Measuring Comparability of Conformation, Heterogeneity, and Aggregation with Circular Dichroism and Analytical Ultracentrifugation

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Outline

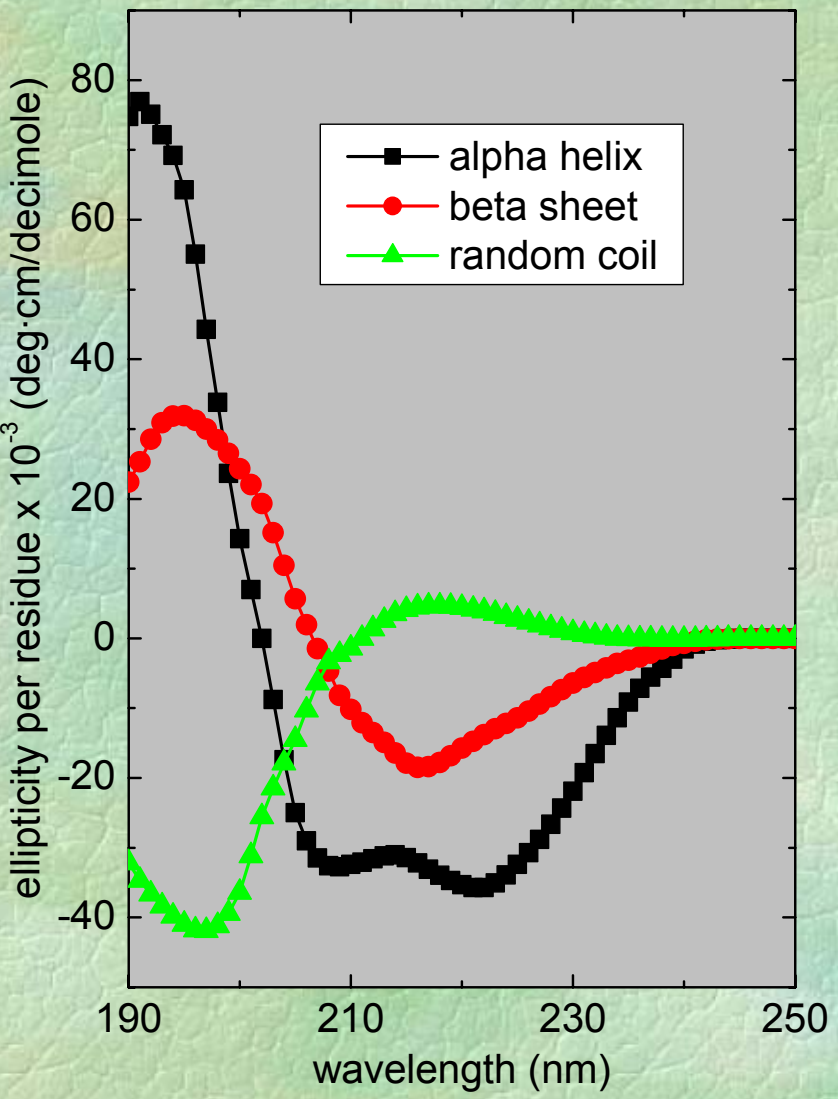
- Circular dichroism---what does it do and how can we apply it for comparability?
- Introduction to analytical ultracentrifugation: sedimentation velocity and sedimentation equilibrium
- Sedimentation equilibrium examples:
  - does my protein have the correct quaternary structure?
- Characterizing protein conformation and aggregation by sedimentation velocity

Circular dichroism (CD) spectroscopy

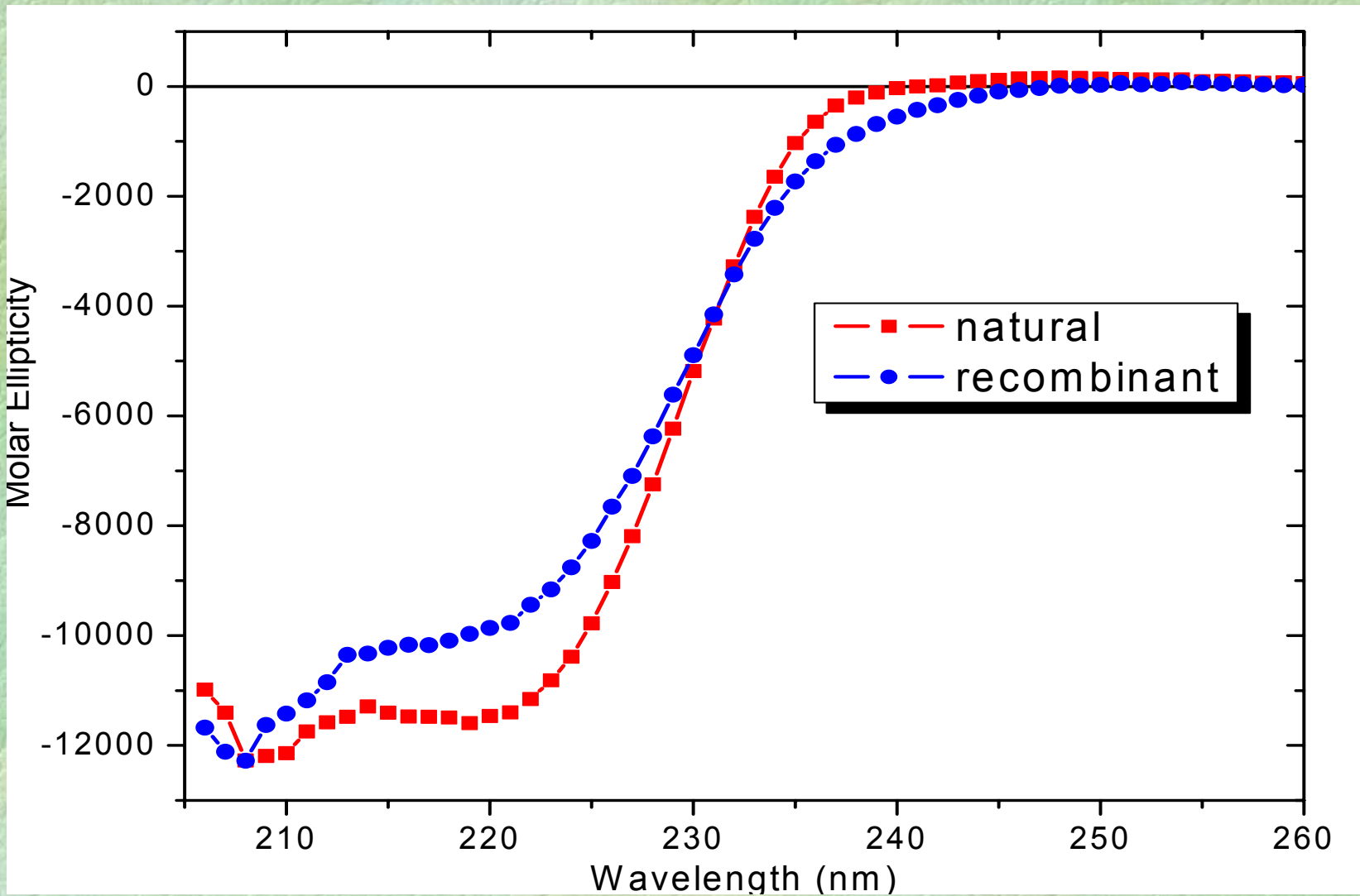
- ★measures the absorbance difference between right-handed and left-handed circularly polarized light
- ★sensitive to chirality or asymmetry around chromophores

Far-UV CD

- ★ 'far-UV' protein spectra (190-240 nm) are sensitive to secondary structure
- ★ by spectral fitting one can estimate percentage α-helix, β-sheet, etc.
- ★ **our view**: most useful as comparative spectral fingerprint



