

Some Biophysical Methods to Demonstrate Comparability of Conformation and Aggregation

John S. Philo* and Tsutomu Arakawa, Alliance Protein Laboratories

The bioactivity of protein or RNA-based pharmaceuticals depends on their conformation (secondary and tertiary structure). They also often form inappropriate intermolecular interactions (aggregates), and such formation of oligomers/aggregates may alter activity, PK, immunogenicity, *etc.* Therefore demonstrating comparability requires good methods to assess both conformation and aggregation.

Sedimentation velocity can be a powerful method for demonstrating comparability. Sedimentation coefficients are highly sensitive to small differences in molecular conformation, and thus provide an excellent quantitative measure of comparability. Sedimentation velocity is also a powerful tool for assessing heterogeneity, such as the presence of aggregates. Data for antibodies will show that recently developed sedimentation velocity techniques can detect and quantitate aggregates at levels well below 1%, while covering a far wider range of masses than is possible by SEC.

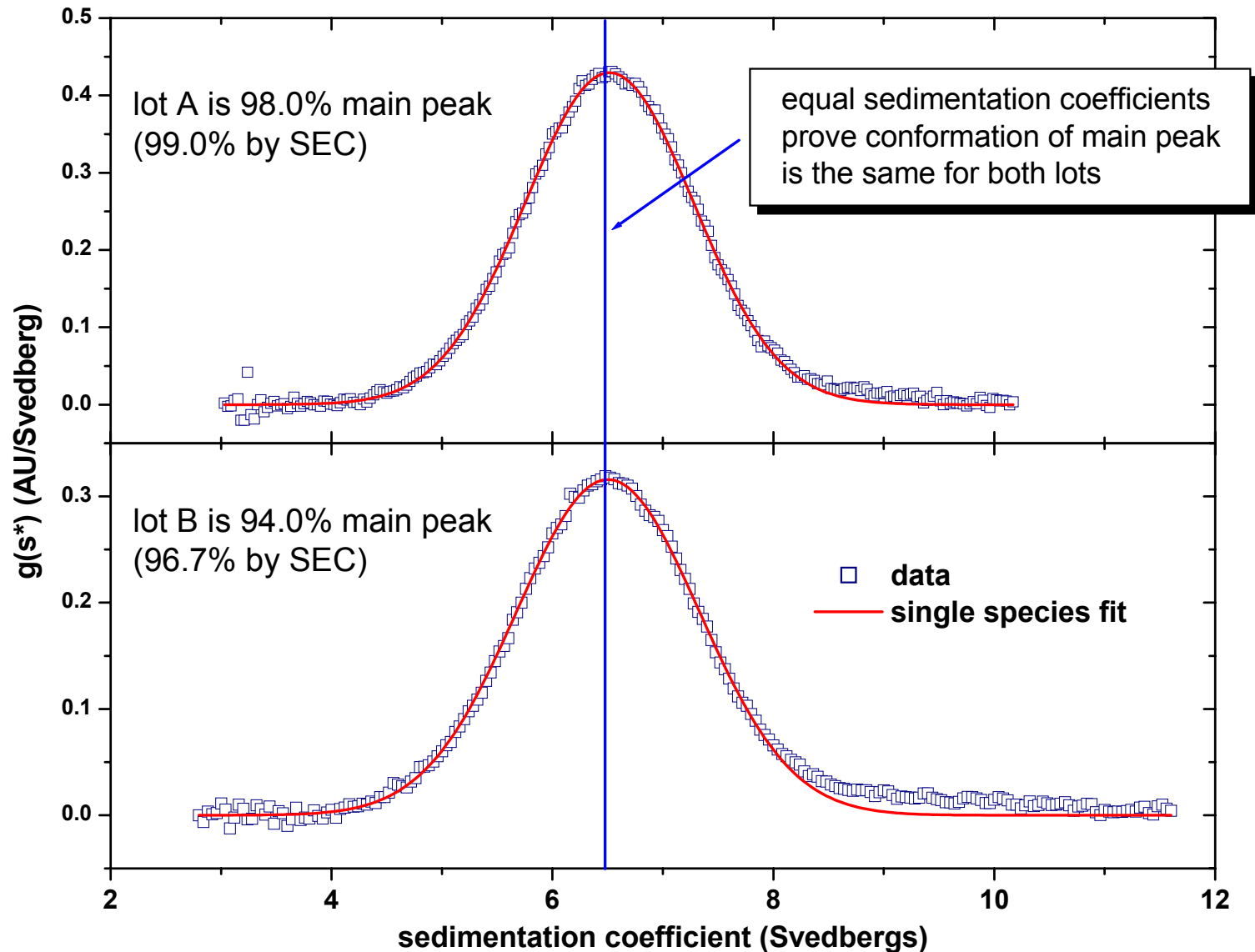
Circular dichroism (CD) spectroscopy is another useful tool for measuring biomolecular conformation. Protein spectra in the far-UV region are sensitive to secondary structure, while their near-UV spectra depend on the tertiary structure around aromatic residues and disulfides. Thus both spectral regions provide a spectral ‘fingerprint’ for showing comparability of conformation. As we will show, however, the often-neglected near-UV spectrum can be more sensitive to subtle conformational differences. CD is also very useful for measuring thermal unfolding transitions, which can be measured quantitatively for lot-to-lot comparisons.

