Analyzing Aggregates of Different Sizes and Types: SEC *vs.* Analytical Ultracentrifugation *vs.* Light Scattering

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Outline

- ★ Different types of oligomers and aggregates
- ★ Some problems with SEC
- ★ Applications and advantages/drawbacks of:
  1. sedimentation velocity
  2. sedimentation equilibrium
  3. SEC with on-line classical light scattering
  4. dynamic light scattering
- ★ Some recommended applications for these biophysical methods

I can't really cover this broad topic in 20 min so I'm going to omit some background and summaries but…

1. See poster P-30-W
2. An expanded presentation can be downloaded from the APL web site at http://www.ap-lab.com/further_reading.htm
3. Also see our web site [www.ap-lab.com] for more method background and applications

The word "aggregate" covers a wide spectrum of types and sizes of associated states

1. rapidly-reversible non-covalent small oligomers (dimer, trimer, tetramer…)
2. irreversible non-covalent oligomers
3. covalent oligomers (*e.g.* disulfides)
4. "large" aggregates (> 10-mer)
   - ★ could be reversible if non-covalent
5. "very large" aggregates (diameter ~50 nm to 3 μm)
   - ★ could be reversible if non-covalent
6. visible particulates
   - ★ probably irreversible

Whether aggregates are "irreversible" or "reversible" depends on the context

- solvent components
  - salts, sugars, other excipients
  - organic modifiers (alcohols, acetonitrile)
- pH
- temperature
- how long you wait

The kinetics of non-covalent association and dissociation (half-times) can vary from milliseconds to days

- metastable oligomers with lifetimes of hours to days occur frequently
  - for an antibody example see J.M.R. Moore *et al.* (1999) *Biochemistry* **38**: 13960-13967
- it may take hours to days for a protein to re-equilibrate its association after a change in concentration, solvent conditions or temperature

Any given protein sample may contain multiple aggregate forms with widely-varying properties

- ★both covalent and non-covalent
- ★different types of non-covalent:
  - ❉both rapidly-reversible and irreversible
  - ❉both rapidly- and slowly-dissociating
- ★even the same size of oligomer may exist in multiple forms

We must be aware that any given analysis technique may not detect all the aggregate sizes or types that are present

- Separation methods (SEC, sedimentation velocity) typically will not resolve individual oligomer species for a system in rapidly-reversible association equilibrium
  - for associations to oligomers larger than dimer, from theory multiple peaks may be seen even for infinitely-fast kinetics, but those peaks usually do not represent single oligomers
  - what is detected may depend on the rates of association and dissociation compared to the speed of separation

The measurement technique itself may perturb the distribution of aggregates that was initially present

★ dissociation or loss of aggregates can be caused by:

1. dilution ← **SEC**
2. change of solvent conditions ← **SEC**
3. adsorption to surfaces (*e.g.* column resin) ← **SEC**
4. physical filtration (*e.g.* column frit) ← **SEC**
5. physical disruption by shear forces ← **SEC**

★ creation of new aggregates can be caused by:

1. change of solvent conditions
2. surface or shear-induced denaturation
3. concentration on surface of column resin (IEX)

SEC

problems & issues with the industry workhorse

Some shortcomings of SEC

1. **change in solvent may change the aggregate distribution**
2. **limited range of sizes; larger aggregates often unresolved**
3. often does not detect rapidly-reversible non-covalent association
4. aggregates may be filtered out by column
5. elution position not reliable indicator of molecular mass
6. limited sensitivity; difficult to resolve and detect species at or below ~0.1%

SEC issue #1: solvent-induced changes

- Achieving good resolution and high recovery often requires extreme solvent conditions that can alter the distribution of non-covalent aggregates
  - high ionic strength
  - addition of organic modifiers (alcohol, acetonitrile)

I have seen cases where the SEC elution buffer completely dissociated the non-covalent aggregates, and also cases where it induced substantial amounts of new aggregates.

The things chromatographers are taught to do to achieve "good" and robust methods often exacerbate this problem!

1. want to add organics or salts to improve resolution, recovery, peak symmetry
2. want to pre-dilute the samples with the elution buffer ← **this can cause false conclusions!**
3. want to validate the method by spiking with pure aggregate
   - ★ but you cannot a pure sample of non-covalent aggregates
   - ★ end up optimizing and validating for <u>covalent</u> aggregates

"Good" chromatography is often in direct conflict with good <u>relevance</u> for measuring non-covalent aggregates!

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SEC issue #2: limited size range

- ★ Often the largest aggregates are not resolved, and elute in a single peak or shoulder at or near the exclusion limit
- ★ Therefore we often cannot tell whether the large aggregate species present in different formulations or different manufacturing lots are actually similar in size

Thus SEC alone may not distinguish samples with significant differences (*e.g.* immunogenicity)

SEC issue #5: incorrect masses

- inaccuracy due to dependence on molecular shape and/or undesirable interactions with the column matrix
  - many, many examples of wrong stoichiometry for native proteins
- absolute accuracy may be unimportant; often all we want is fraction main peak

But is that "dimer" peak really a dimer, or is it a partially-unfolded monomer (which might be much more immunogenic)?

Analytical Ultracentrifugation (AUC)

sedimentation equilibrium

sedimentation velocity

Both AUC methods are sensitive to all types of aggregates, but…

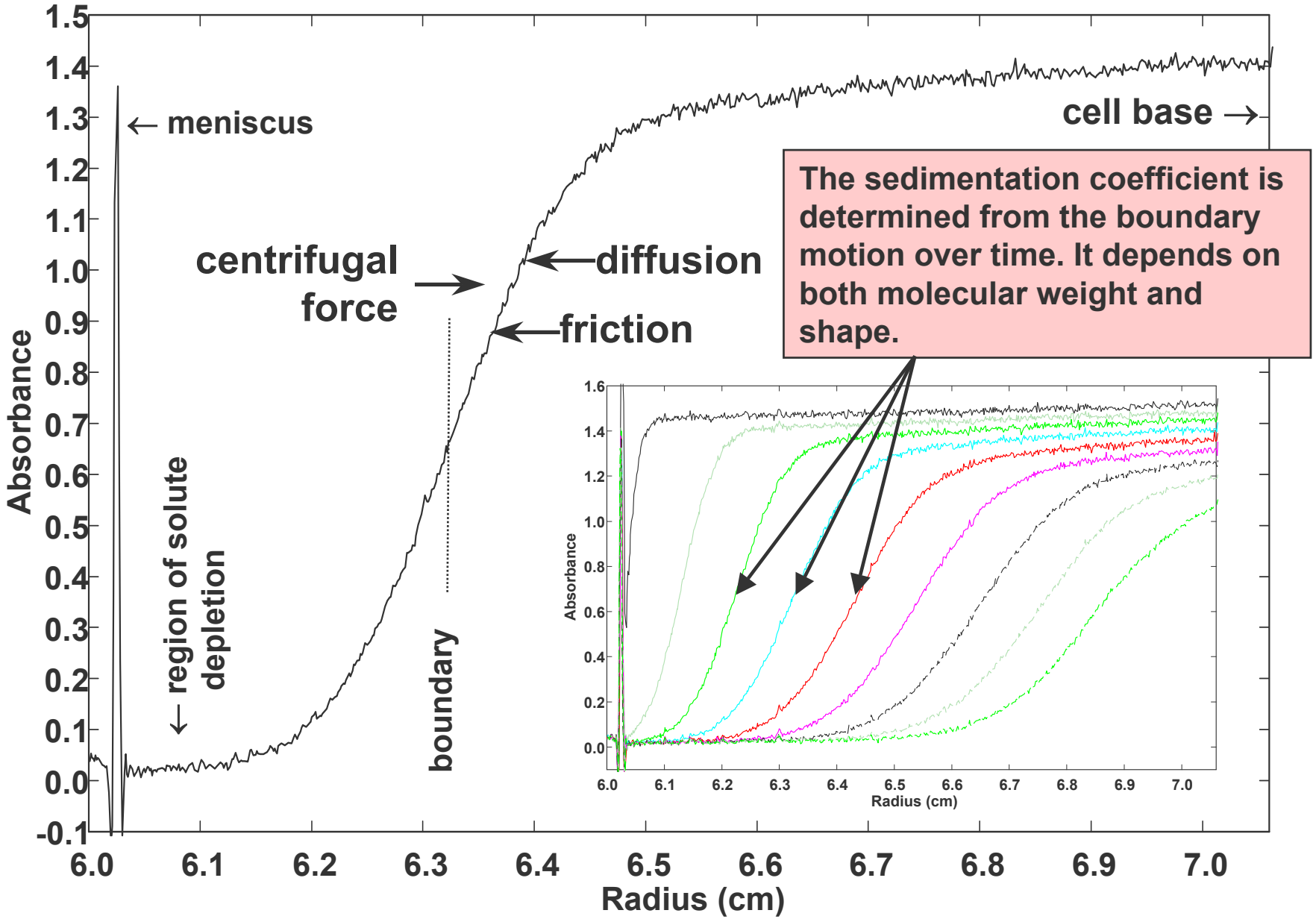
- ★ Sedimentation equilibrium is primarily a thermodynamic tool for studying rapidly-reversible self-association (equilibrium constants and stoichiometry)
- ★ Sedimentation velocity is much more a separation method and is particularly useful for characterizing irreversible (covalent or non-covalent) and relatively stable reversible non-covalent aggregates

Both sedimentation methods are "first principle" methods

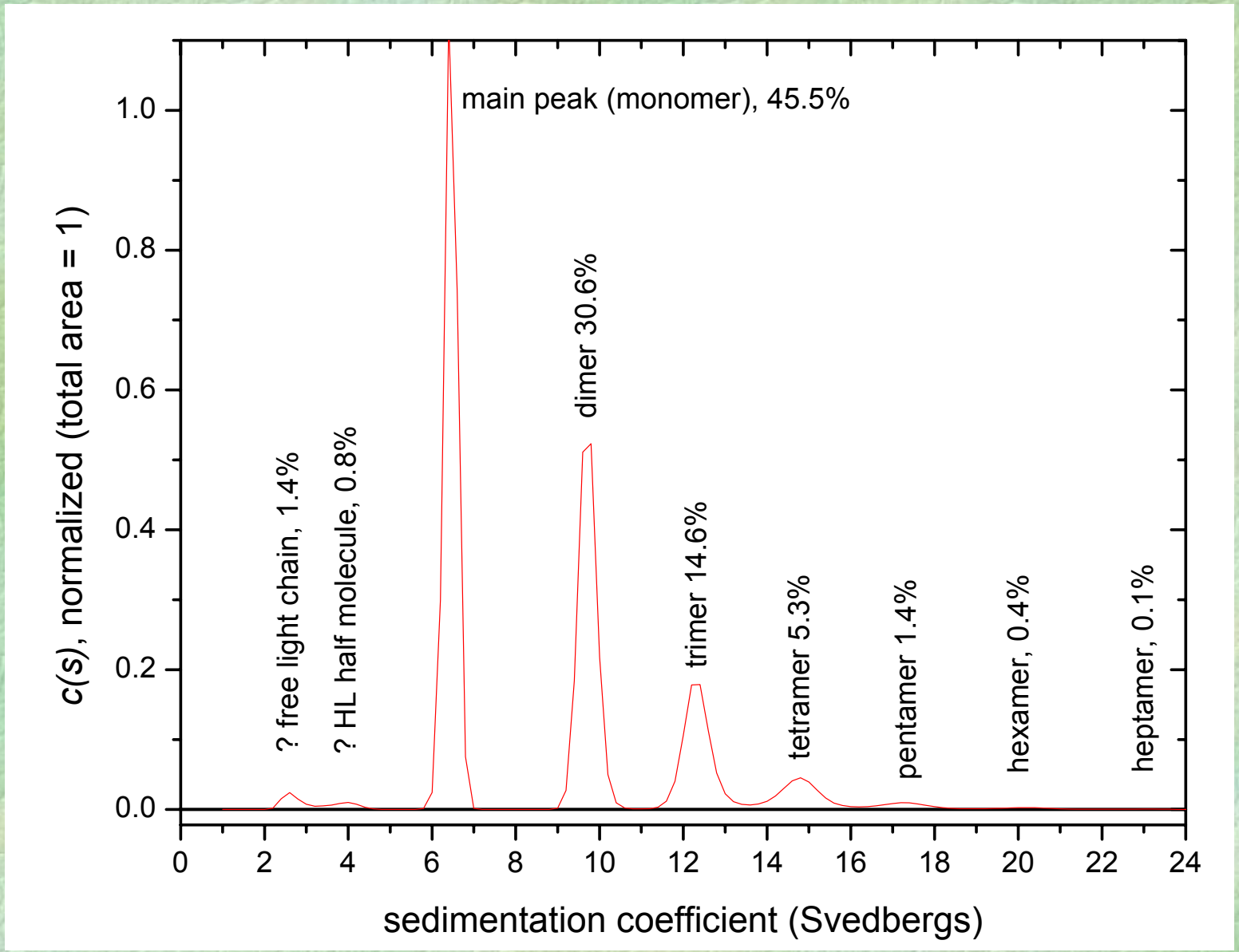
- based on fundamental physical laws
- theory is well understood
  - true for dilute solutions; concentrations > 10 mg/ml become complex and difficult
- require no standard molecules for calibration
  - calibration is based only on fundamental units of distance, time, and temperature