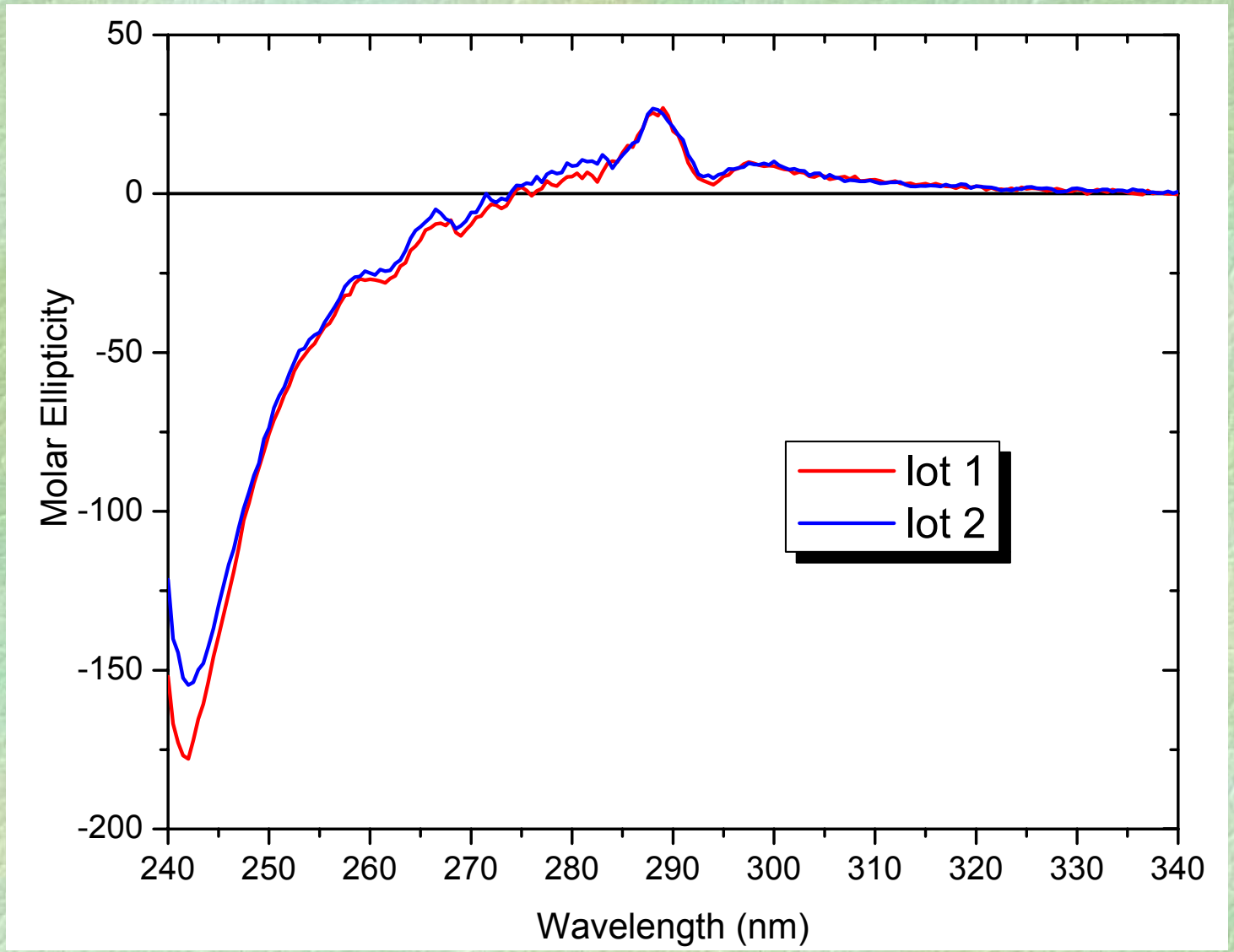
Differences in far-UV CD for natural *vs.* recombinant forms of an enzyme (which also have different enzymatic activity)



Near-UV CD

- 'near-UV' protein spectra (240-340 nm) are sensitive to local tertiary structure around aromatic residues and disulfide bonds
  - proteins lacking regular tertiary structure show zero near-UV signal (*e.g.* "molten globules")
- signals can be either positive or negative
  - sometimes they nearly cancel
- strictly a fingerprint---no direct structural interpretation
- **our view:**
  - under-utilized
  - usually more sensitive to subtle conformational differences than far-UV

Differences in near-UV circular dichroism between stable and unstable lots of a monoclonal antibody



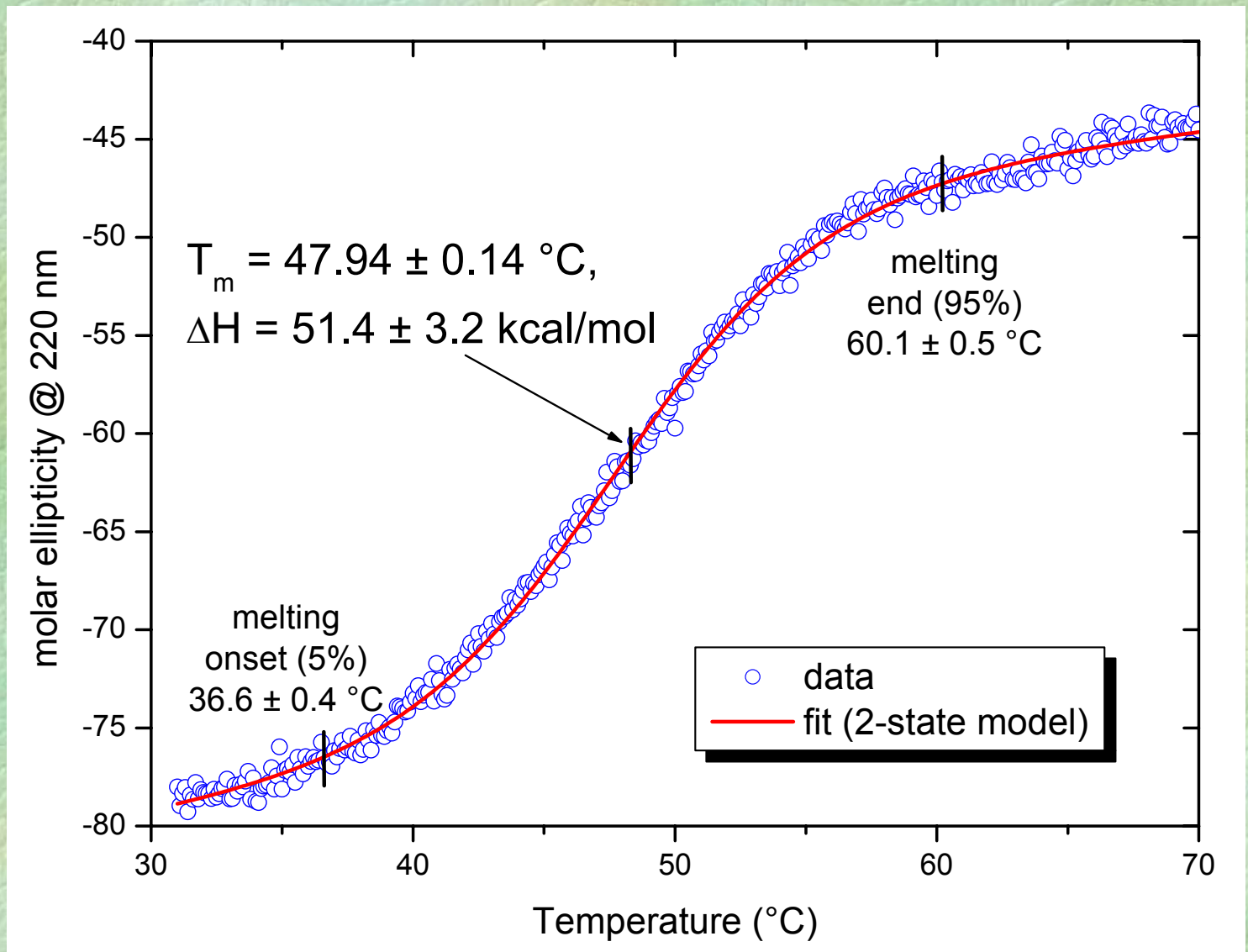
Drawbacks to CD Spectral Analysis

1. difficult to quantitate similarity or differences
   - ★ what is "comparable" is often a judgment call
2. like all spectroscopy, if there is heterogeneity all you see is an average
   - ★ unlikely to detect minor components
3. certain buffer components absorb strongly in the far-UV and can cause interference

Measuring thermal stability (thermal unfolding) by CD

- Another conformational measure than can be used for comparability
- Very similar uses as DSC
  - can be done at much lower concentrations
  - can tell how the structure is changing, not just that something is unfolding
- Generally done by monitoring a single wavelength in the far-UV *vs.* temperature

