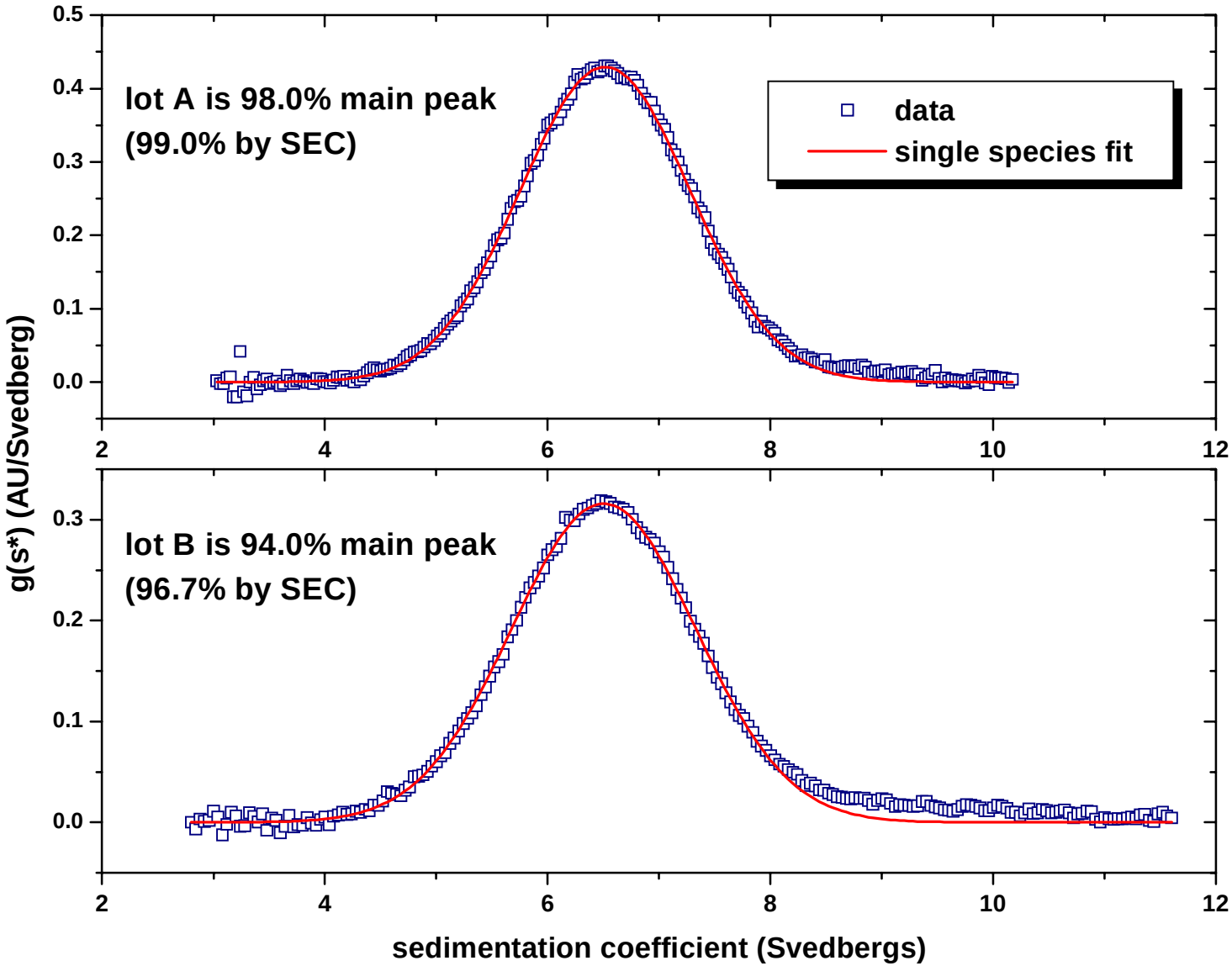
Further interpretation is possible by fitting individual species as Gaussians; this is an example for a homogeneous antibody sample



Results

- **Sedimentation velocity was used to study comparability between different manufacturing processes for a monoclonal antibody as well as lot-to-lot variability**
  - the results showed previously undetected lot-to-lot variability in aggregate content, but the difference between processes was no larger than variations within each process
  - since sedimentation coefficients are very sensitive to subtle changes in conformation, the fact that the main peaks from both processes have the same sedimentation coefficient proves equivalence of solution conformation
- **Note that analysis of these samples by size-exclusion chromatography (SEC) underestimates the true aggregate content**
  - this appears to be due to the aggregates sticking to the SEC column