Sedimentation velocity

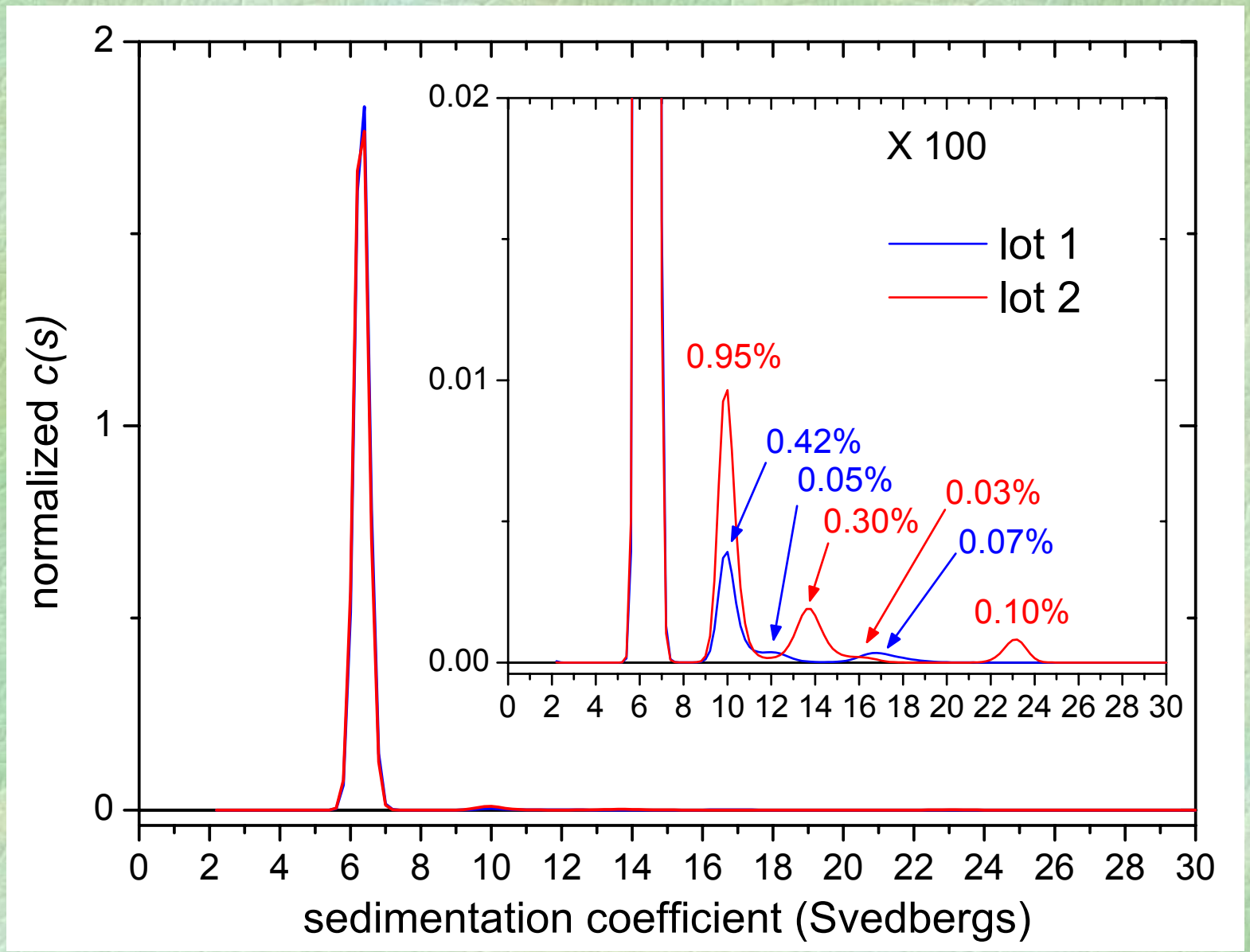
The fundamentals of sedimentation velocity



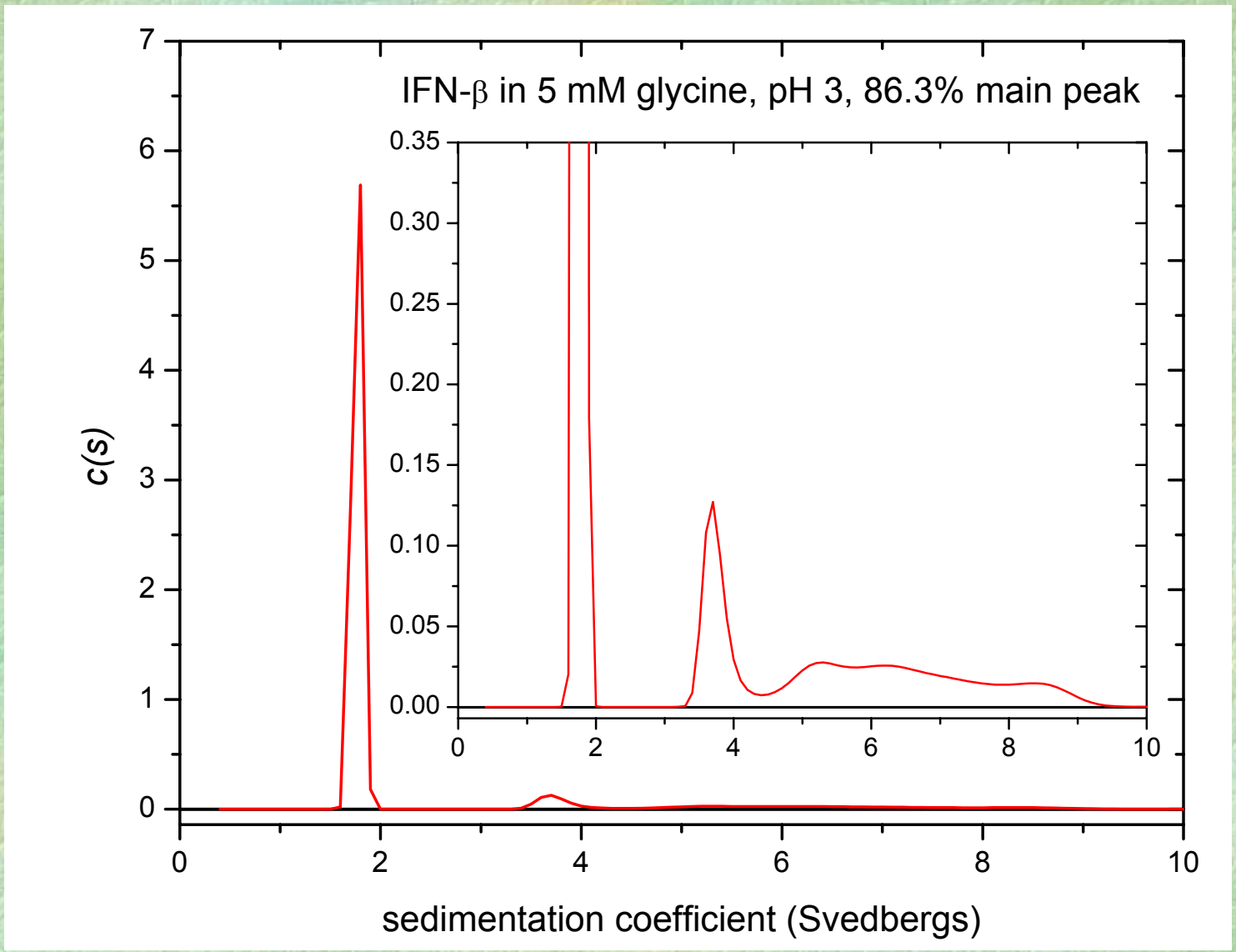
High resolution analysis of a highly stressed antibody sample resolves 6 aggregate peaks plus 2 fragments



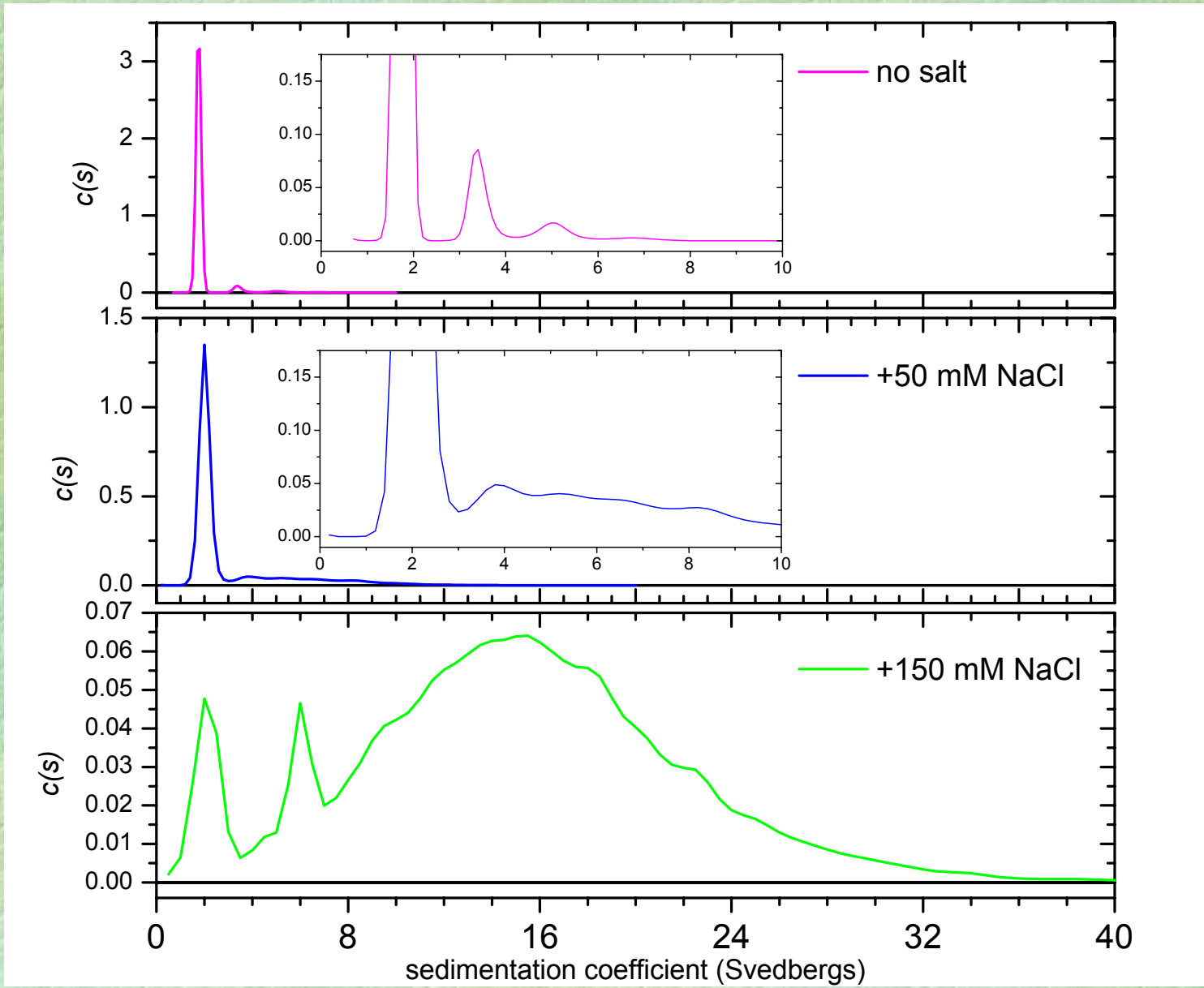
Comparability of a monoclonal antibody; detecting aggregate peaks at levels below 0.05%