Quantitative analysis of thermal stability from CD data

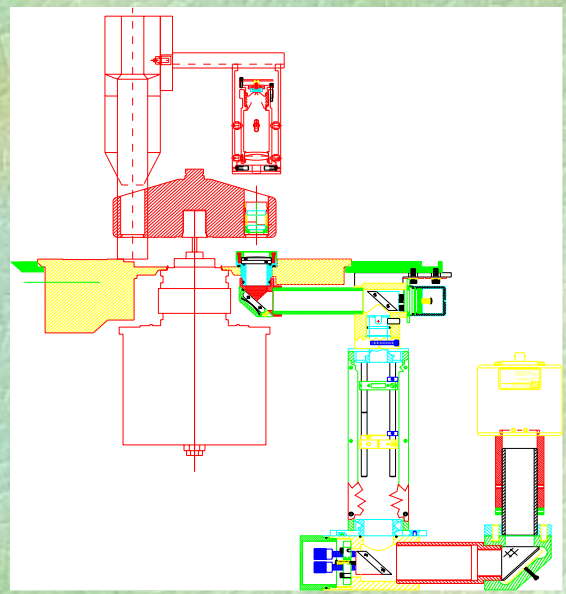


Analytical Ultracentrifugation

The modern analytical ultracentrifuge

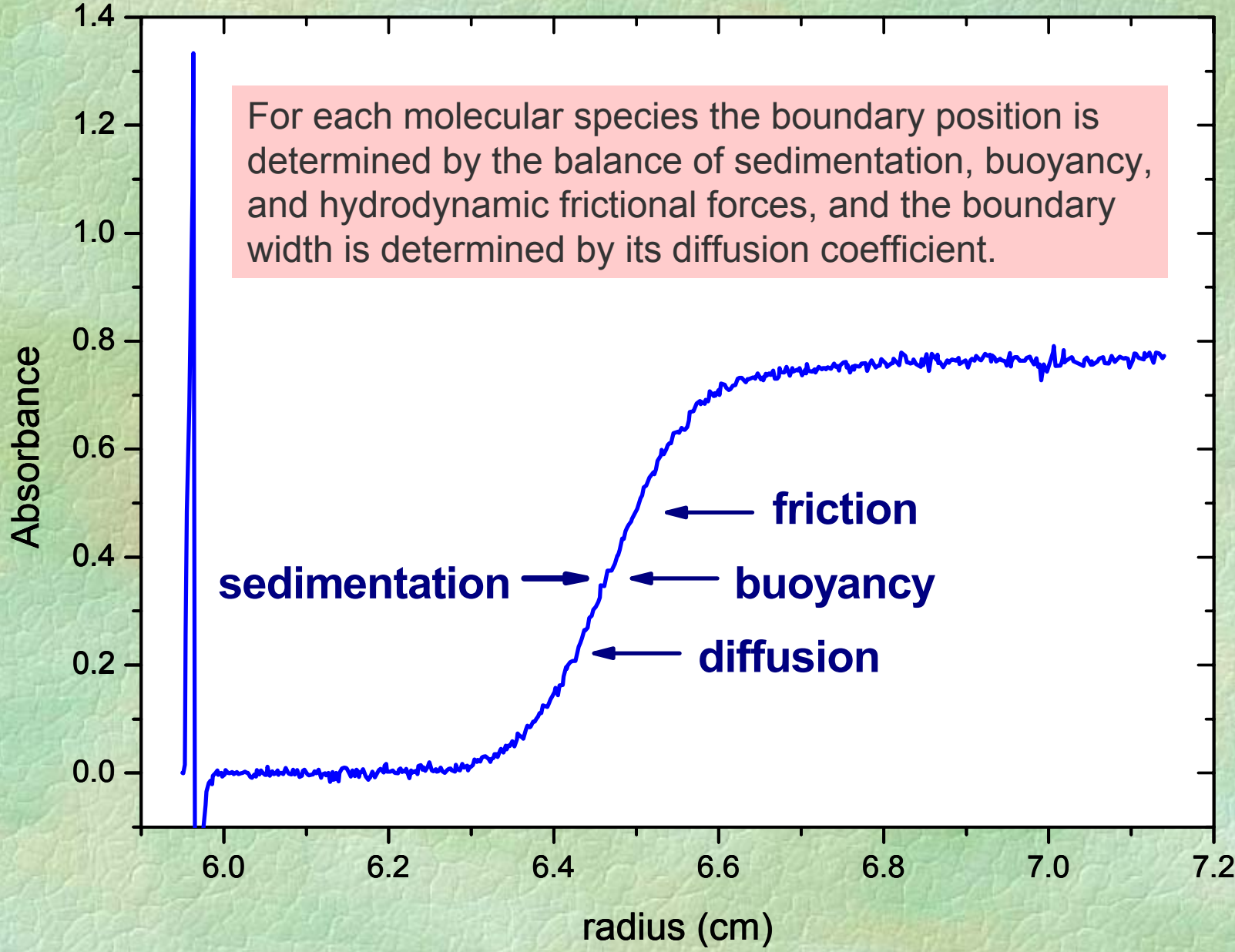
a preparative ultracentrifuge

+ optical systems, special rotors, and sample cells

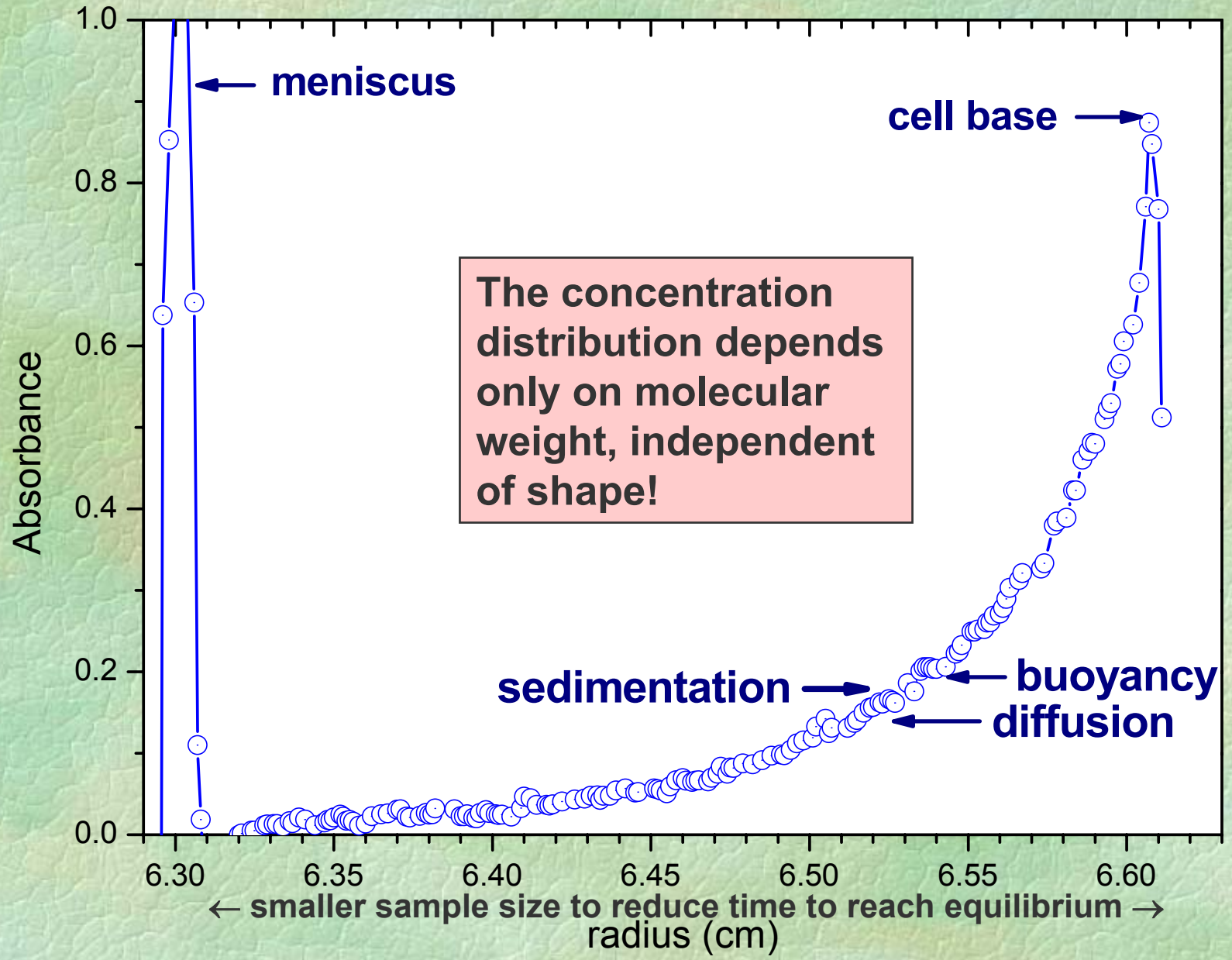


+ computerized control, data acquisition, and analysis