Light scattering techniques provide another comparability tool. When combined with SEC, classical light scattering identifies the mass of each aggregate peak, in an absolute manner, independent of molecular shape. Dynamic light scattering directly determines the hydrodynamic size of biopharmaceuticals, which depends on both mass and conformation. It is also an excellent method for detecting trace amounts (<0.01%) of very large aggregates.

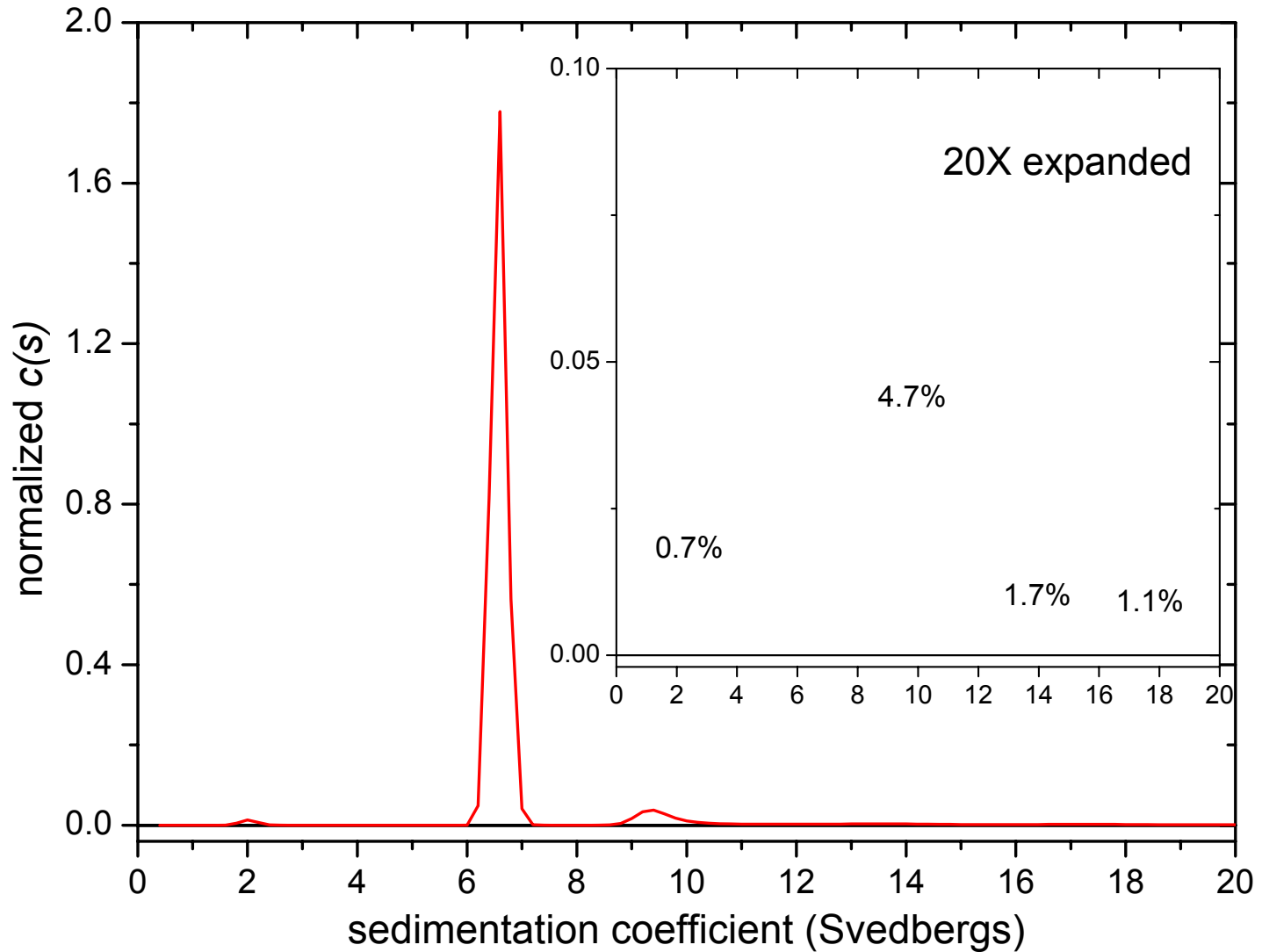
Sedimentation velocity

- The most quantitative way to compare molecular conformation
 - sedimentation coefficients are highly sensitive to small differences in conformation
 - they can be measured to a precision of 0.5% or better
- An excellent tool for measuring heterogeneity, such as that due to aggregates
 - aggregates can be detected at levels of $\sim 0.2\%$
 - covers a much broader range of sizes than any SEC column
- Can usually be done directly in formulation buffers