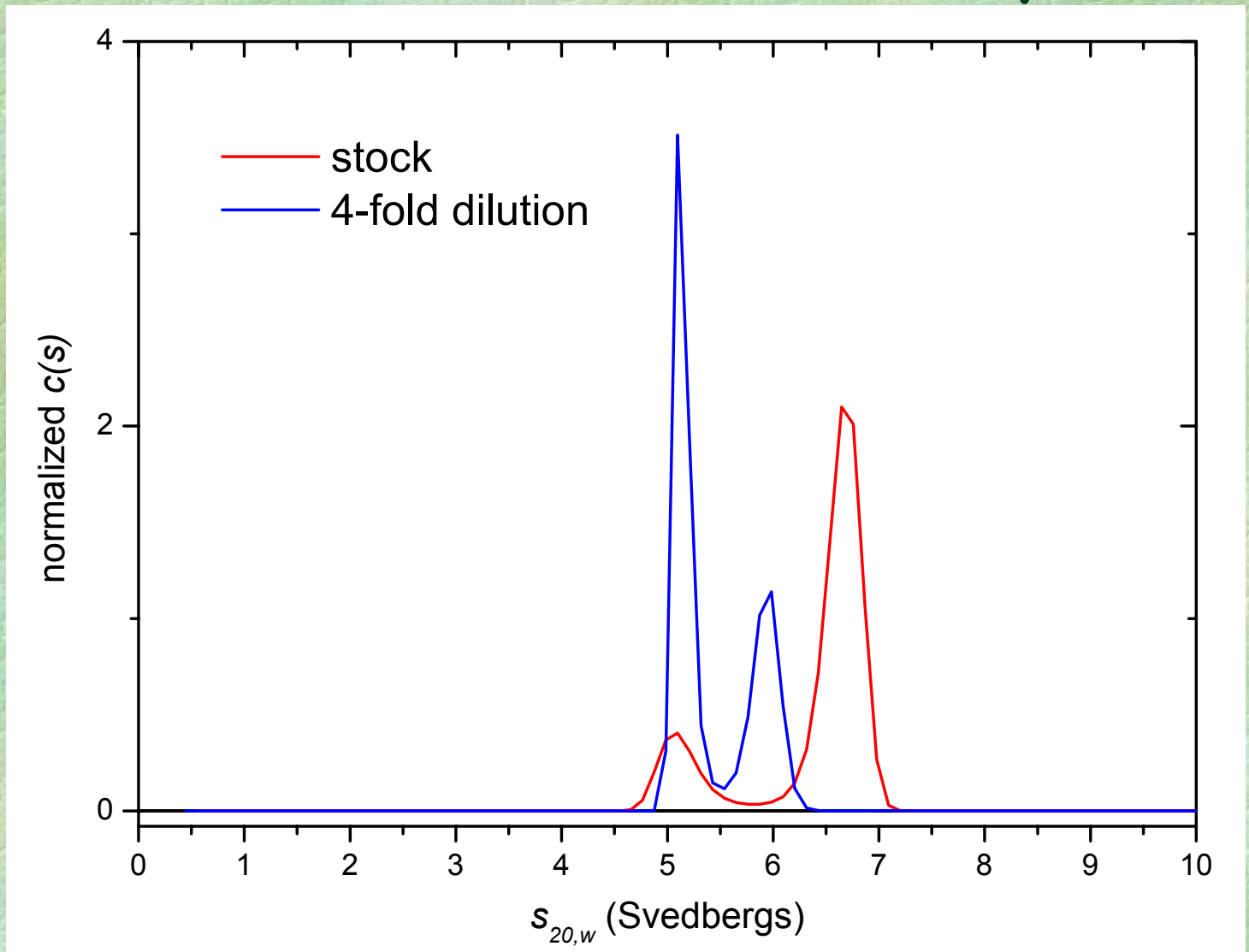
This interferon-β sample is 13.7% non-covalent aggregate; by SEC (in 30% acetonitrile + 0.2% TFA) it would be pure monomer



Adding NaCl to interferon-β formulations leads to a broad distribution non-covalent aggregates out to ~100-mers



We must study a range of concentrations to check for reversible association; this is a monomer-dimer-tetramer system



Strengths of sedimentation velocity

1. high resolution (generally better than SEC)
2. covers very large range of masses in a single experiment (much larger than SEC)
3. detects both covalent and non-covalent aggregates
4. generally can be done directly in formulation buffers
5. little dilution of sample (~25%)
6. strong theory; "first principles" method

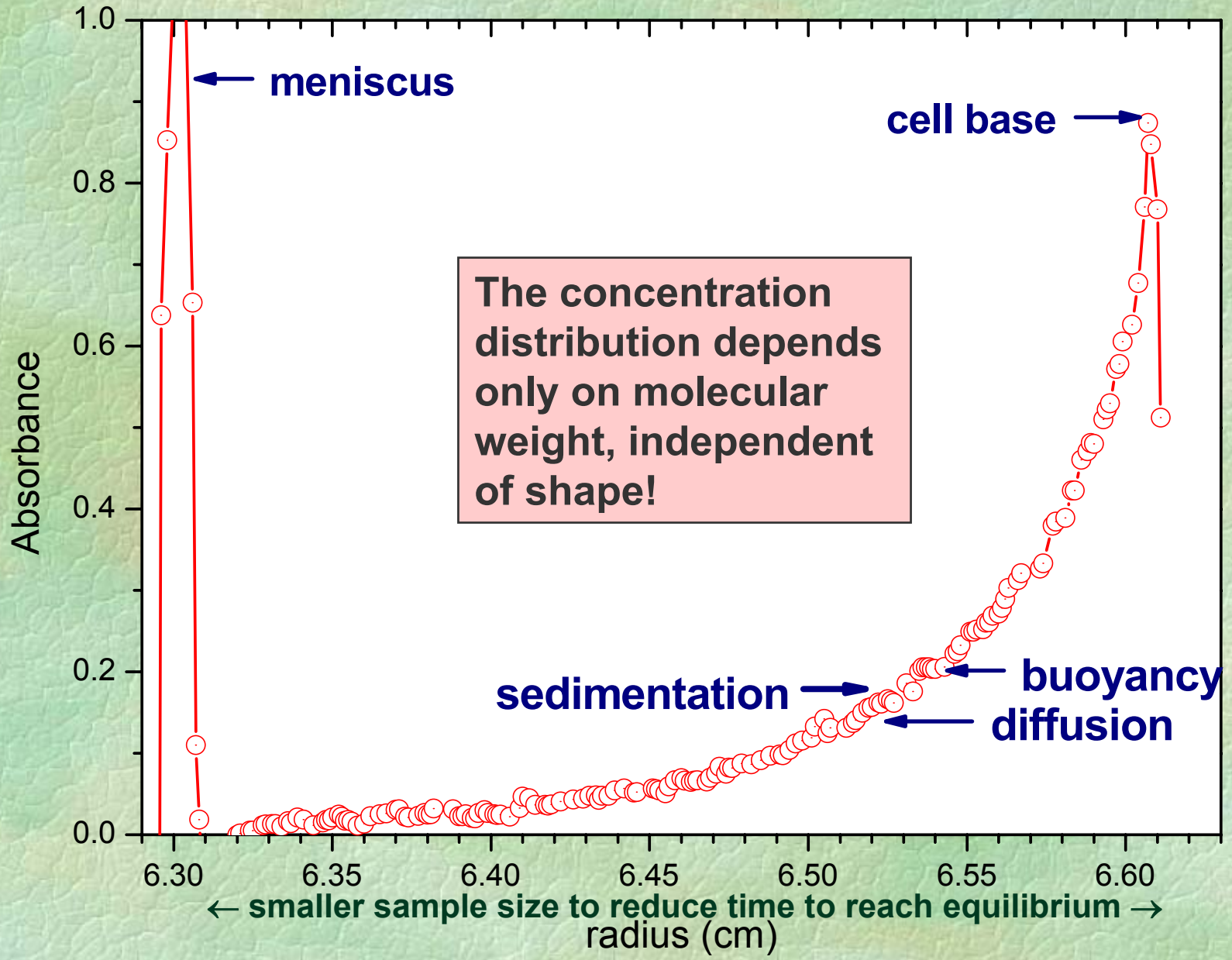
Weaknesses of sedimentation velocity

1. low throughput; often 3-7 samples/day
2. equipment and data analysis not automated like HPLC; labor intensive
3. expensive equipment (~250-300 K$)
4. requires substantial training

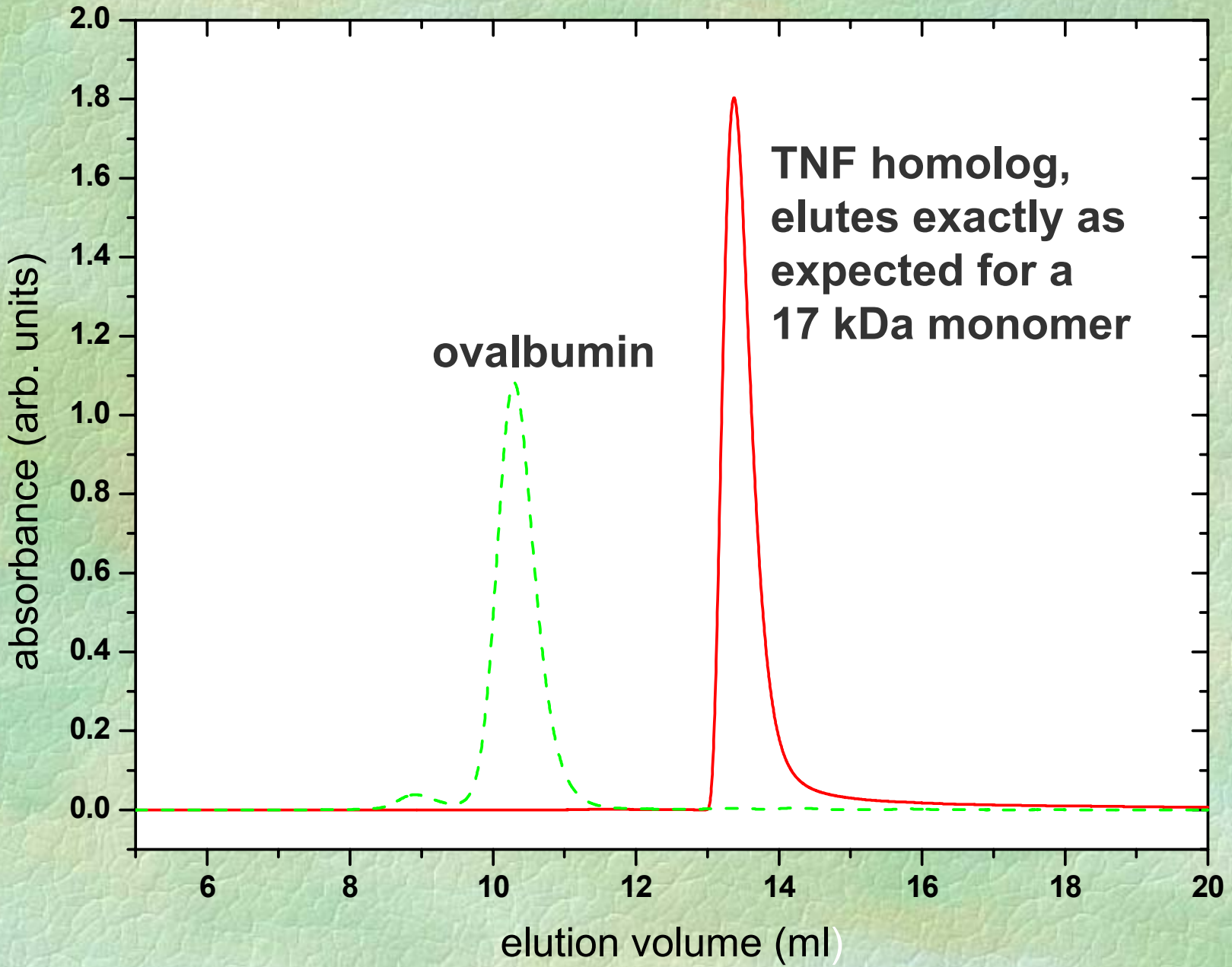
Sedimentation velocity can not replace SEC, but it is an excellent tool to test whether SEC is missing important features. It can also serve as a "gold standard" to help improve SEC methods.