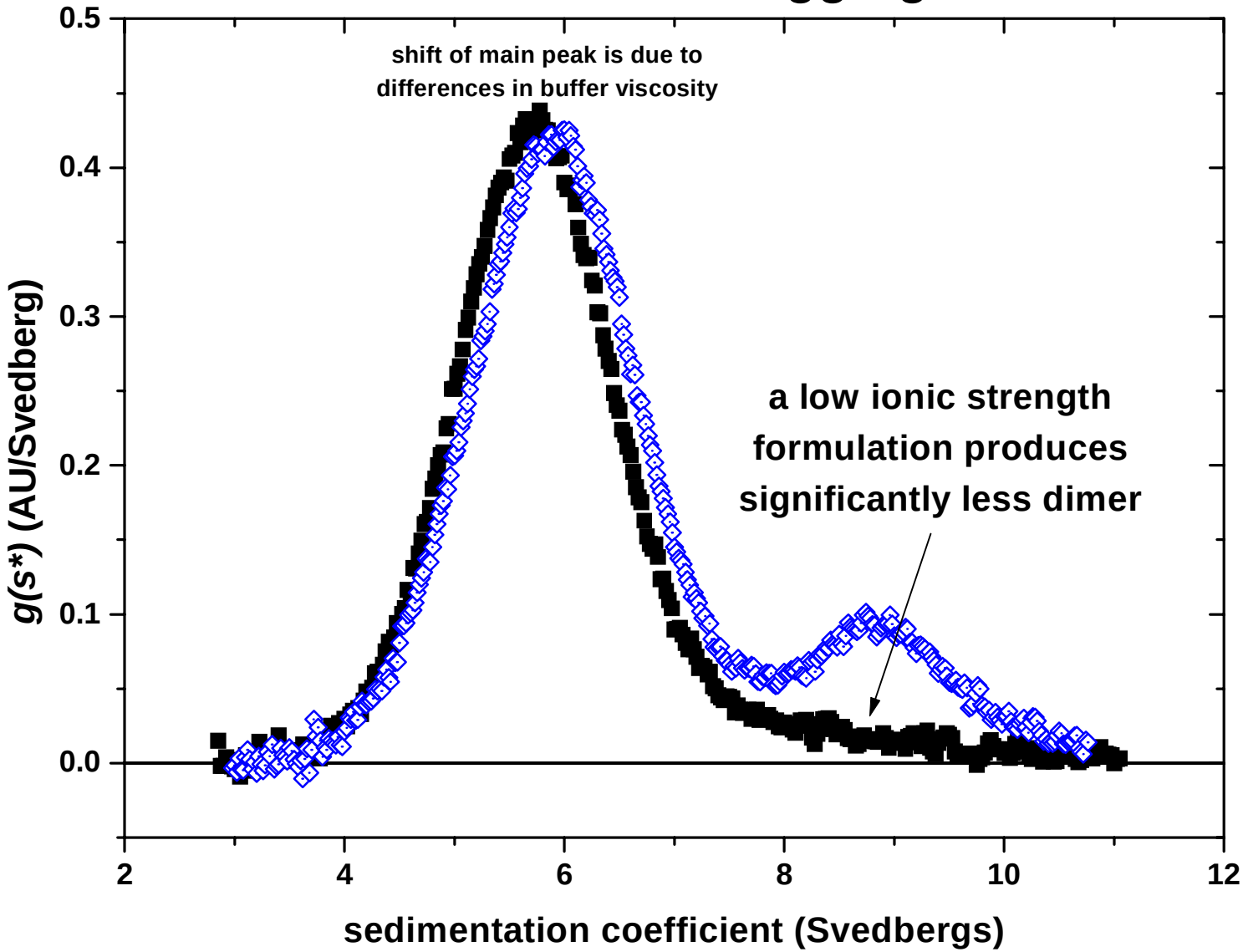
Comparability testing of two manufacturing processes for a monoclonal antibody: the main peaks have identical conformation, but aggregate levels vary lot-to-lot