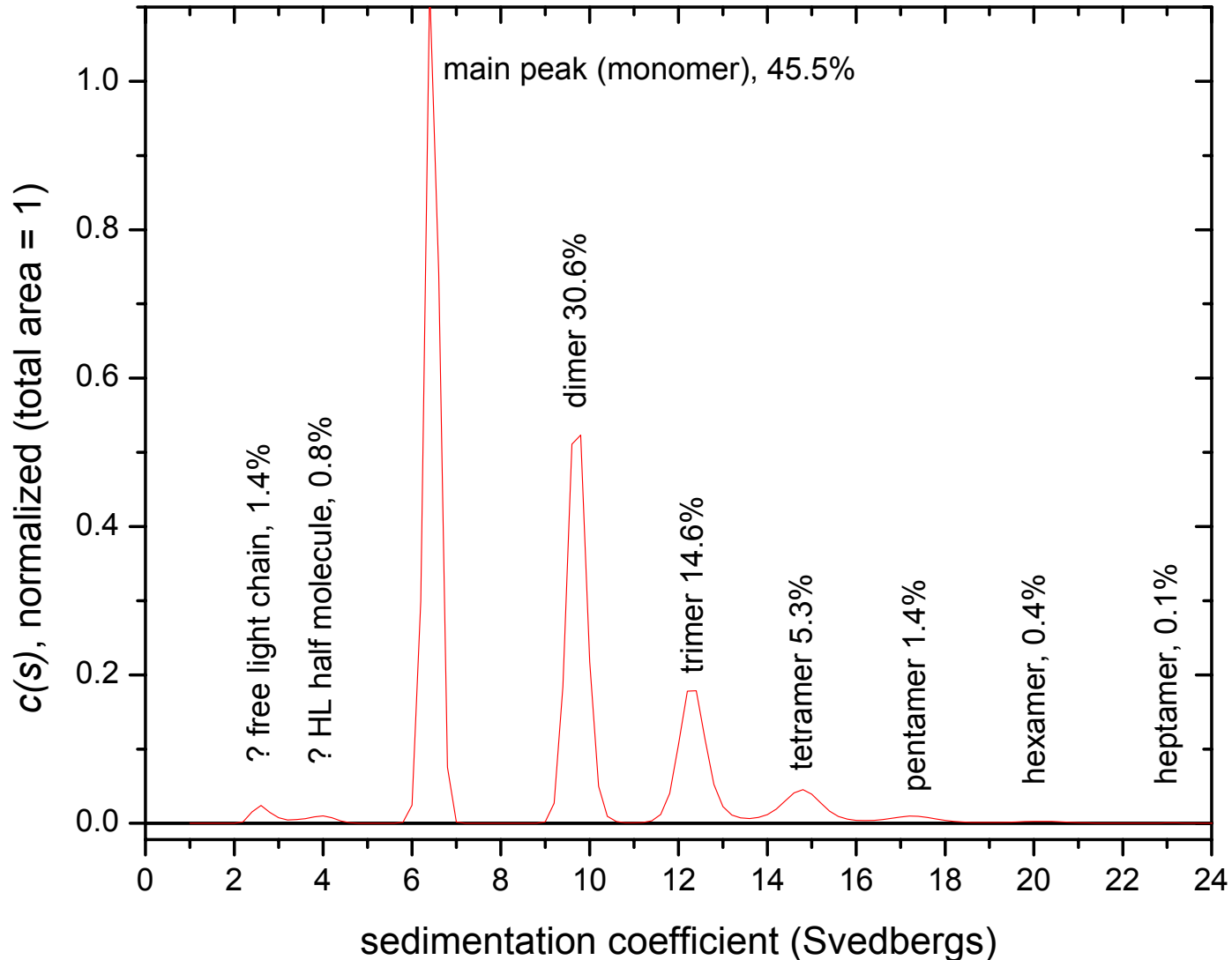
Comparability testing by sedimentation velocity: two manufacturing processes for a monoclonal antibody give main peaks with identical conformation, but aggregate levels vary lot-to-lot



A new high-resolution analysis method is excellent for resolving and quantifying minor components