Non-covalent self-association by sedimentation equilibrium

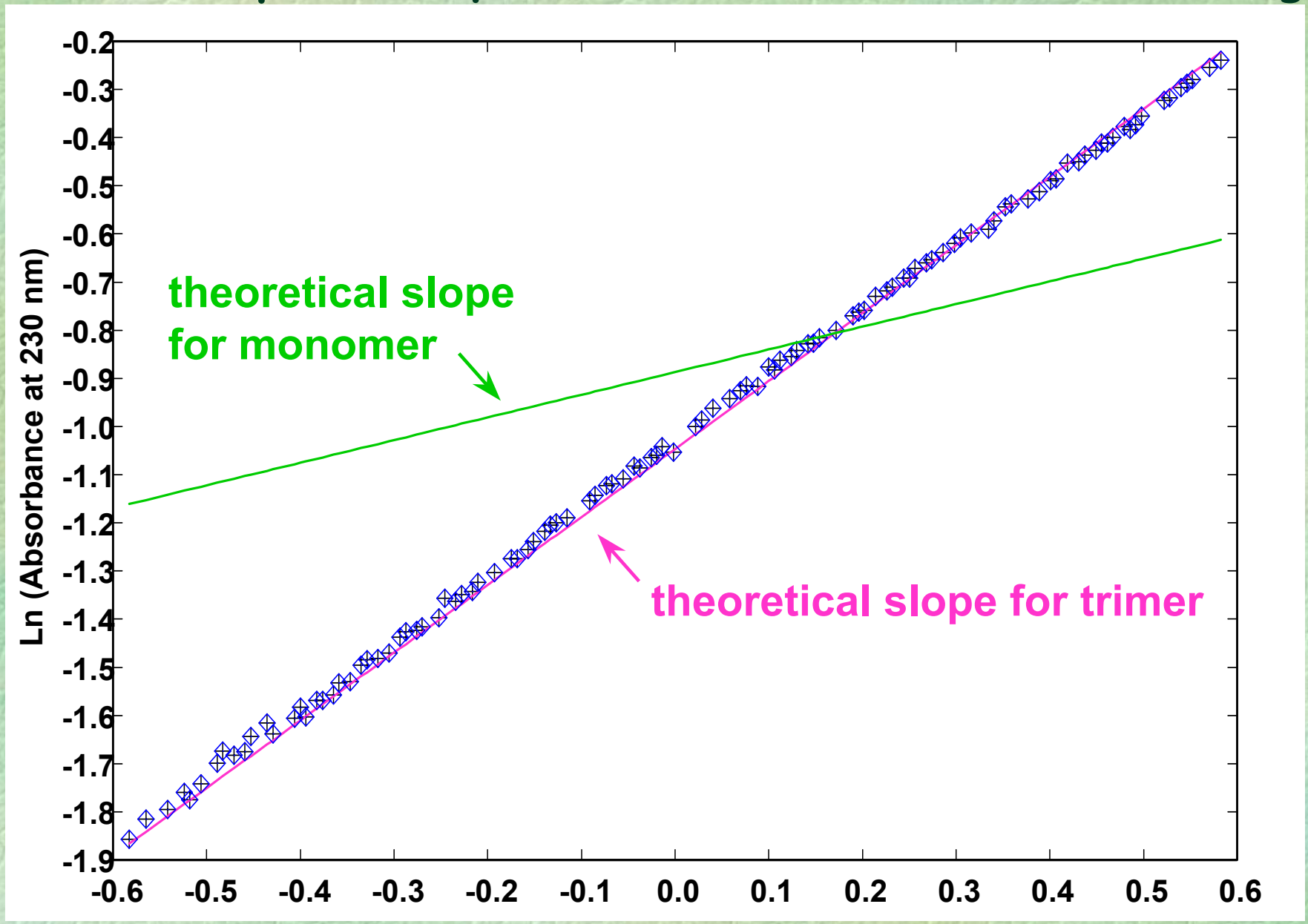
The fundamentals of sedimentation equilibrium



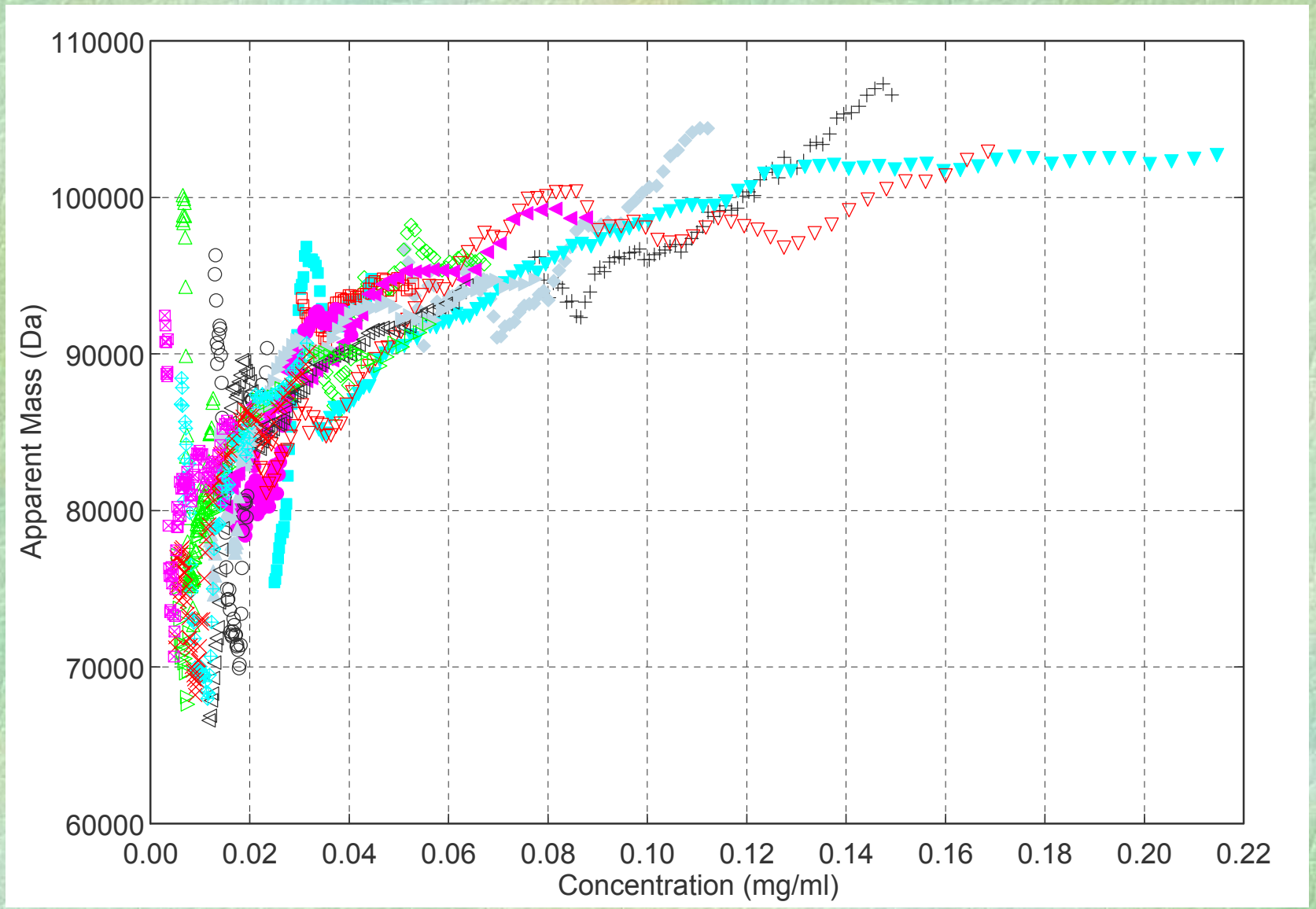
Size-exclusion chromatography of a TNF homolog



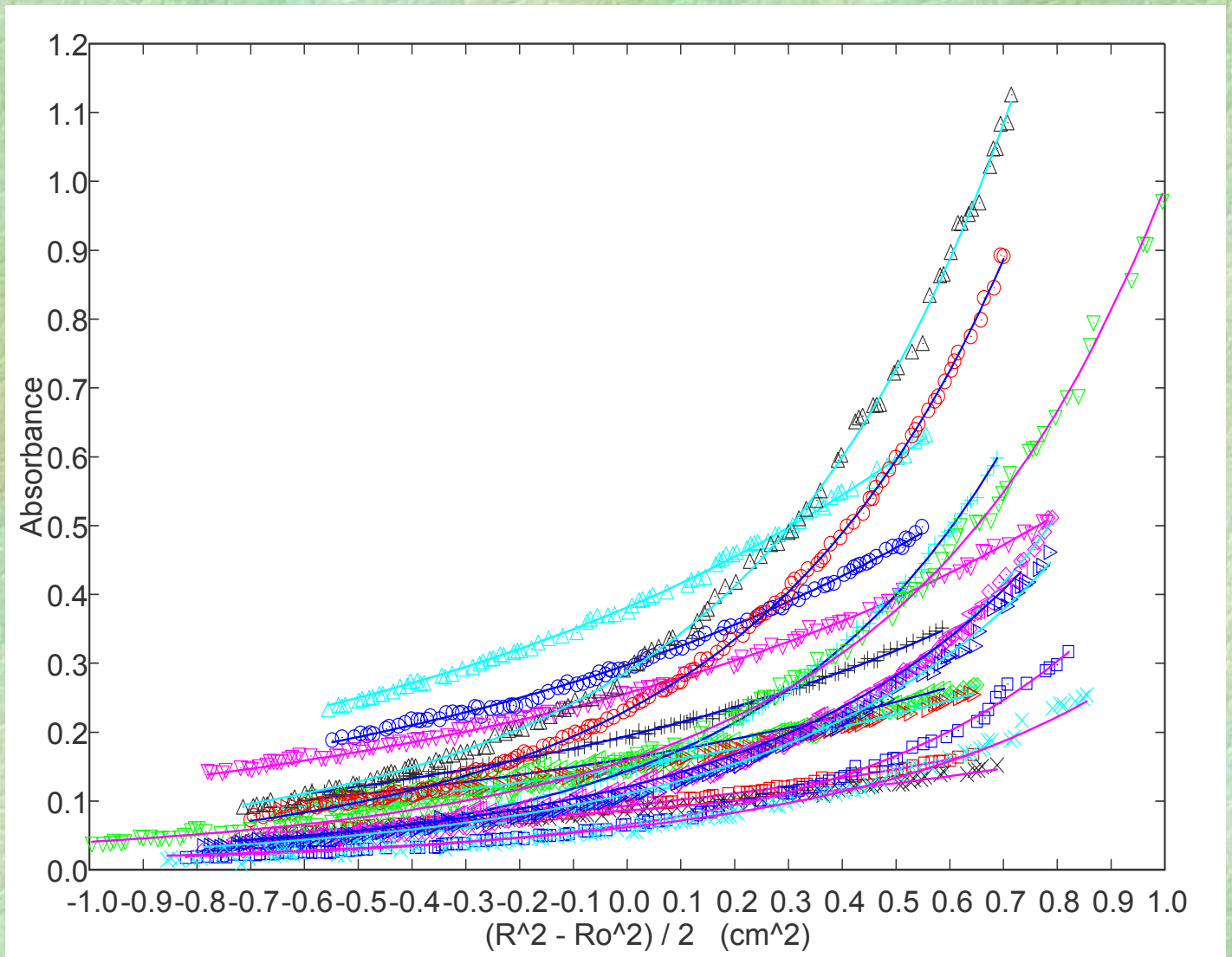
Linearized plot of equilibrium data for the TNF homolog