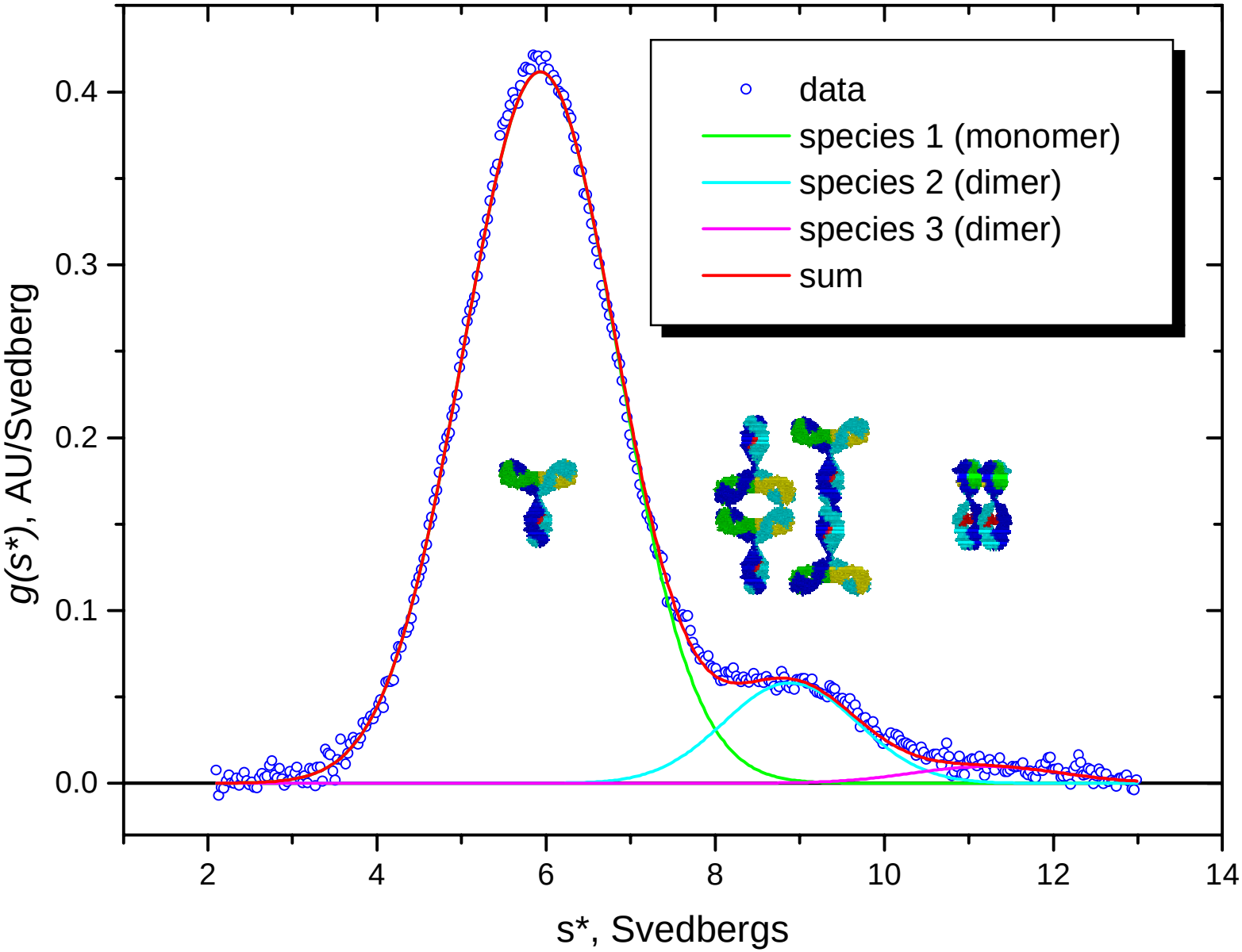
Results (continued)

- **Comparison of different formulations of an antibody showed that one contains significantly less dimer**
  - The low ionic strength of the better formulation would not allow SEC studies under those formulation conditions
- **A new software method developed in our lab indicates there are actually two types of antibody dimer in these samples:**
  - a slowly-sedimenting extended conformation (probably end-to-end)
  - a rapidly-sedimenting compact conformation (probably side-by-side)

Velocity analysis of two different formulations of an antibody, each analyzed in its own formulation buffer, reveals differences in aggregation