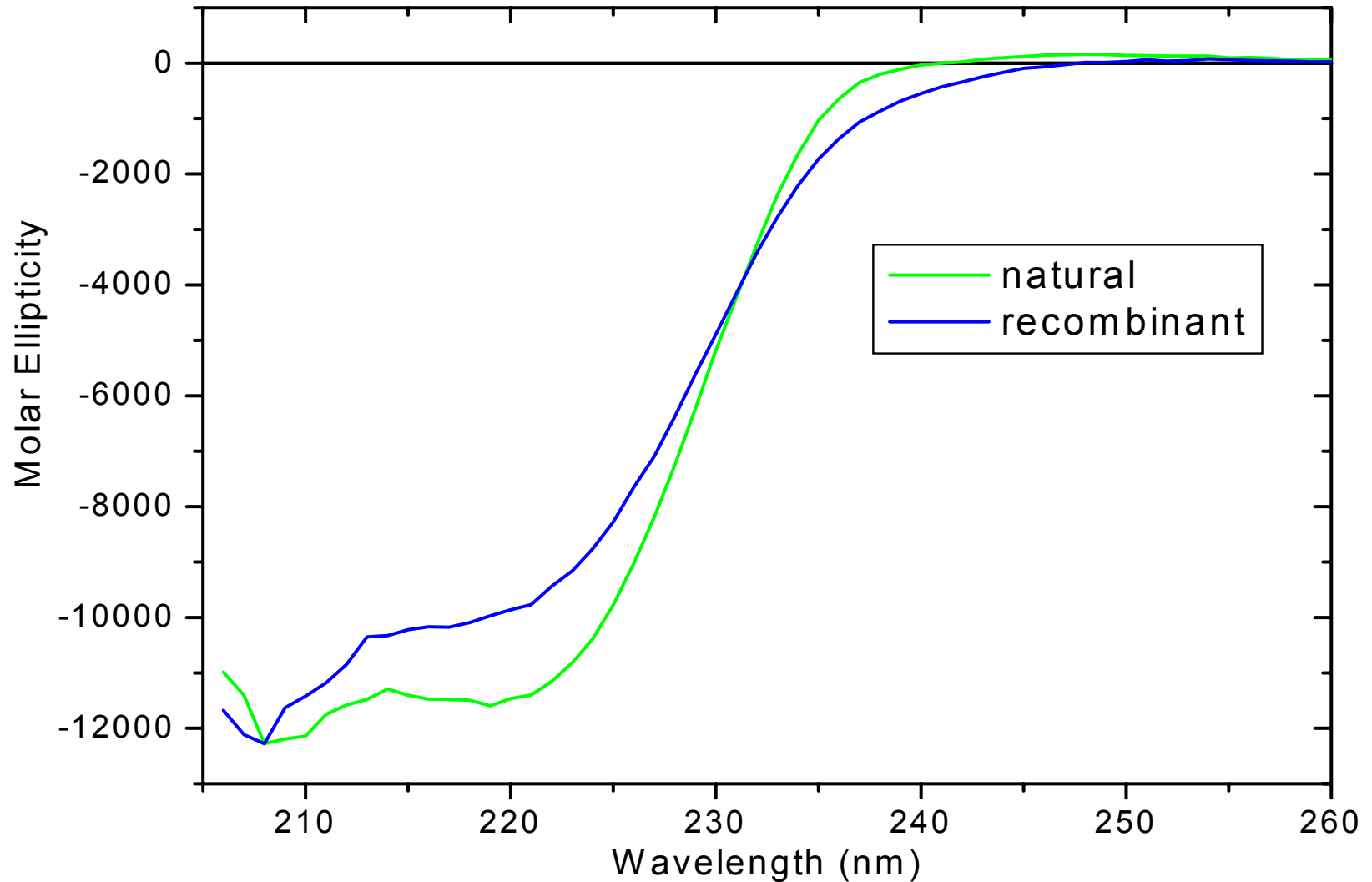
This highly-stressed antibody sample shows a well-resolved series of oligomers up to heptamer, as well as fragments.