Apparent mass *vs.* concentration for the soluble extracellular domain of the atrial natriuretic peptide receptor (monomer mass 58 kDa)



Global analysis of all 18 samples gives a good fit to a monomer-dimer association model with K_d = 520 +/- 20 nM (ΔG = -8570 +/- 25 cal/mol)



Strengths/weaknesses of sedimentation equilibrium

Strengths

1. equilibrium method; all forms of association are detected
2. wide choice of solvent conditions
3. strong theory; "first principles" method

Weaknesses

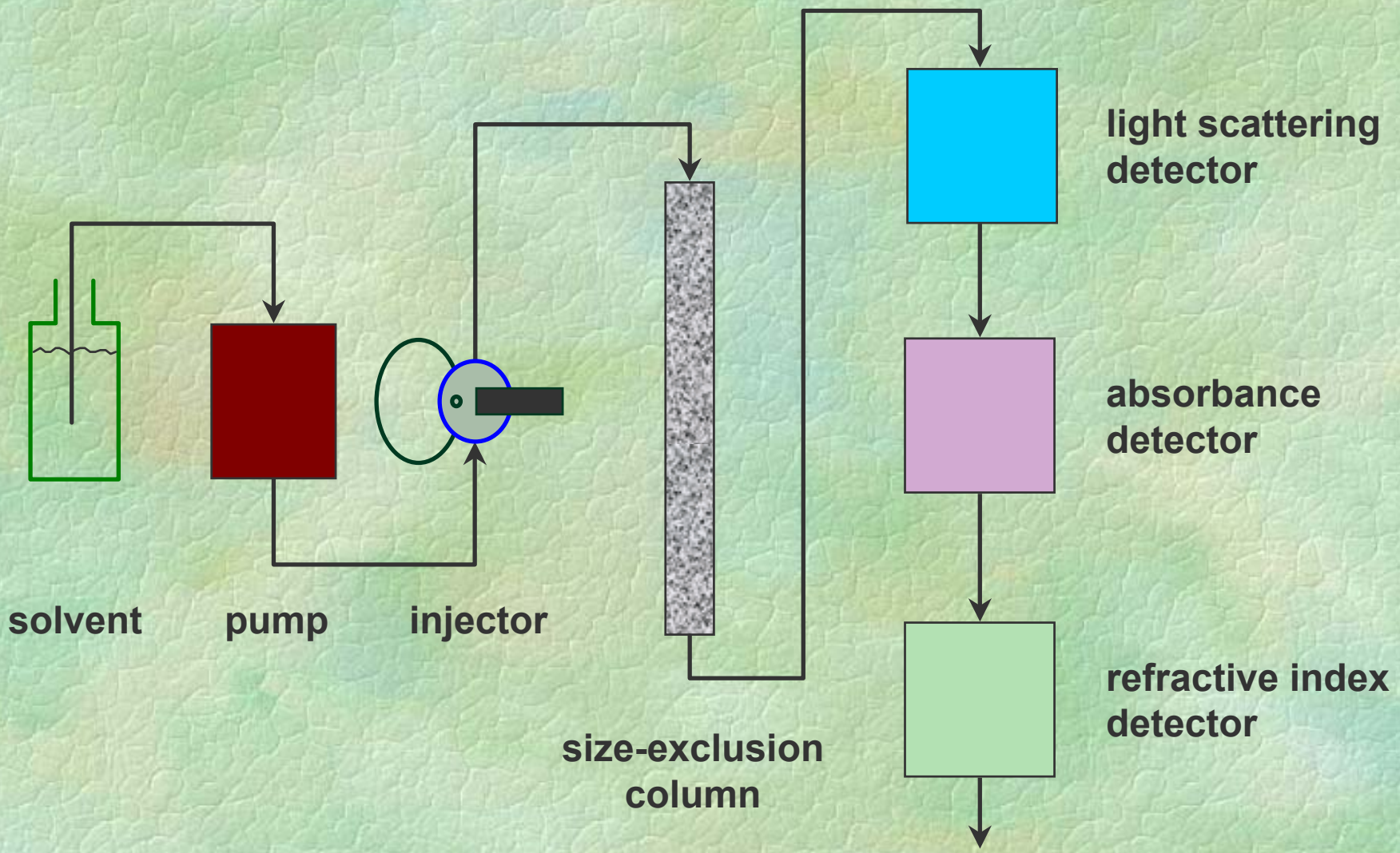
1. difficult to quantitate irreversible aggregates
2. low throughput (9-21 samples/day typical)
3. expensive equipment
4. difficult data analysis
5. requires highly trained personnel

How can we validate AUC methods for non-covalent aggregates given we can't spike in pure species?

- ★ In part these methods are validated by over 60 years of experience and tens of thousands of publications
- ★ More significantly, we have a theory, and we can create test data sets *in silico* (including realistic noise levels) and submit them to the analysis software to evaluate the ability to quantitate various species

"Classical" or "static" light scattering used on-line with SEC

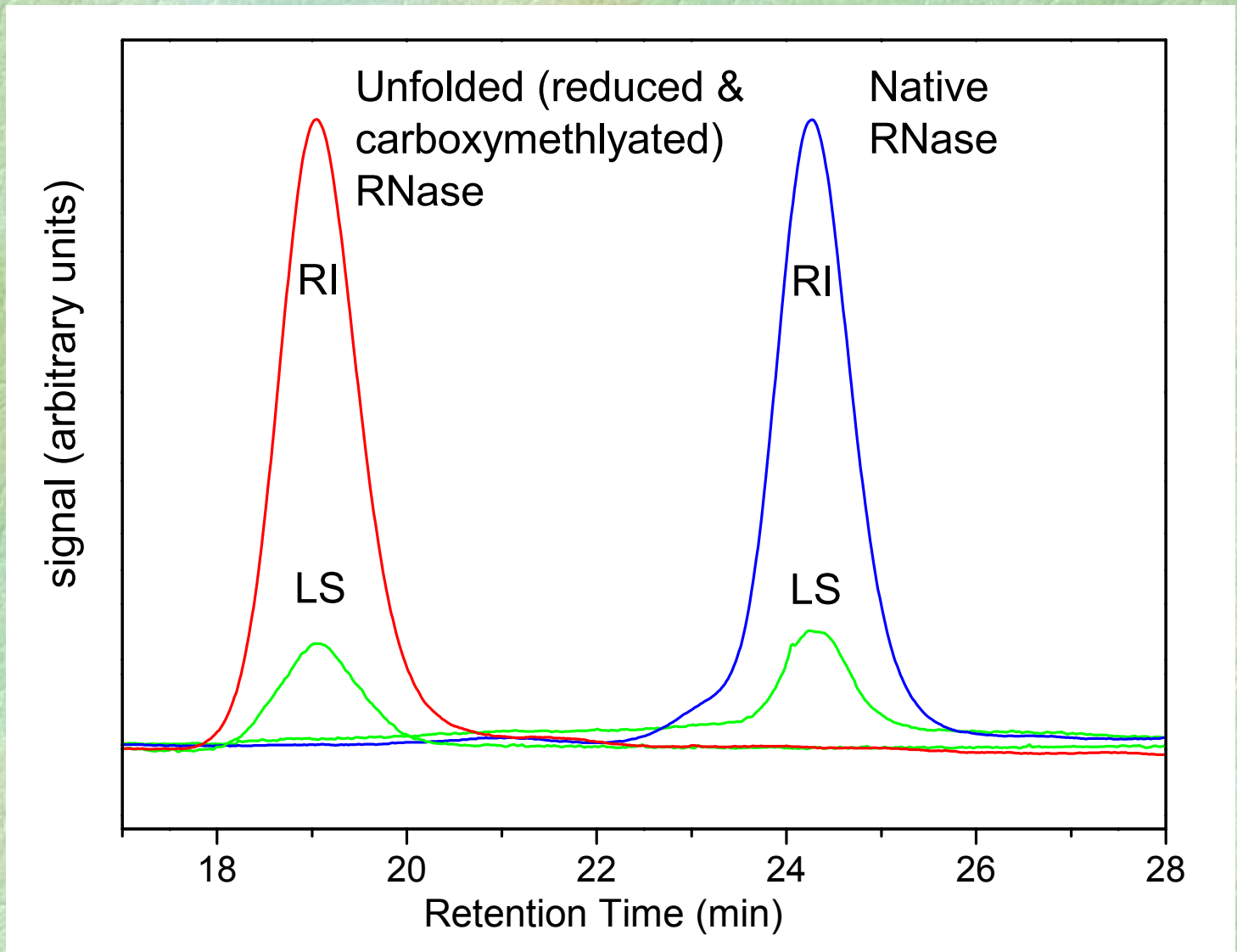
Typical setup for size-exclusion chromatography with on-line light scattering detection



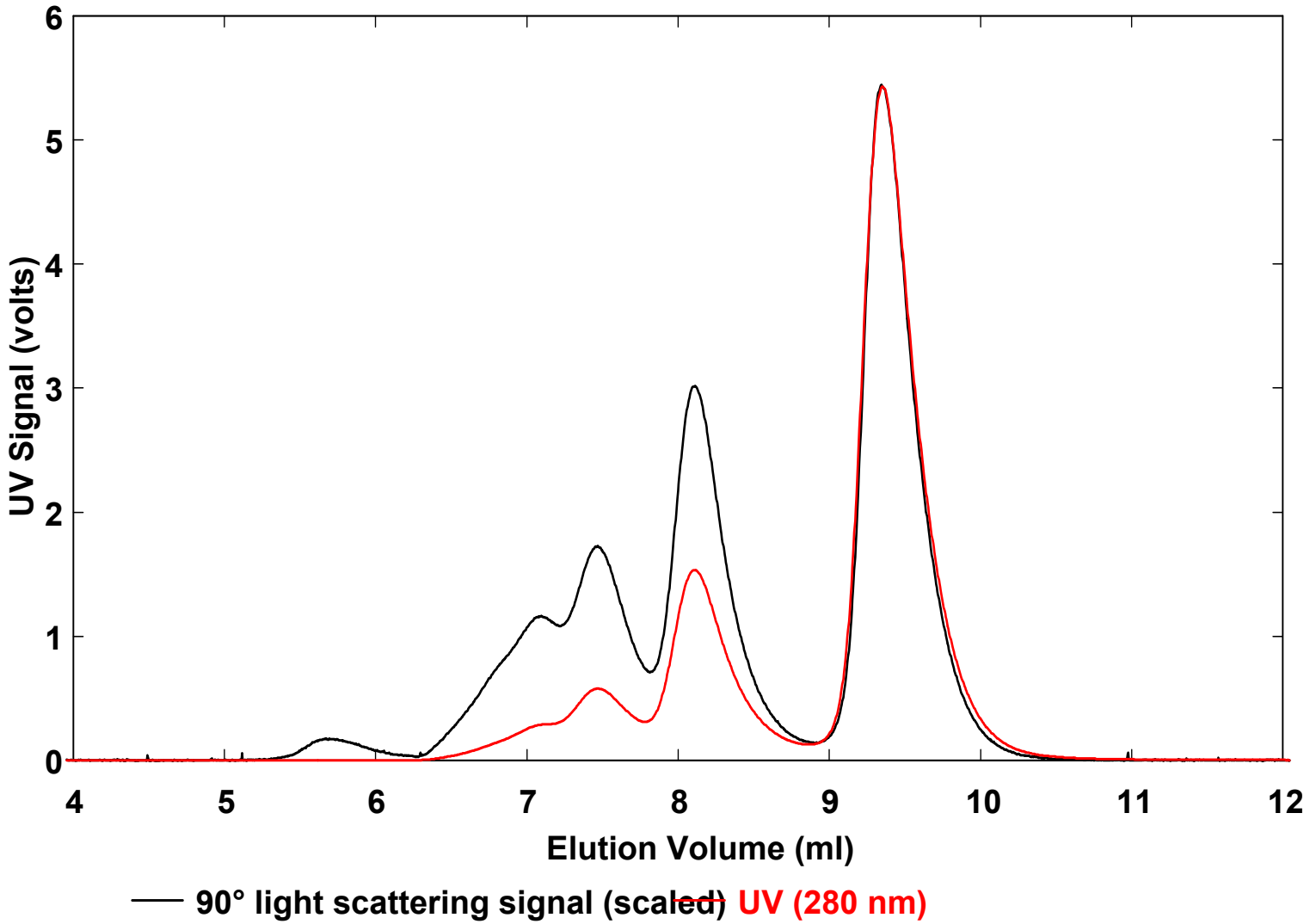
Getting molecular mass from static light scattering: the basic idea