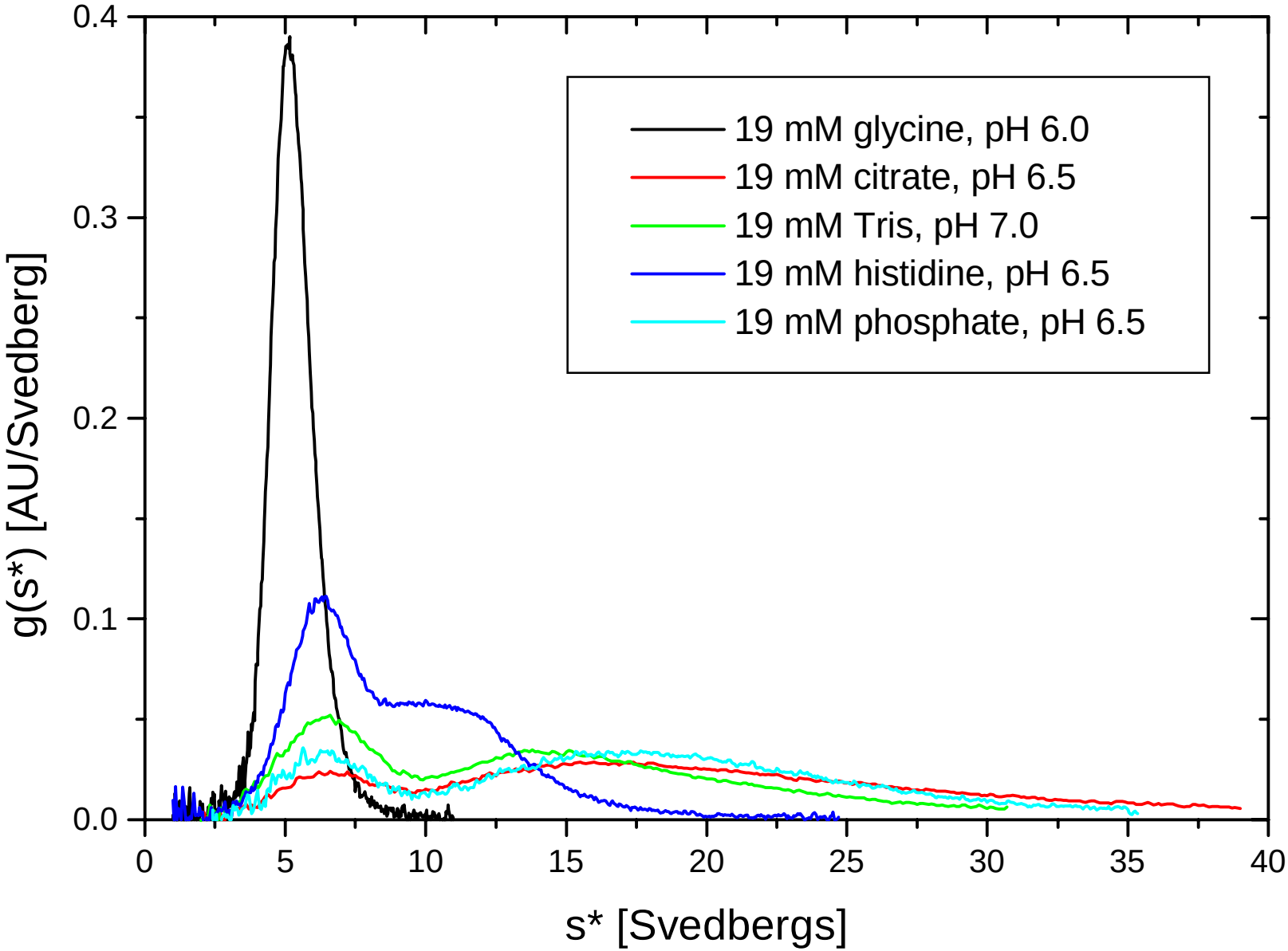
Detailed analysis of the heterogeneous antibody sample reveals 3 species. Because light scattering data shows that only monomer and dimer are present, we believe there are two different conformations of dimer