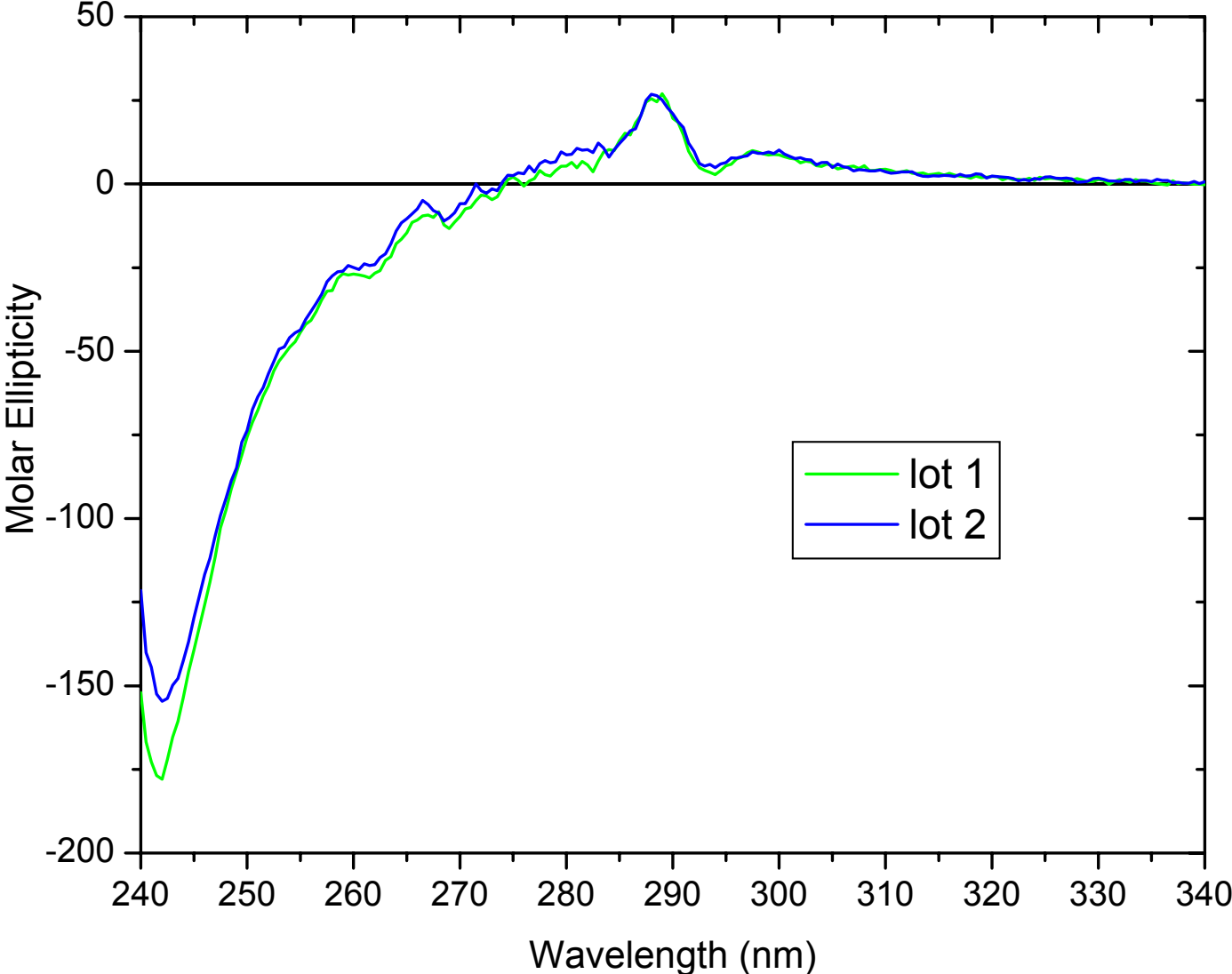
Circular dichroism (CD) spectroscopy

- Spectra provide a ‘fingerprint’ for comparing conformation
 - ‘far-UV’ spectra (190-240 nm) are sensitive to secondary structure
 - ‘near-UV’ spectra (240-340 nm) are sensitive to local tertiary structure around aromatic residues and disulfide bonds
- In our experience near-UV spectra are often more sensitive to subtle conformational differences than far-UV spectra
- Temperature-dependent spectra can quantitate and compare thermal stability
 - variations in conformation will lead to variation in the thermal unfolding profile
 - CD studies can usually be done at lower concentration than DSC, reducing problems with irreversible unfolding and thus giving data that is more relevant

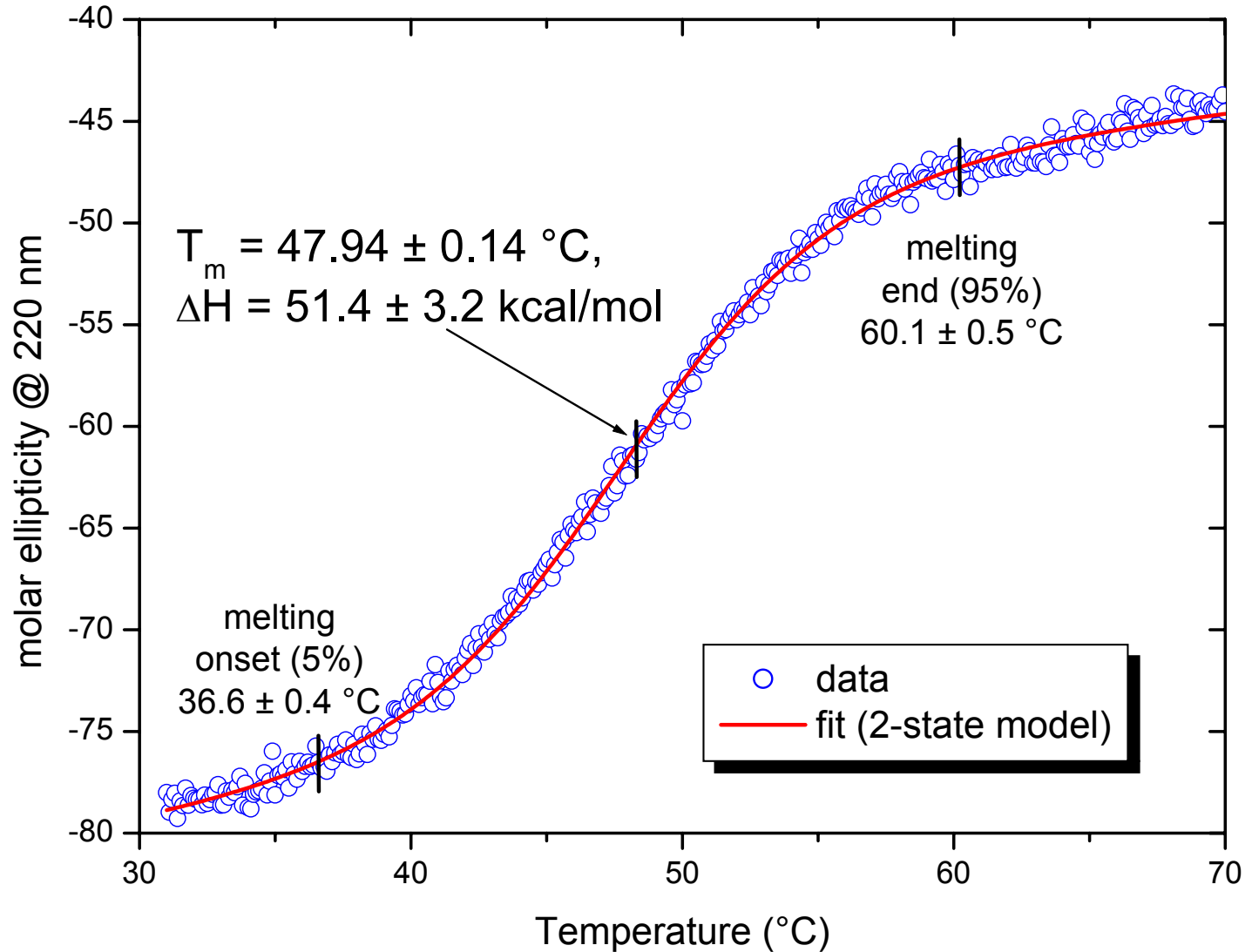
Differences in far-UV CD for natural vs. recombinant forms of an enzyme (which also have different enzymatic activity)