- ★ the light scattering signal is proportional to the product $c \times M$
- ★ we measure c simultaneously with a UV or RI detector
- ★ then the ratio of the scattering to concentration signals will be proportional to M
- ★ masses obtained this way are absolute, and independent of conformation and elution position

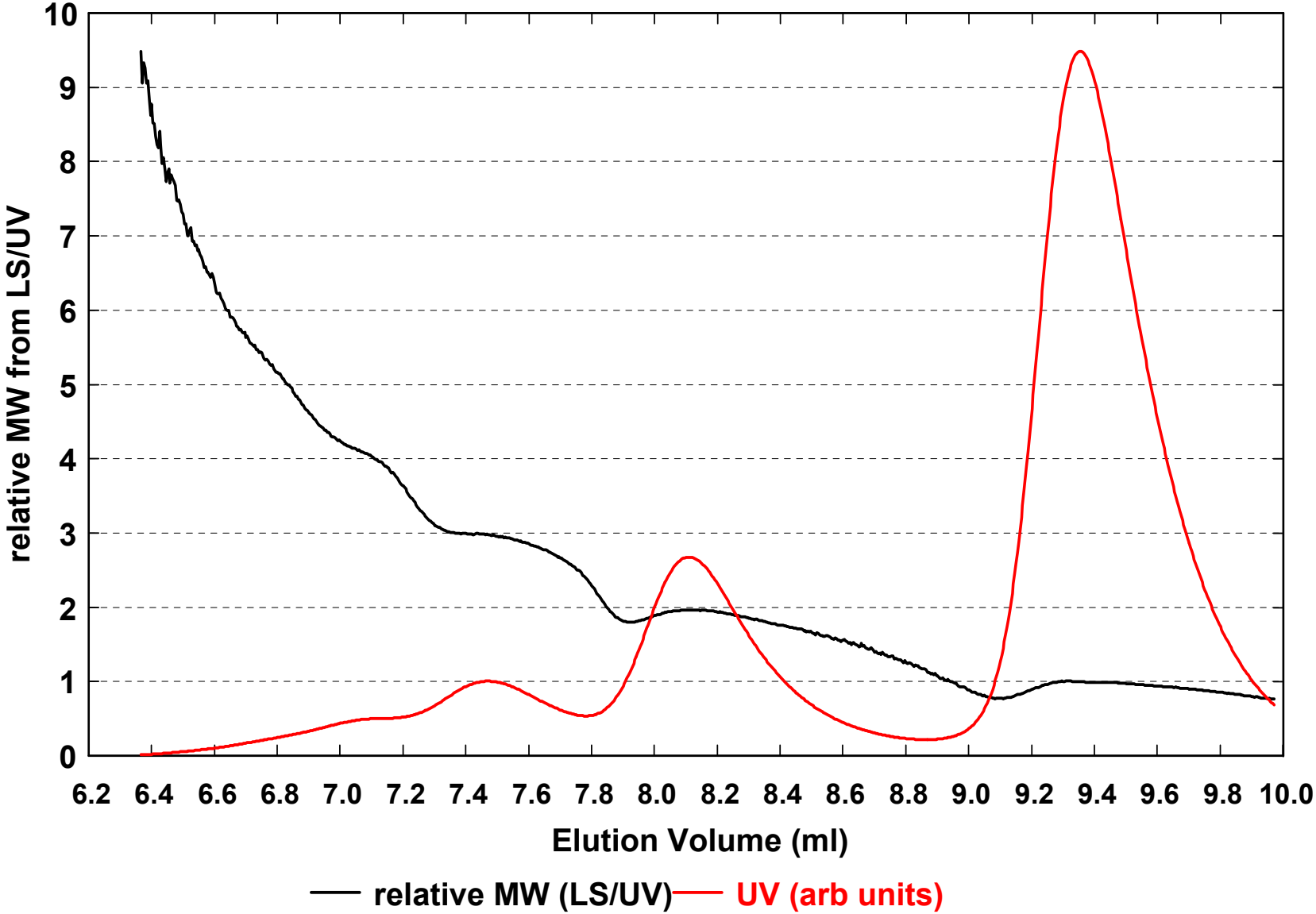
Demonstrating that scattering is independent of elution position and molecular conformation: the ratio of LS to RI signals is the same even for an unfolded protein



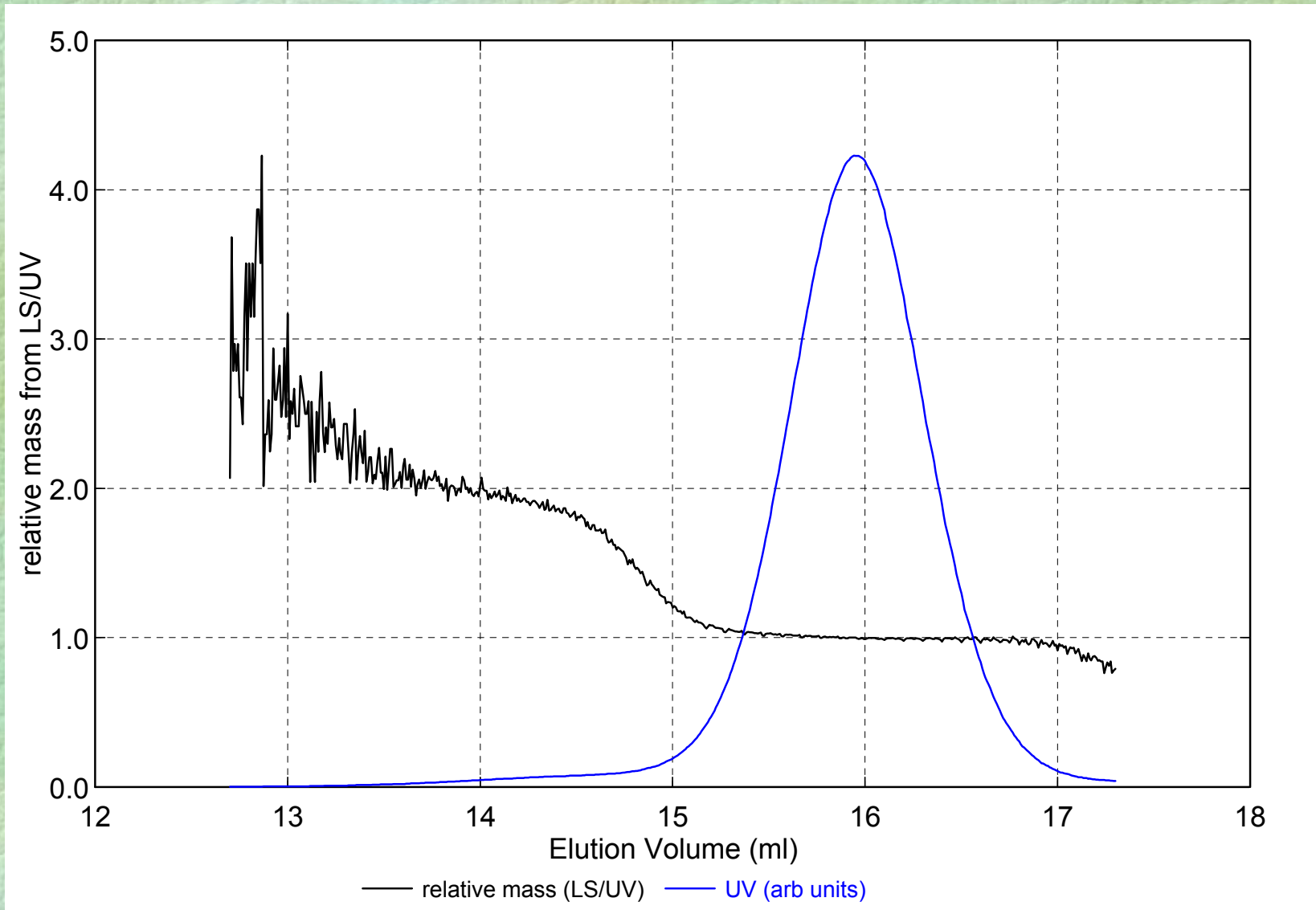
An example for an Fc-fusion protein: the aggregate signals are much stronger in 90° scattering than in the UV chromatogram



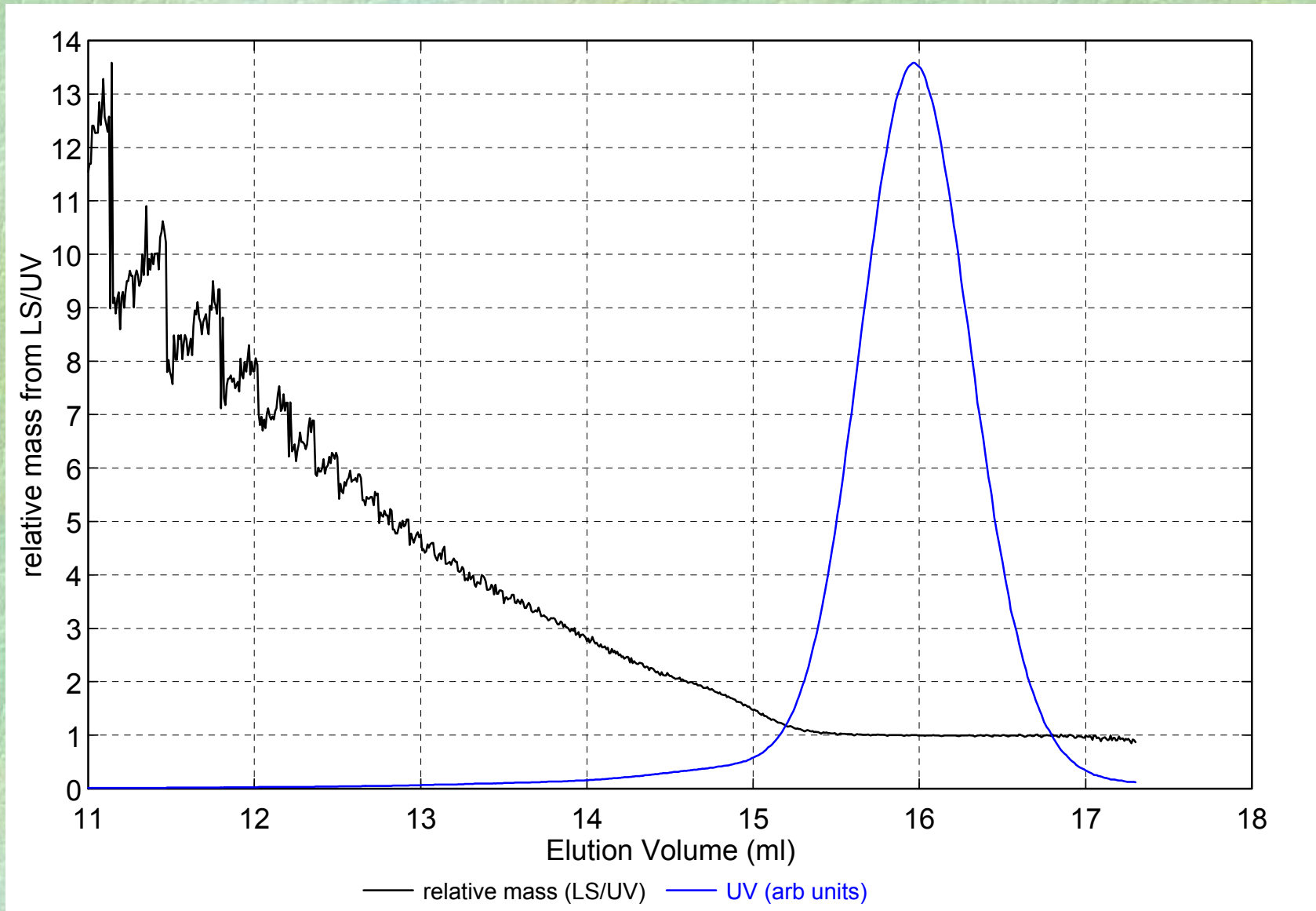
"Oligomer hunting": display the absolute molecular weight from LS in units of monomers