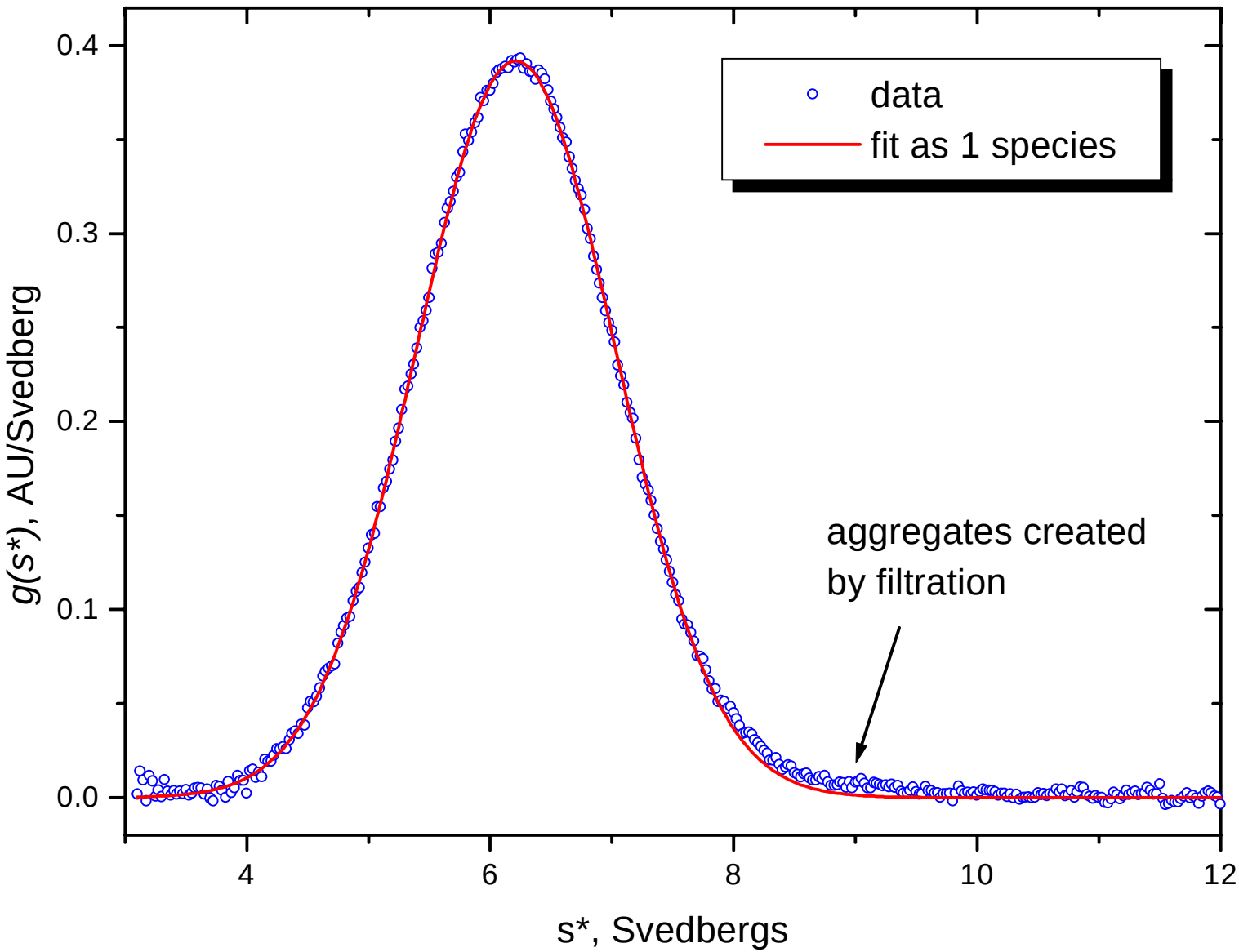
Results (continued)

- Studies of monoclonal samples after thermal stress reveal strong differences among buffers in formation of soluble aggregates
  - The glycine formulation which gives the least aggregation also produces a significantly different sedimentation coefficient for the main peak (different conformation); whether this conformational difference is directly related to the improved stability is not clear.
- Sedimentation velocity also detected formation of soluble aggregates after filtration (presumably induced by shear forces or exposure to the large filter surface)

Formulation applications: analysis of antibody samples after accelerated stability studies in various buffers



Assessing effects of physical stress: the homogenous antibody sample shows aggregates after filtration



Conclusions

- Sedimentation velocity is a very useful method for detecting protein aggregates (both covalent and non-covalent), characterizing their size, and quantitating the aggregate content
- Sedimentation velocity is also very useful for demonstrating comparability of solution conformation for the main species
- A major advantage of this method over size-exclusion chromatography is that the analysis can usually be done in the actual formulation buffer, and there is no concern about loss of aggregates to a column matrix
- The utility of this technique has grown considerably with modern instrumentation and improved software tools