The fundamentals of sedimentation velocity



The fundamentals of sedimentation equilibrium

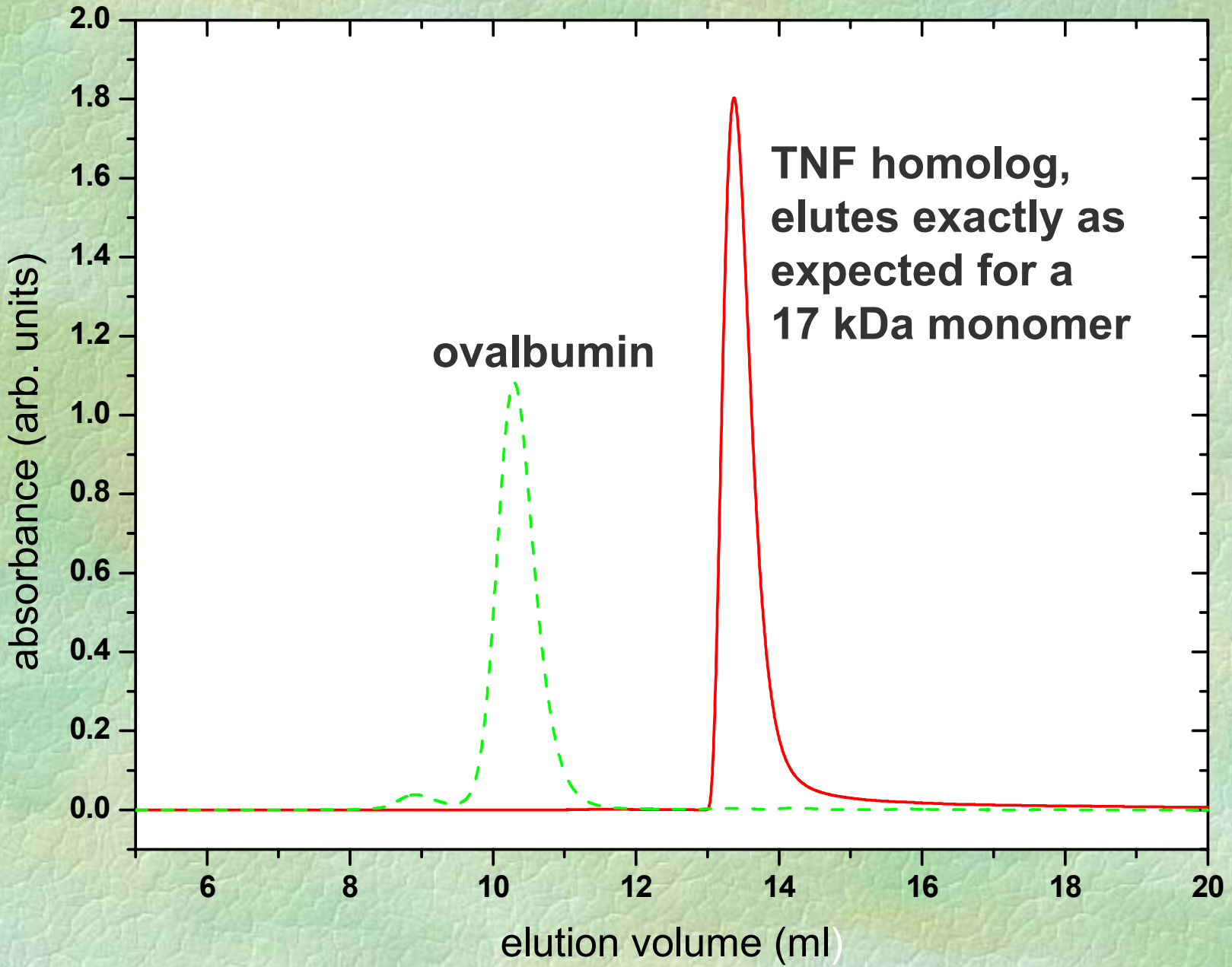


Both sedimentation methods are "first principle" methods

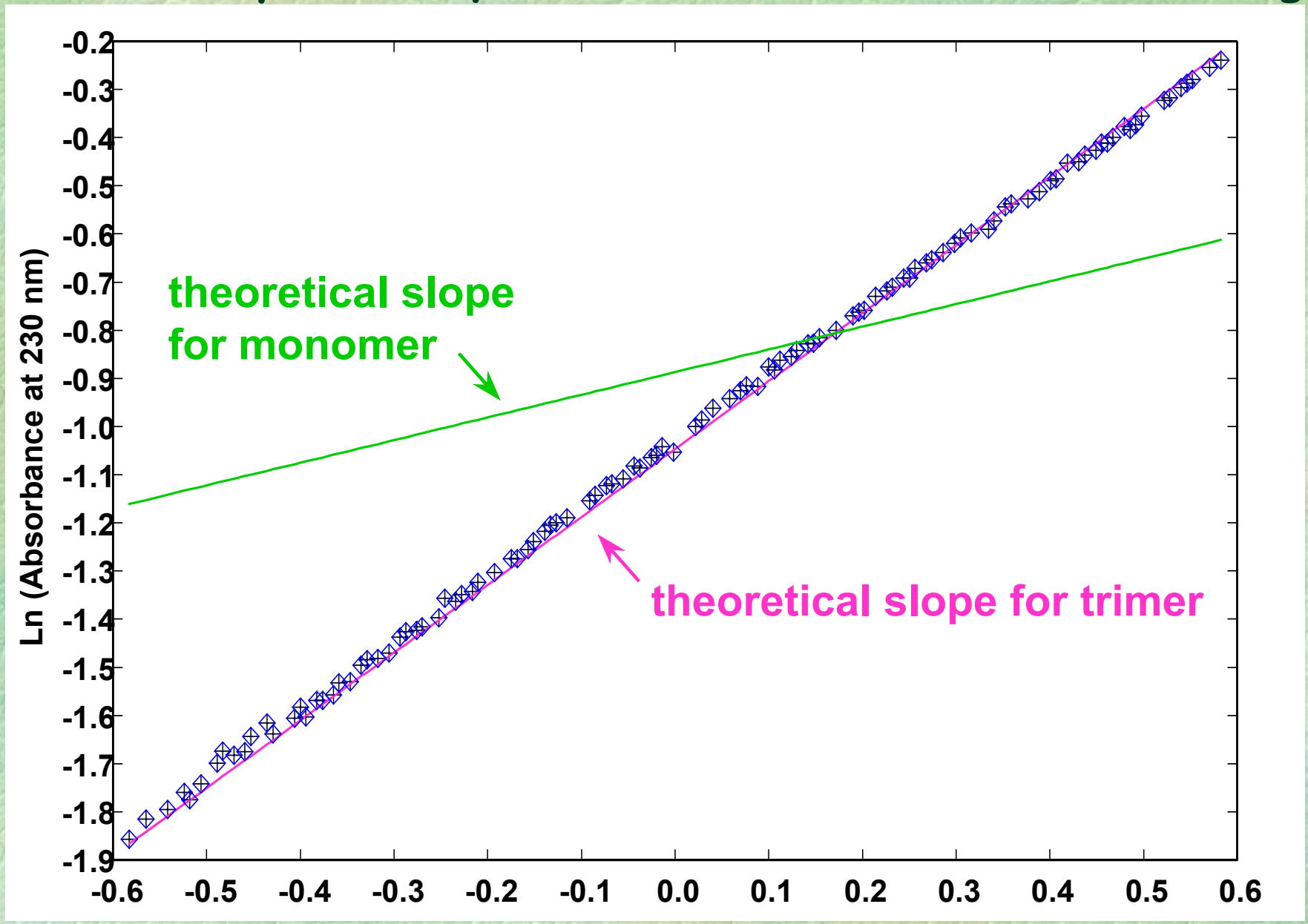
- based on fundamental physical laws
- require no standard molecules for calibration
  - calibration is based only on fundamental units of distance, time, and temperature