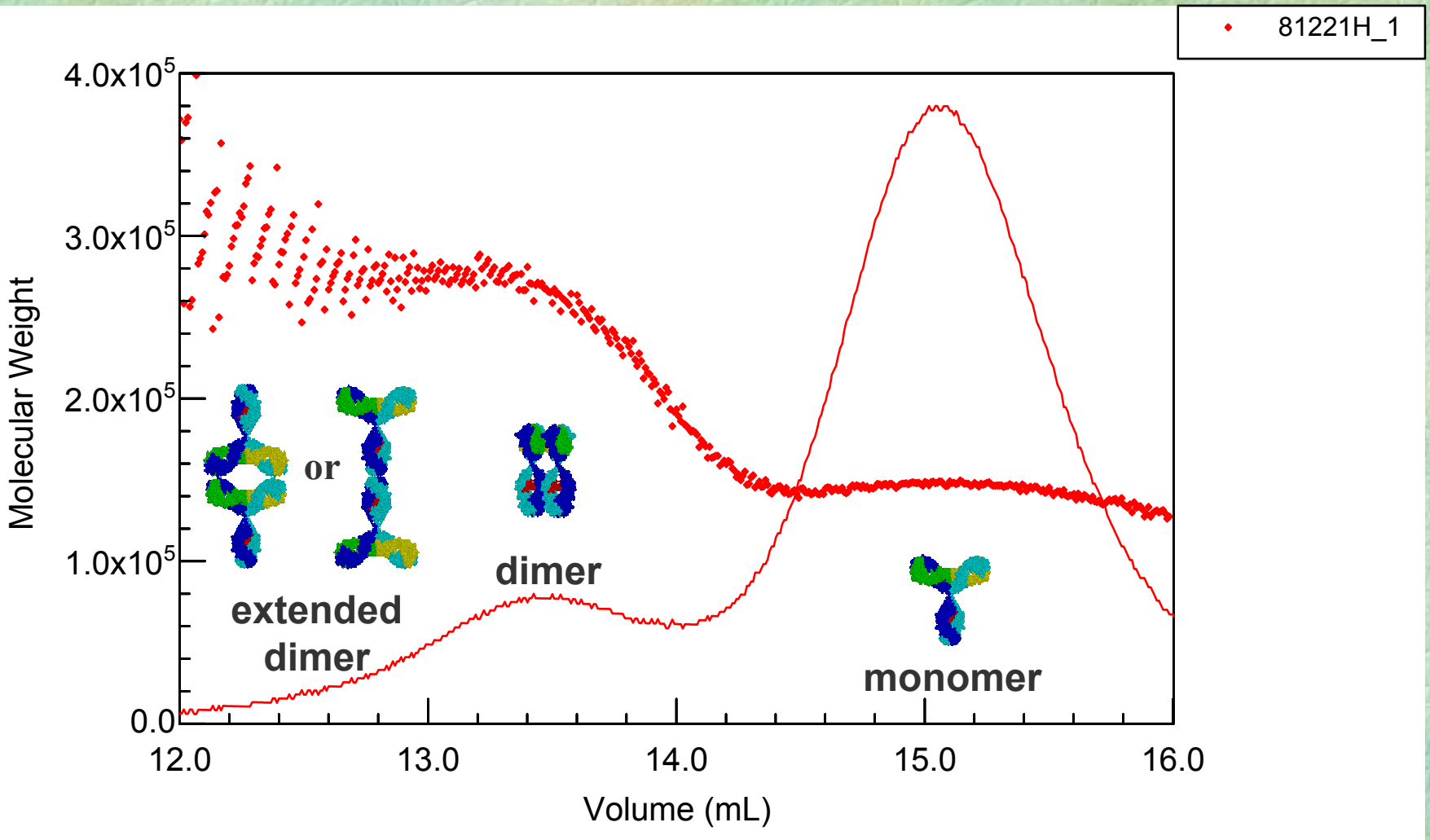
This antibody sample has traces of dimer and trimer



A different lot contains more higher oligomers, and they are so sticky that even dimer is no longer resolved



SEC/LS shows an antibody sample contains only monomer and dimer, but dimer elutes at more than one position



Strengths of SEC + classical LS

1. absolute molecular mass, independent of conformation or elution position
2. gives us at least an average mass for the "aggregate" fraction near the exclusion limit
3. helps tell us whether our chromatography is really working properly
4. strong theoretical background; "first principles" method
5. high throughput, low cost (less than the HPLC it is used with), fairly easy

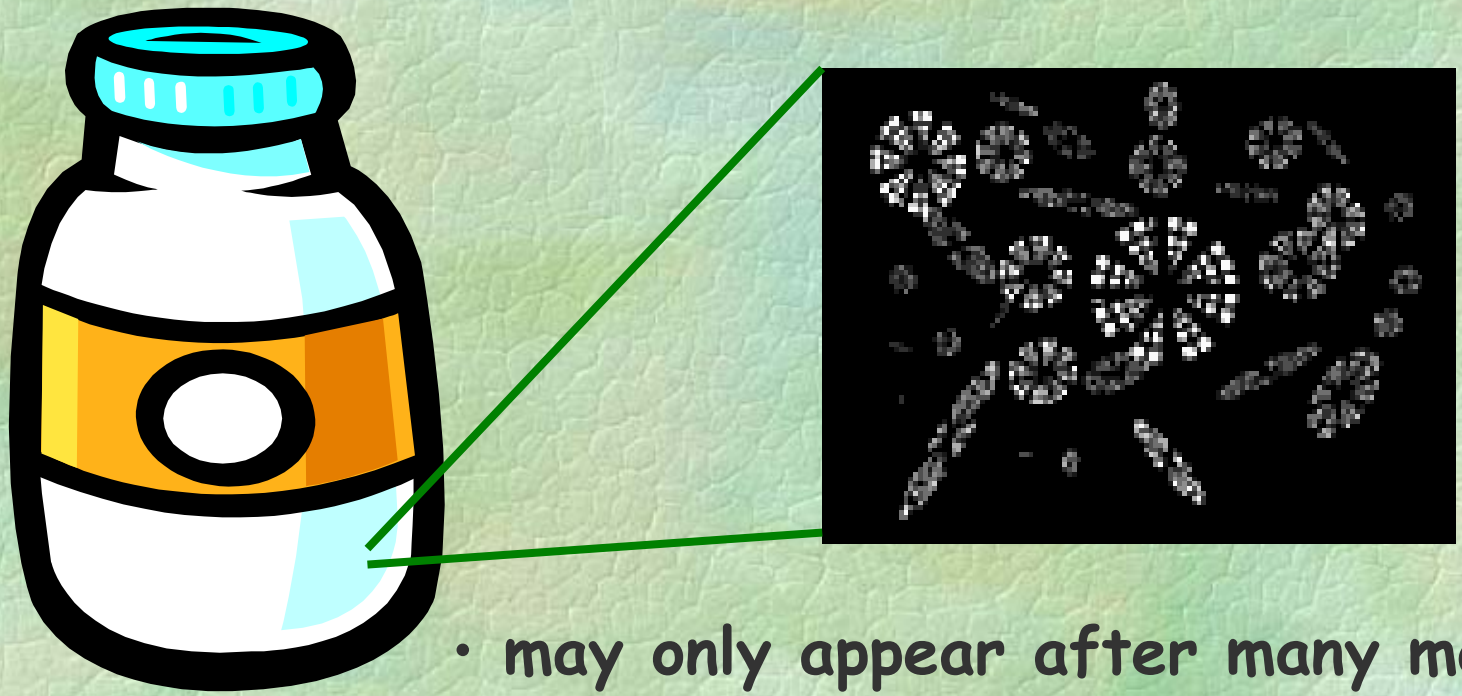
Weaknesses of SEC + classical LS

1. it inherits all the problems of SEC (change in aggregate distribution from dilution, change in buffer, adsorption/filtration, *etc.*)
2. while it is very sensitive to high MW aggregates, quantitation of % by weight still relies on the concentration detector (RI or UV)
3. particles shed from columns may obscure the region near the exclusion limit
4. good signal/noise may require larger injection amounts than normally used

Batch-mode dynamic light scattering (DLS)

also known as quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS)

Often when proteins go bad they develop "snow" (a.k.a. "white amorphous material" [WAM] or "floaters")



- may only appear after many months
- often nucleation-controlled reaction
- often ≤ 0.01% of total protein

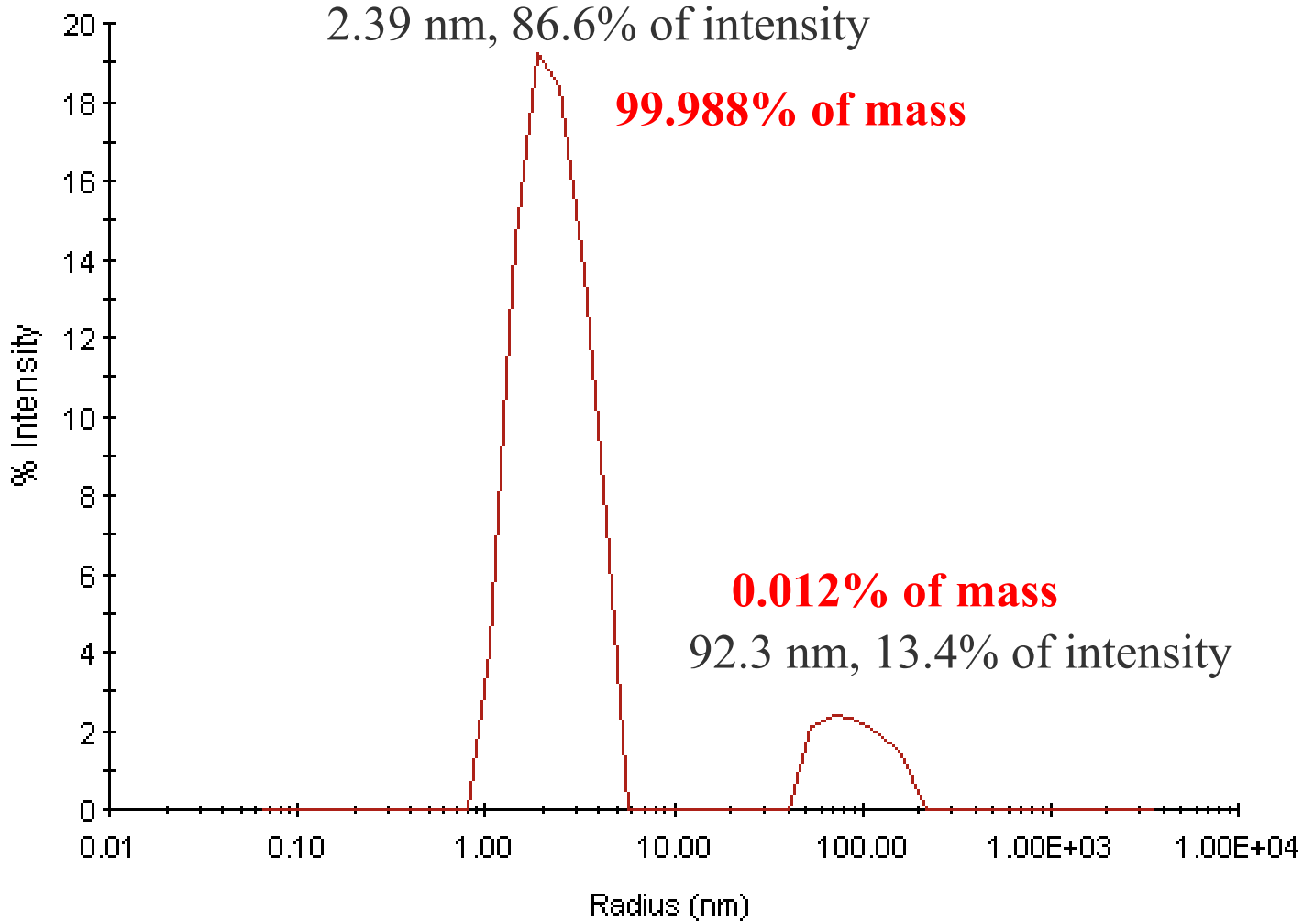
When this happens our valuable protein can only be used for…