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

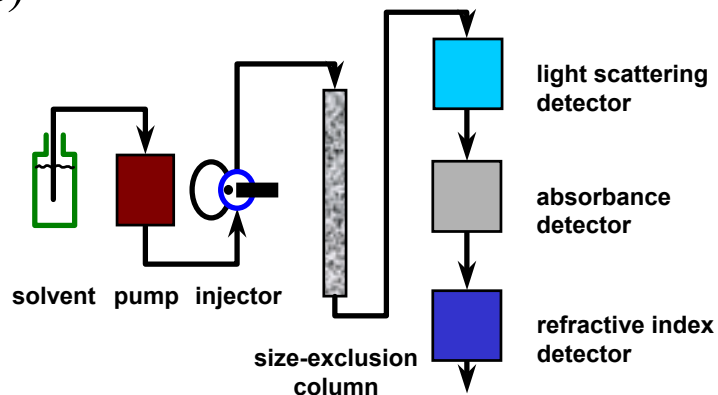


Quantitative analysis of thermal stability from CD data using proprietary A.P.L. software

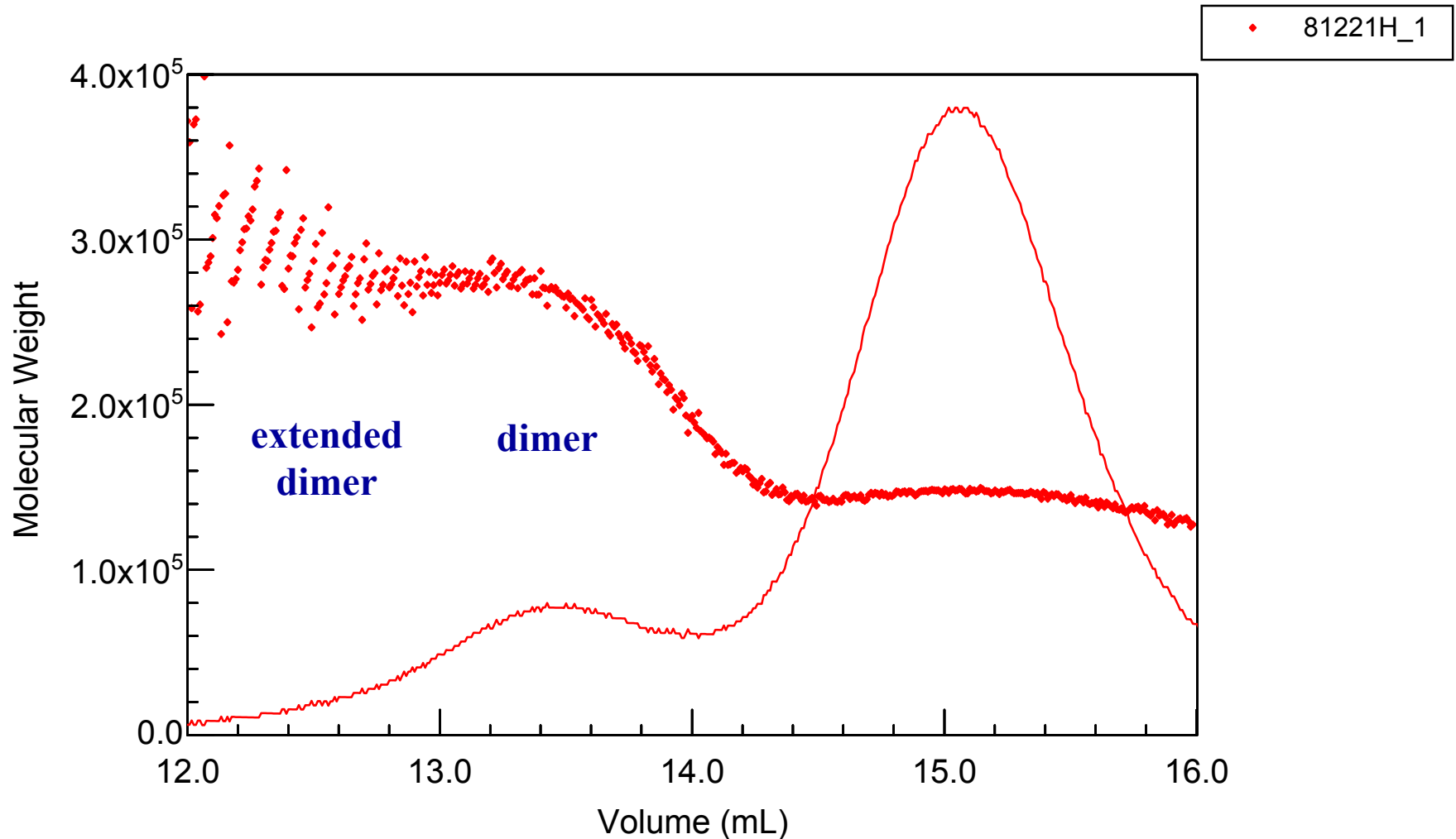


Classical light scattering used on-line with SEC

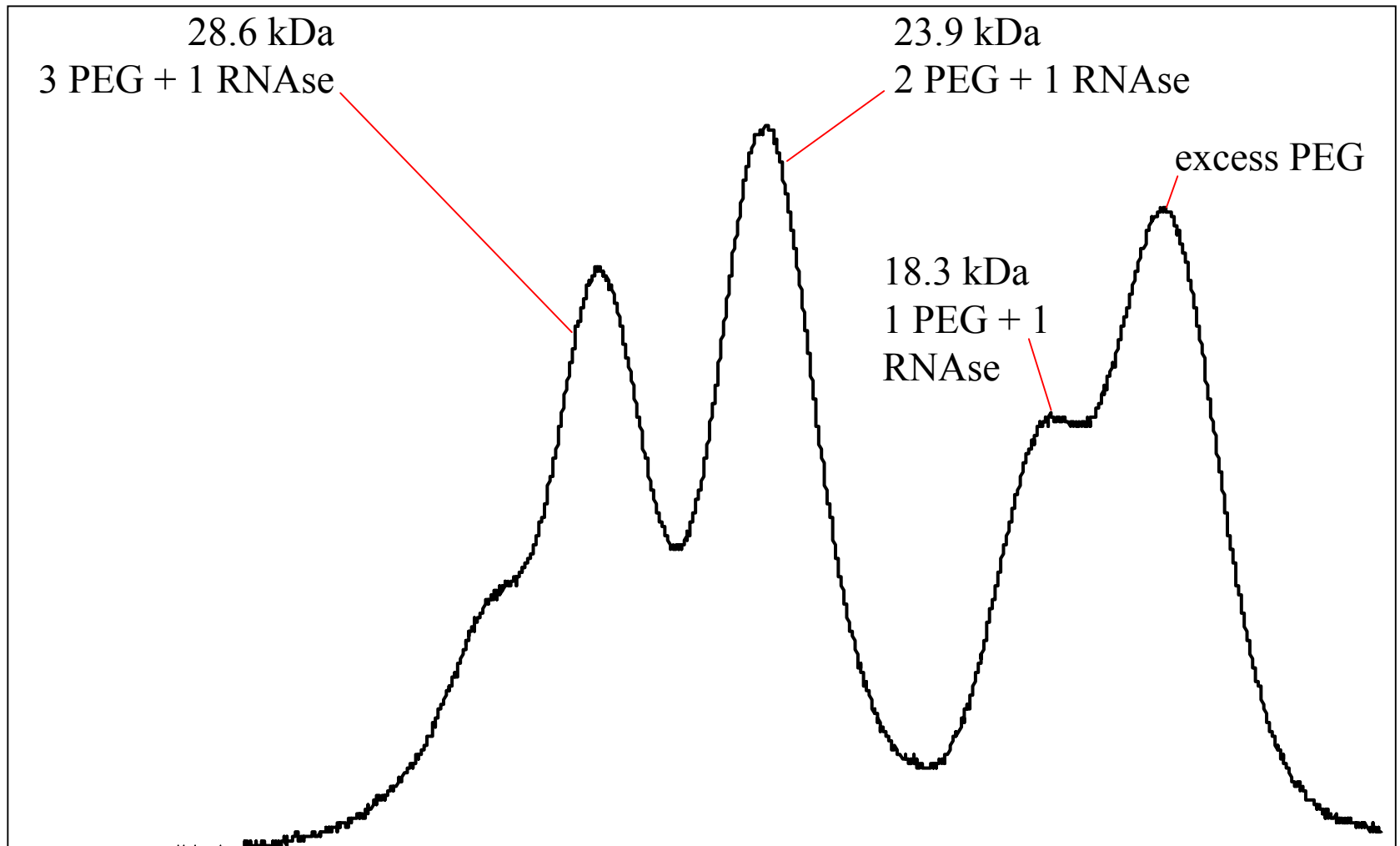
- identifies solution mass of individual peaks from SEC
- mass determination is absolute, independent of molecular shape or interactions with the column matrix
- for comparability studies primary use is demonstrating that the mass of aggregates is comparable (same oligomers are present)
- can measure degree of conjugation for PEGylated proteins
- can distinguish multiple conformations with same mass
 - can distinguish different forms of dimer (see next slide)
 - can distinguish dimer from unfolded monomer (which usually elute at similar positions)



SEC with on-line classical light scattering shows an antibody sample contains only monomer and dimer, but two conformations of dimer are present



Analysis of products from a 5:1 reaction of 5 kDa PEG with ribonuclease A (13.7 kDa)