Characterizing solution mass by sedimentation equilibrium

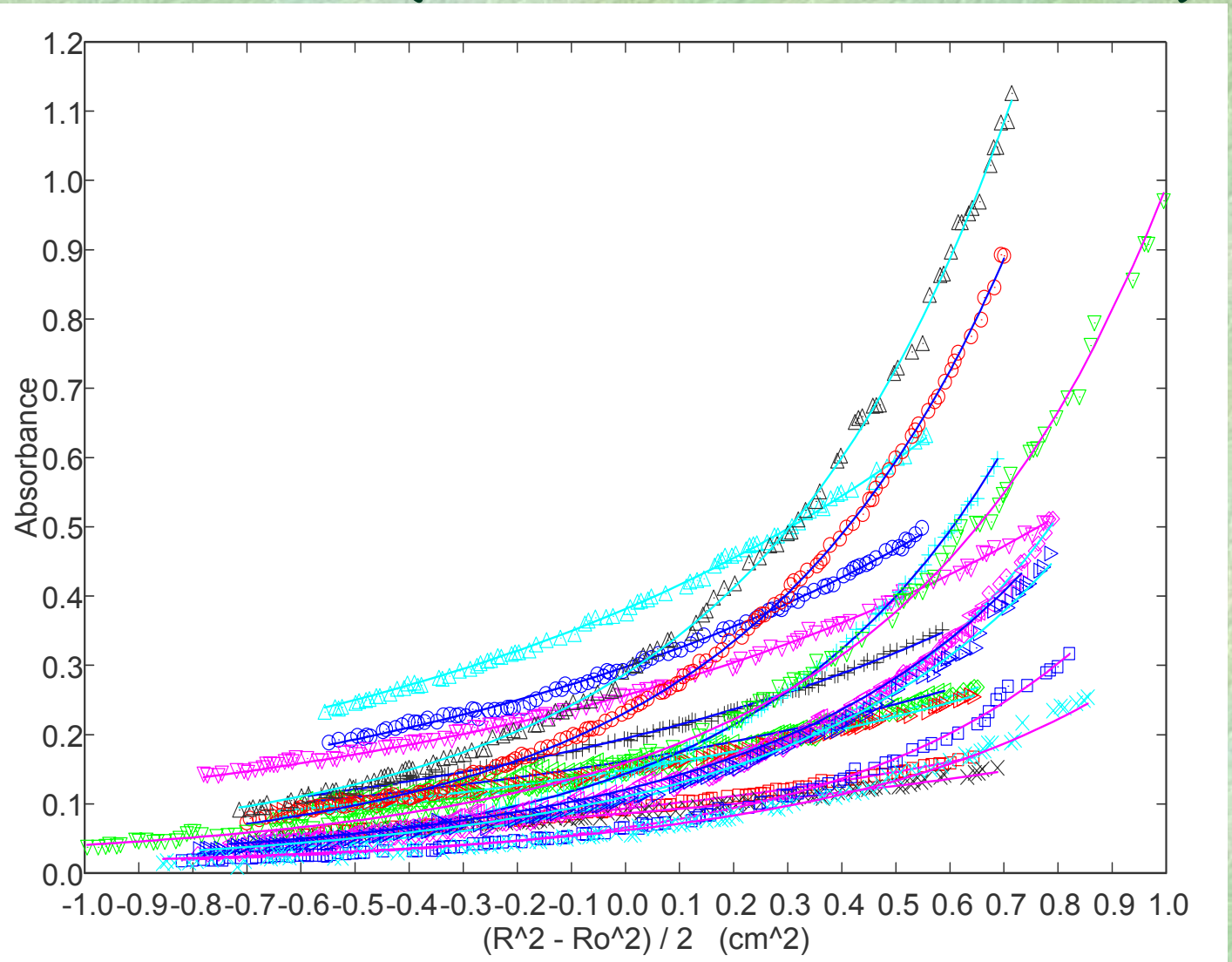
Size-exclusion chromatography of a TNF homolog



Linearized plot of equilibrium data for the TNF homolog

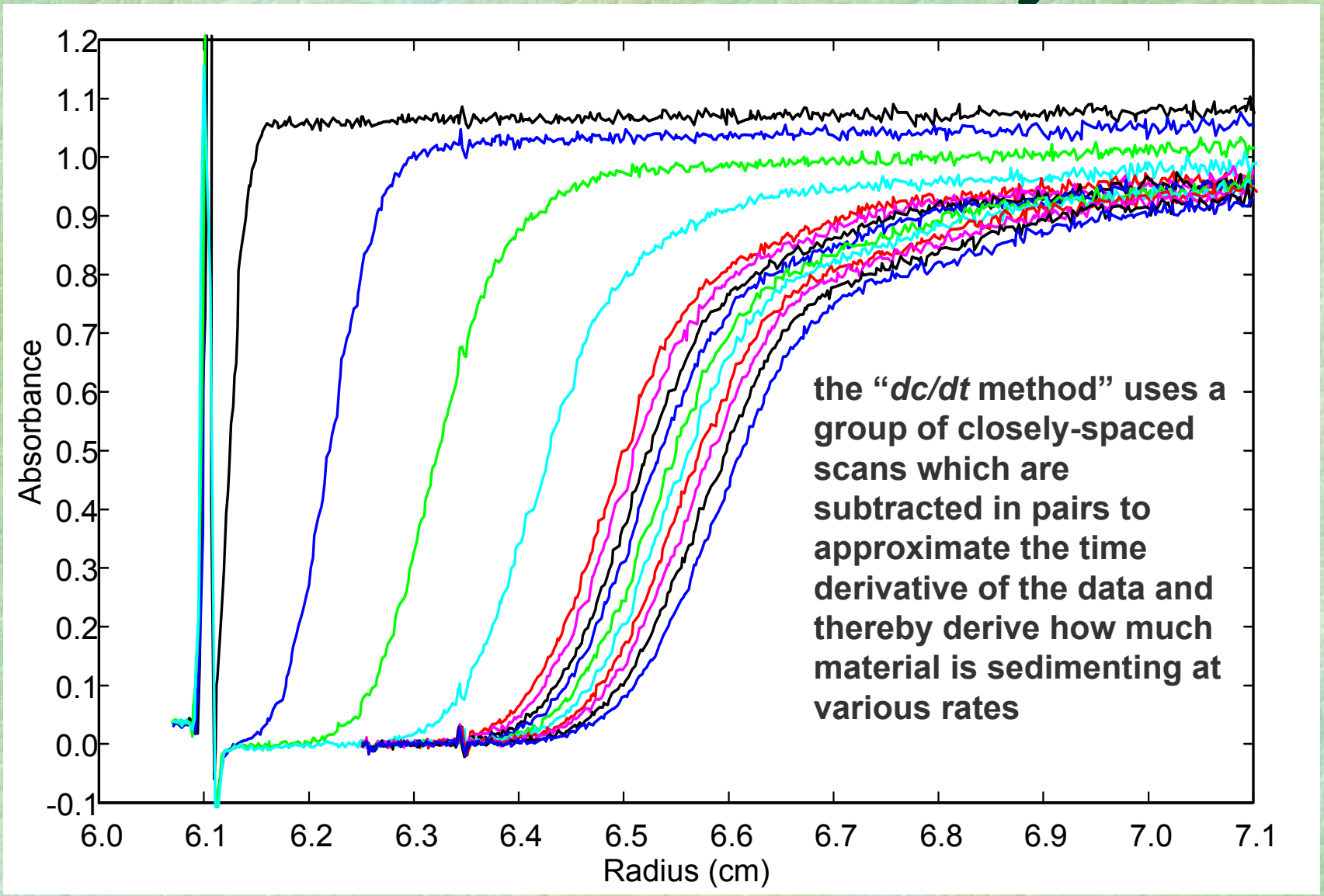


An example of characterizing a protein that self-associates to form dimers. Global analysis of experiments at ~5-250 μg/ml (using multiple wavelengths) gives K_d = 520 +/- 20 nM (ΔG = -8570 +/- 25 cal/mol)

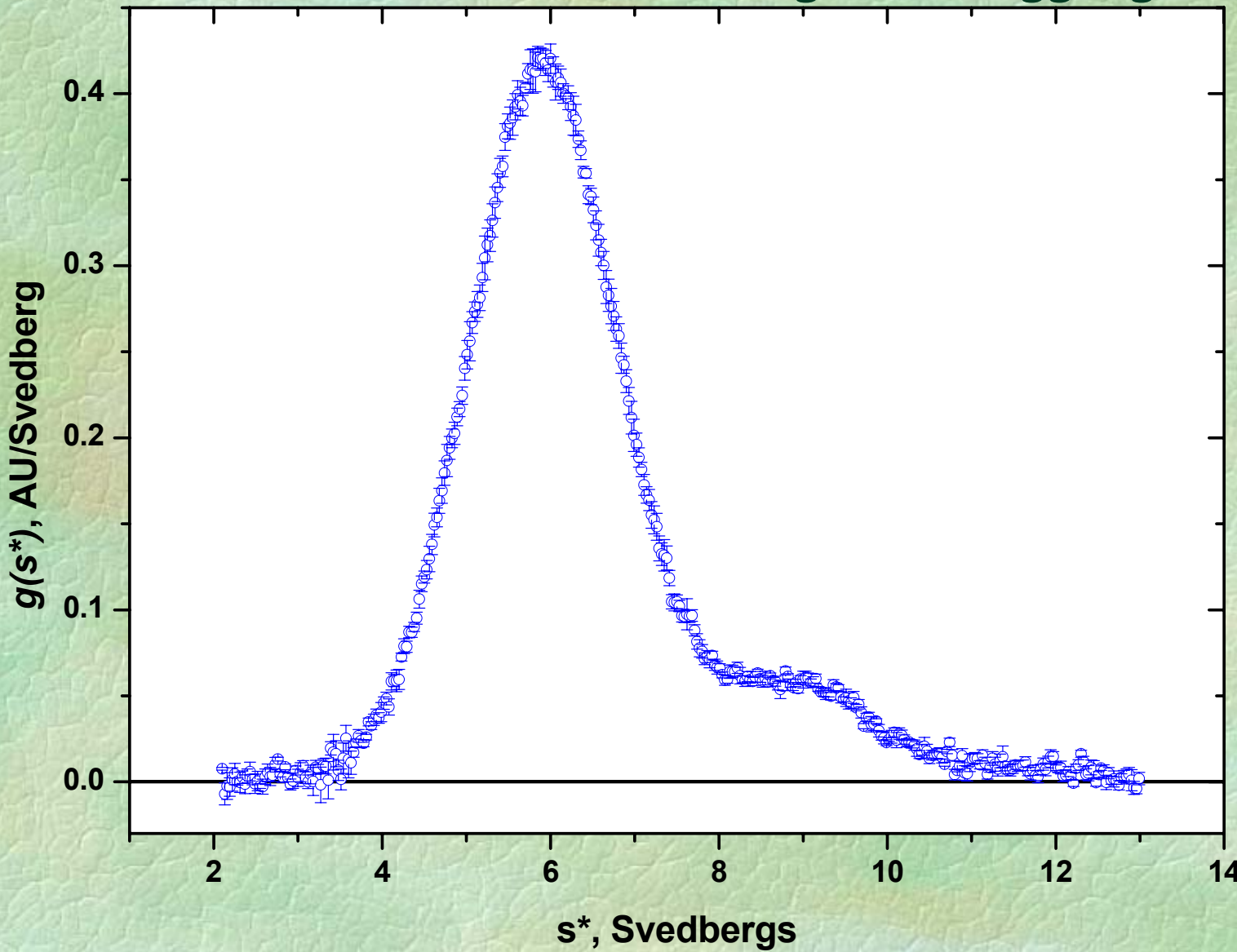


Using sedimentation velocity to characterize protein conformation and aggregation

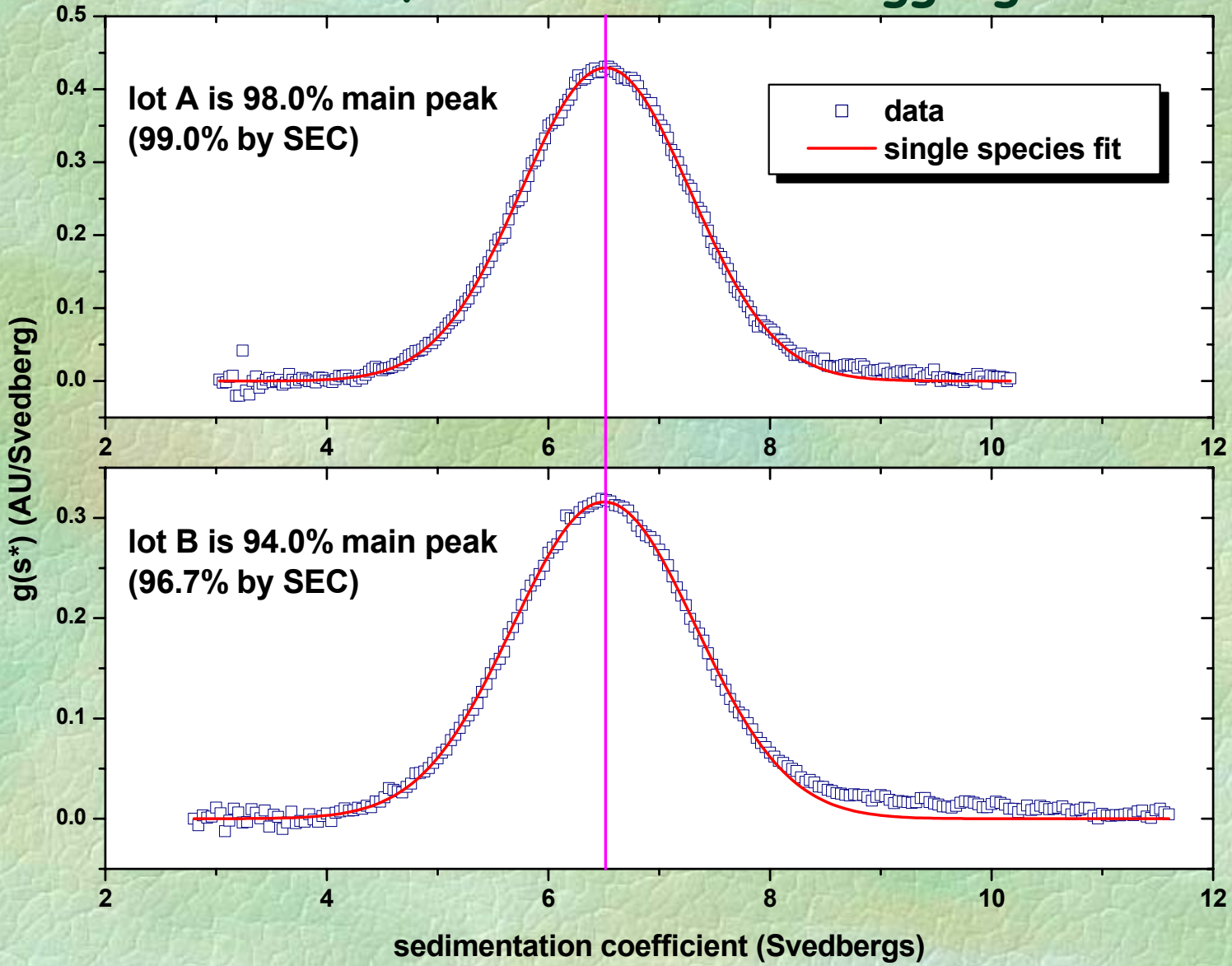
How can we interpret these raw data for an antibody? Time-derivative (*dc/dt*) analysis allows us to convert it into a distribution like a chromatogram



The sedimentation coefficient distribution function for the antibody sample shows it is heterogeneous and contains at least 2 different long-lived aggregates



Comparing lots of antibody from two different purification processes: the conformation of the main peak is identical, but amounts of aggregate differ



How reproducible are sedimentation coefficients for demonstrating comparability?