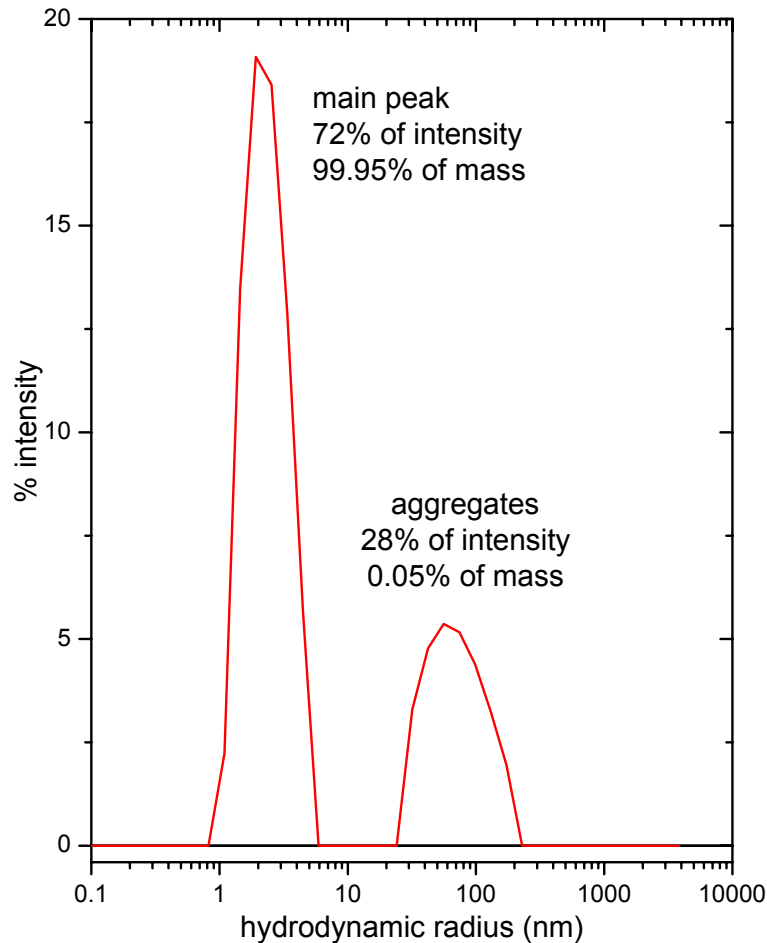


Dynamic light scattering

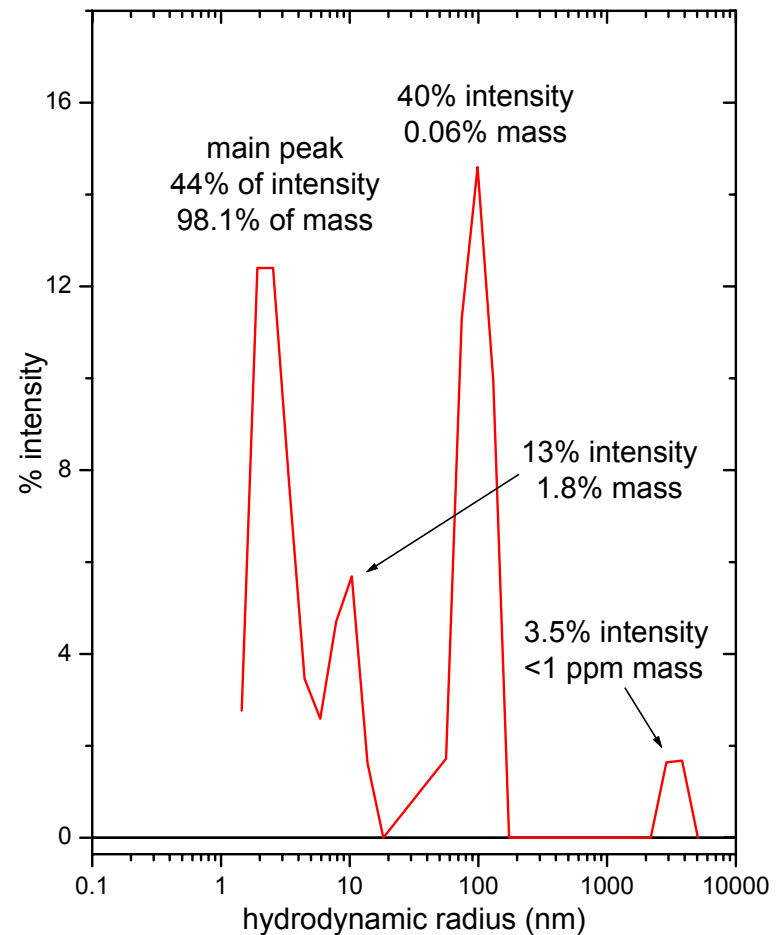
- measures Brownian motion of molecules, which is directly related to their hydrodynamic size (which depends on both mass and conformation)
- data are usually converted to a distribution of hydrodynamic radius graph
- excellent for detecting trace amounts (0.01% to <1 p.p.m.) of very large aggregates
- covers extremely large range of sizes at one time
- works in wide range of solvent conditions
- useful for continuous monitoring of aggregation at elevated temperatures (accelerated stability)

DLS can easily detect large aggregates (~ 100 nm) at levels of $\sim 0.01\%$ and very large ones (~ 1 μm) at ~ 1 ppm

data for “good” lot



data for “bad” lot



What about detecting smaller aggregates with DLS? They are generally not resolved as separate species, but they may shift the distribution toward larger sizes (and increase total scattered light)