- The general rule of thumb is these values should be accurate and reproducible to ±0.5% or better, both run-to-run and even year-to-year
  - precision within the same run should be ~0.1-0.2%
- Some real data for the same reference standard lot measured 5 different times over 18 months:

6.264 ± 0.005 S

6.268 ± 0.005 S

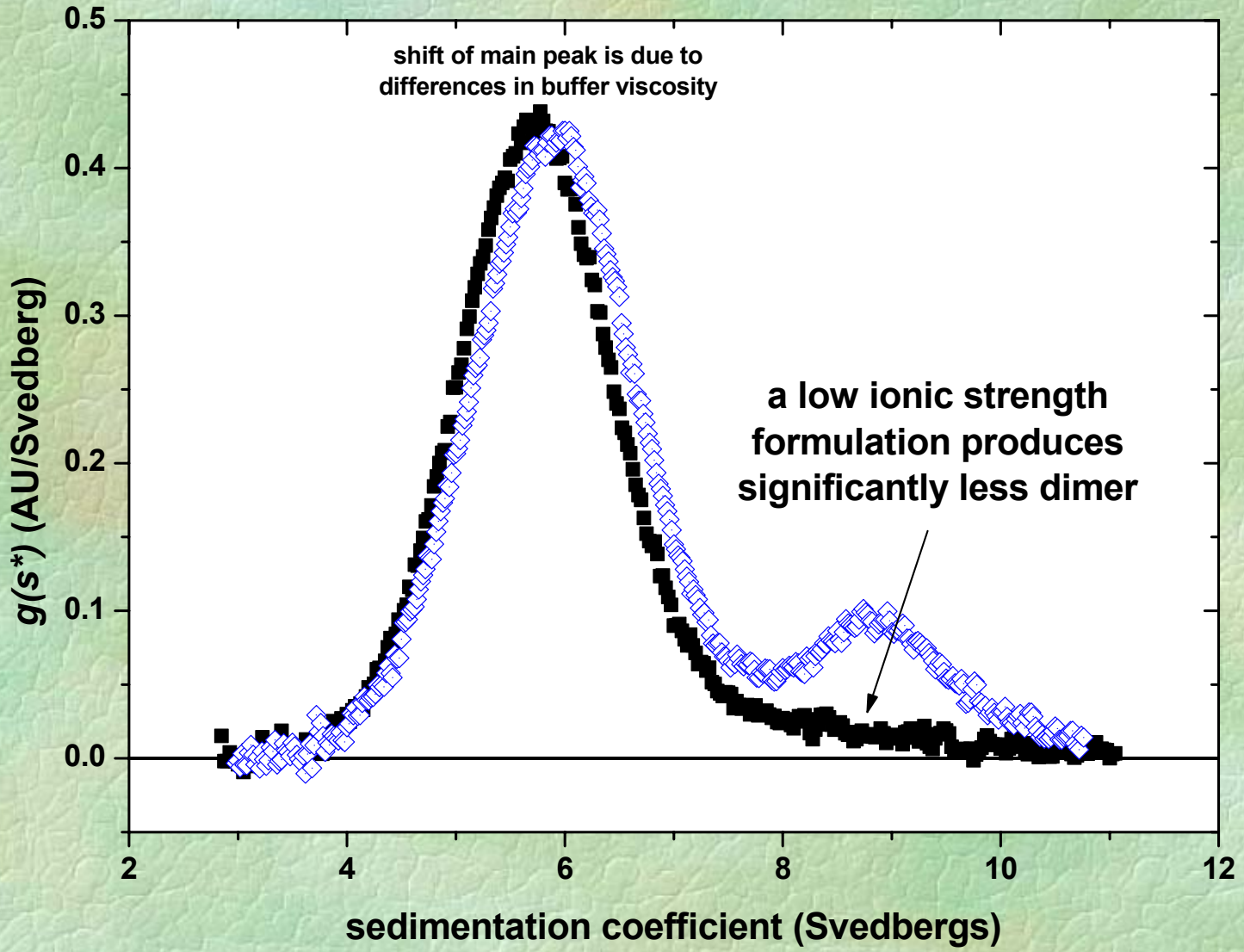
6.270 ± 0.005 S

6.270 ± 0.005 S

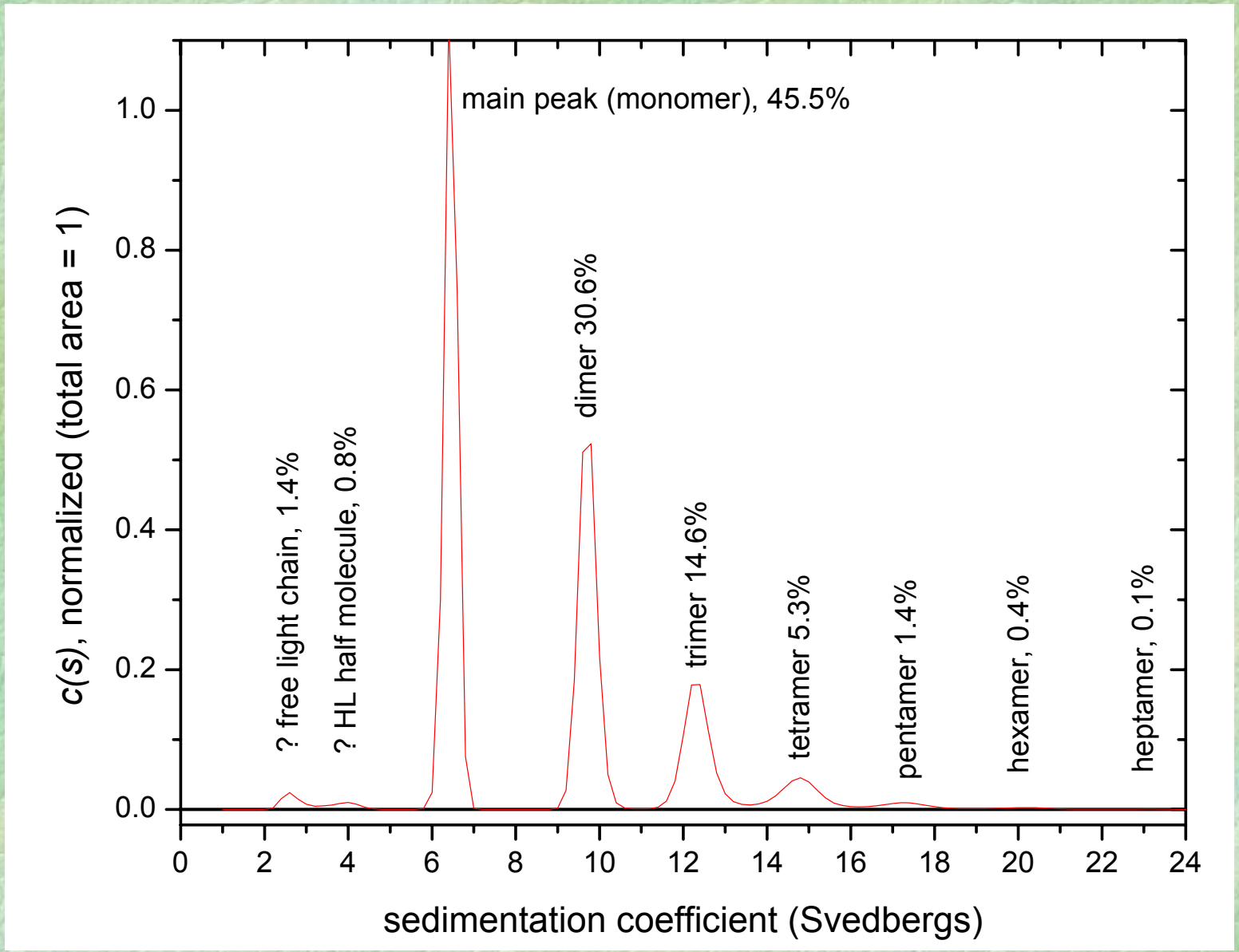
6.265 ± 0.006 S

mean 6.267 ± 0.0028 (± 0.04%)

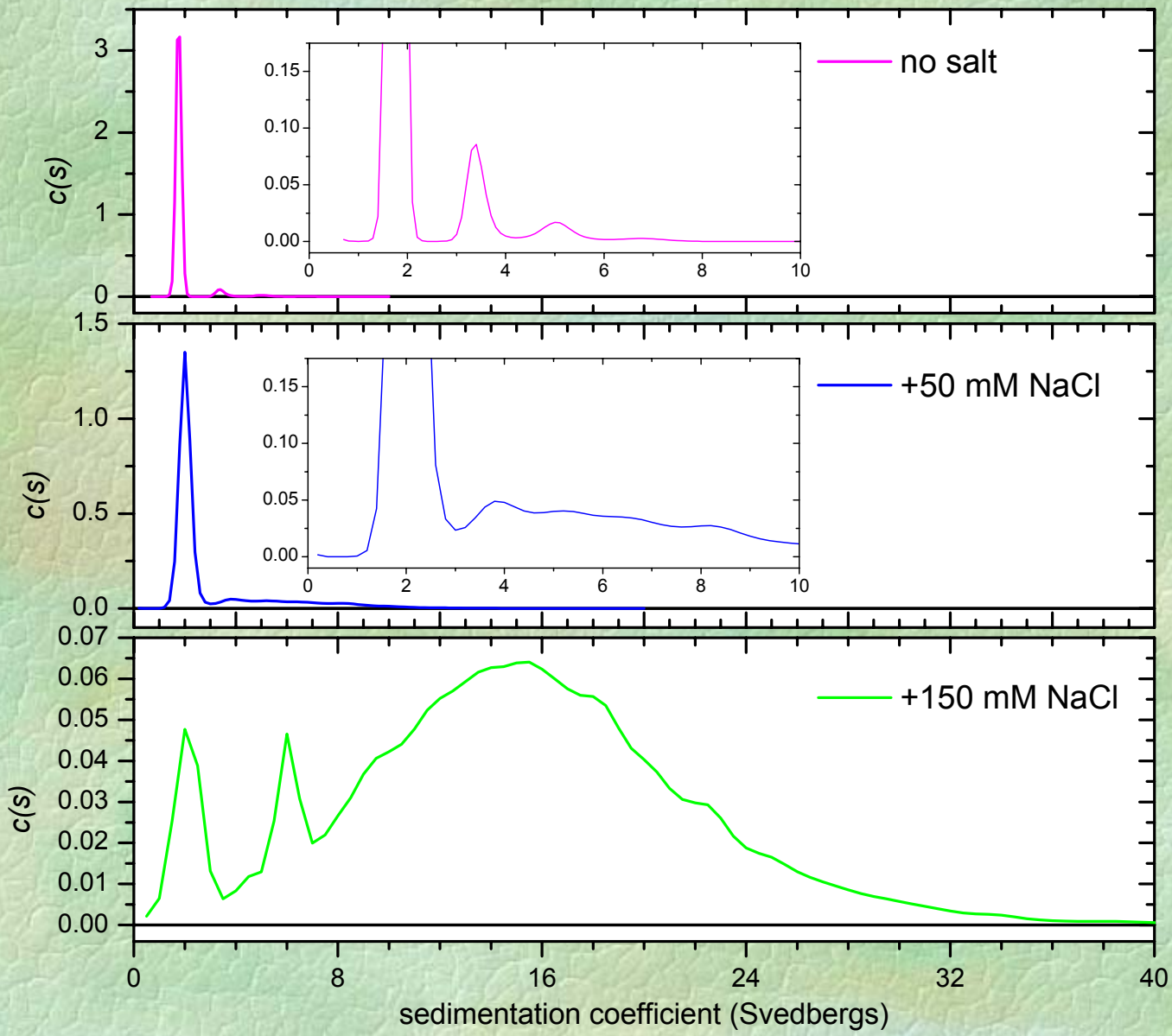
Velocity analysis of two different formulations of an antibody, each analyzed in its own formulation buffer, reveals differences in aggregation



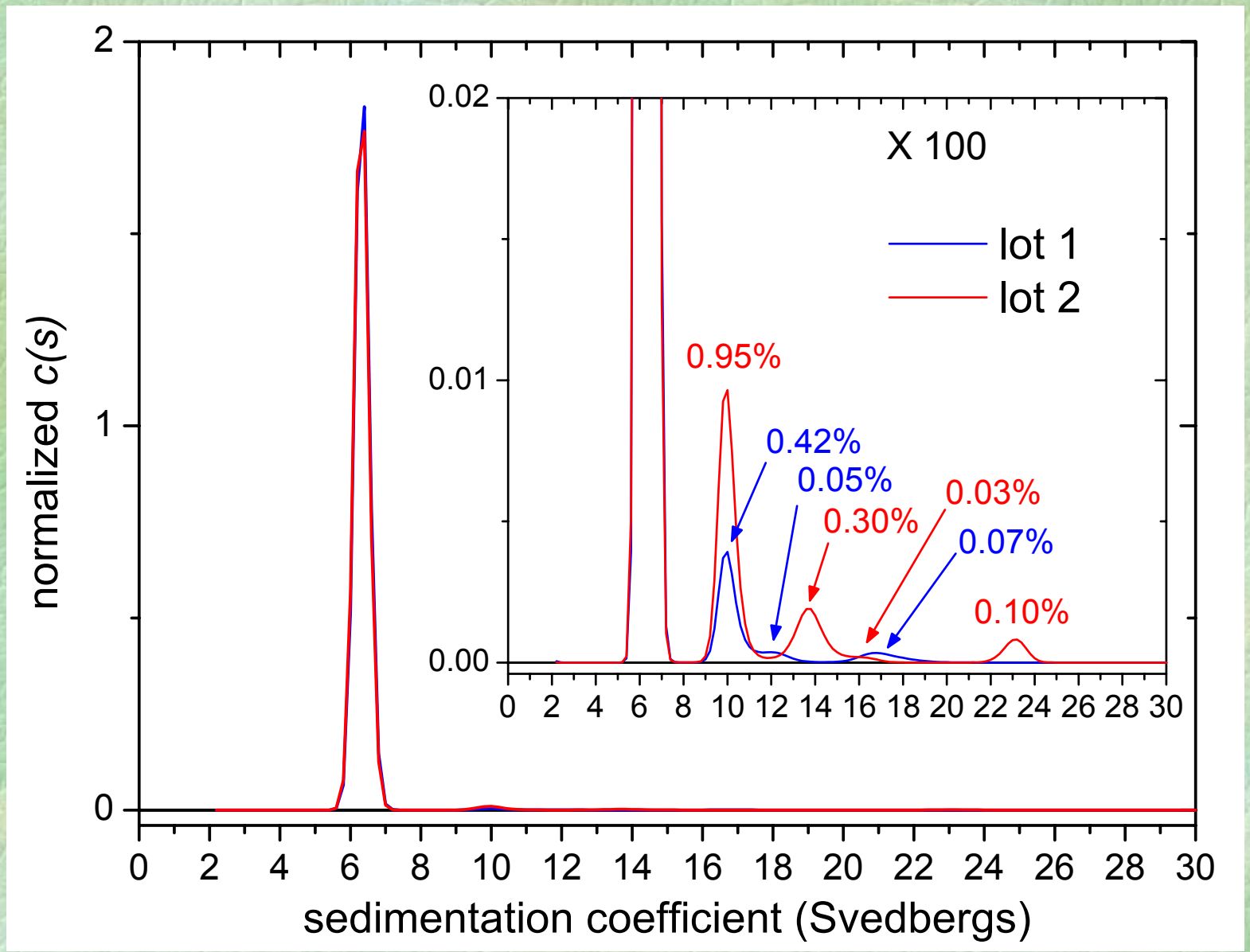
High resolution analysis of a highly stressed antibody sample using the *c(s)* method from Peter Schuck (NIH)



Non-covalent aggregates of a ~20 kDa cytokine: monomer to ~100-mer can be measured in a single analysis



Comparability of a monoclonal antibody by the high-resolution method



"Aggregates" *vs.* self-associated oligomers

- We must be careful to distinguish between rapidly-reversible, self-associated oligomers and irreversible (or at least long-lived) oligomers ("aggregates")
  - distinction more important now that we are seeing so many high concentration formulations
- Oligomers which are reversible, but which dissociate only over hours-days, are much more common than most people realize
- **Our View:** only the long-lived (hours or more) aggregates are likely to have an impact on immunogenicity, PK, or efficacy
  - oligomers which rapidly dissociate upon dilution *in vivo* are unlikely to impact safety or efficacy

These same velocity methods can be applied to other biopharmaceuticals besides pure recombinant proteins

1. protein mixtures such as blood products
2. nucleic acids
   - ★ naked vaccines
   - ★ gene therapy
   - ★ ribozymes
3. whole virus
   - ★ vaccines
   - ★ gene therapy vectors
4. drug-polypeptide conjugates
   - ★ toxin:antibody conjugates
   - ★ small-molecule:poly-amino acid conjugates

Summary

- ★ CD can provide useful spectral fingerprints and thermal unfolding data for measuring conformational comparability
- ★ Sedimentation coefficients are an excellent and highly quantitative way to demonstrate comparability of conformation
- ★ Sedimentation velocity is an excellent method to detect and quantify protein aggregates, with resolution and range far exceeding that of SEC
- ★ Sedimentation equilibrium is a powerful tool for characterizing quaternary structure in solution and protein-protein interactions