Dynamic scattering is one of the few tools that may be able to detect the precursors that eventually form 'snow'

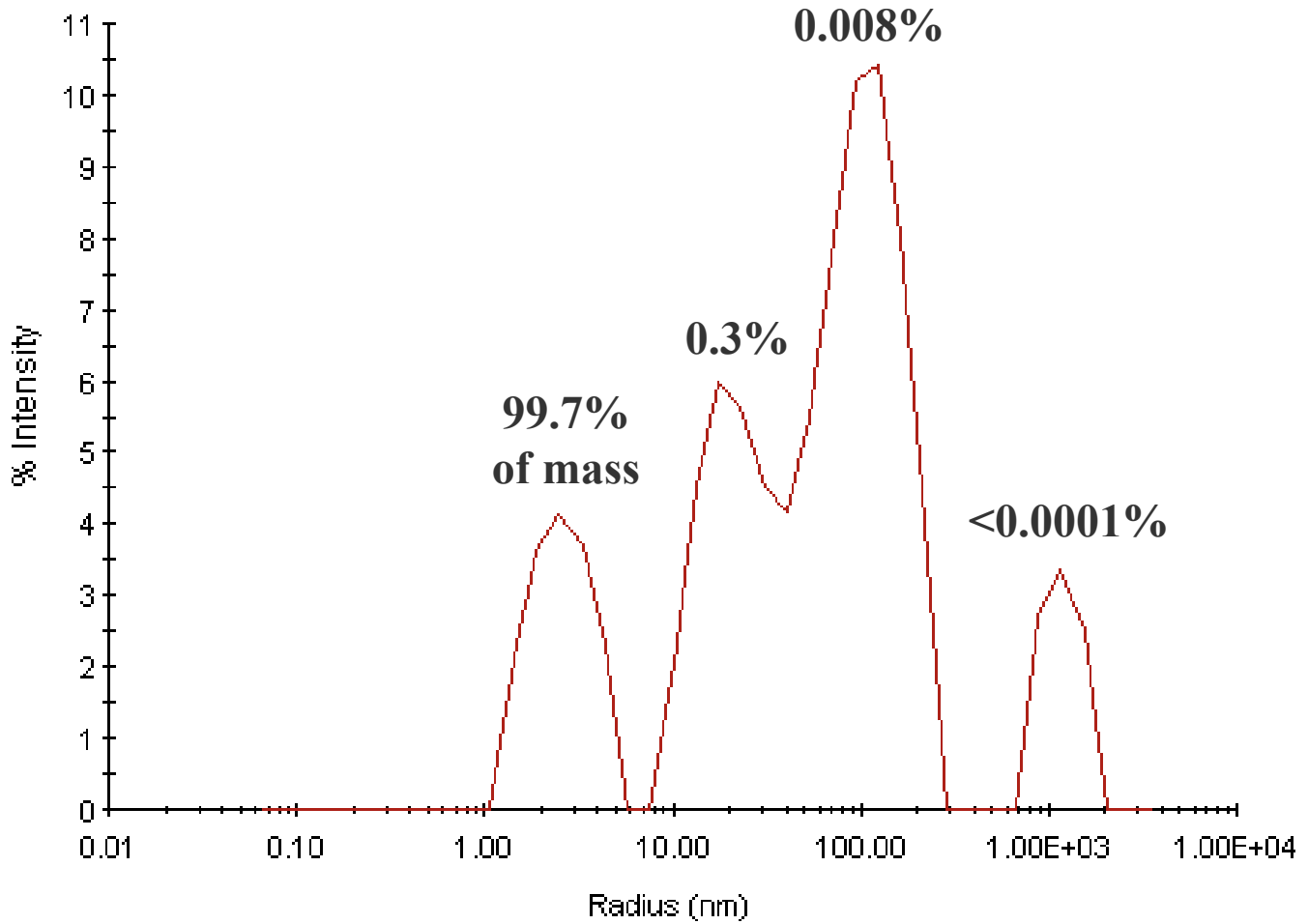
Dynamic light scattering: the basic idea

1. In dynamic scattering we measure the fluctuations in scattering intensity
2. The time scale of those fluctuations depends on the diffusion coefficient of the macromolecule, which in turn depends on its size
3. Like classical LS, the intensity of scattered light is proportional to *M*, so the sensitivity to very large aggregates is very high

Typically the data are transformed into a distribution of hydrodynamic radius; this distribution shows 2 well-resolved peaks



A different lot has 3 aggregate peaks, but they still represent only ~0.3% by weight



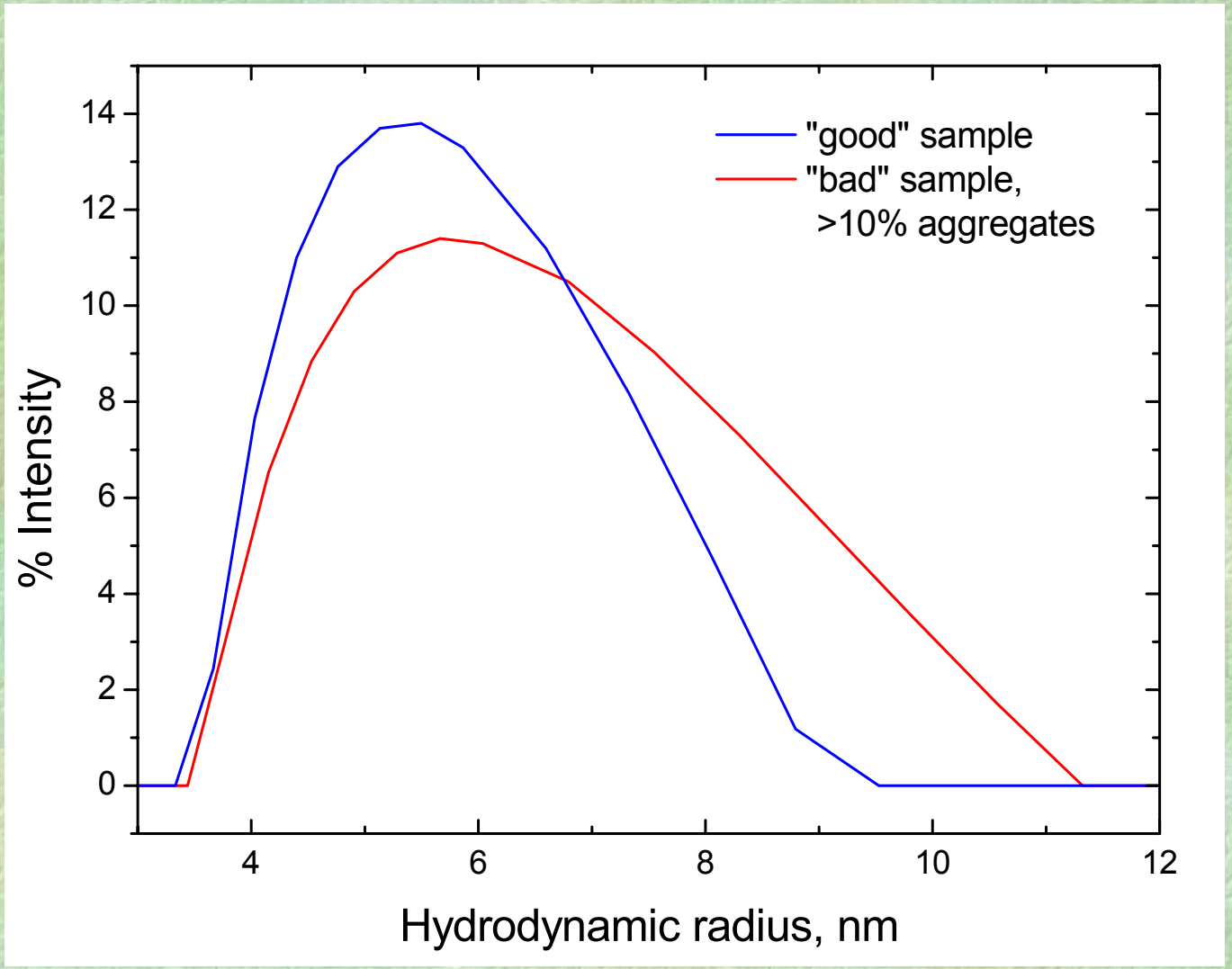
DLS drawback 1: Poor quantitation of mass fractions

- Usually at best the reproducibility of mass fractions is only within a factor of 2
- There is no universally-accepted standard algorithm; different methods can give quite divergent results

DLS drawback 2: Low Resolution

- Generally, to be resolved as a separate species, a second component must be > 2-fold different in R_h and thus > 8-fold in mass
- Thus small oligomers are not resolved, and this is generally a poor method for detecting them
- This limited resolution may simply not be good enough to tell you what you need to know

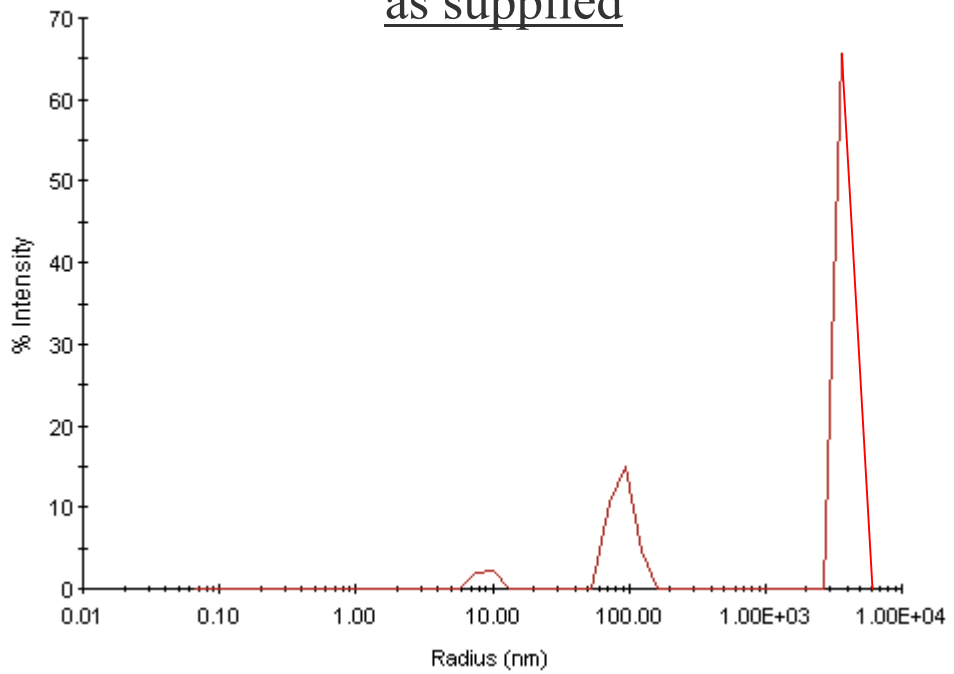
Although small aggregates are generally not resolved as separate species, they do shift the distribution to higher sizes



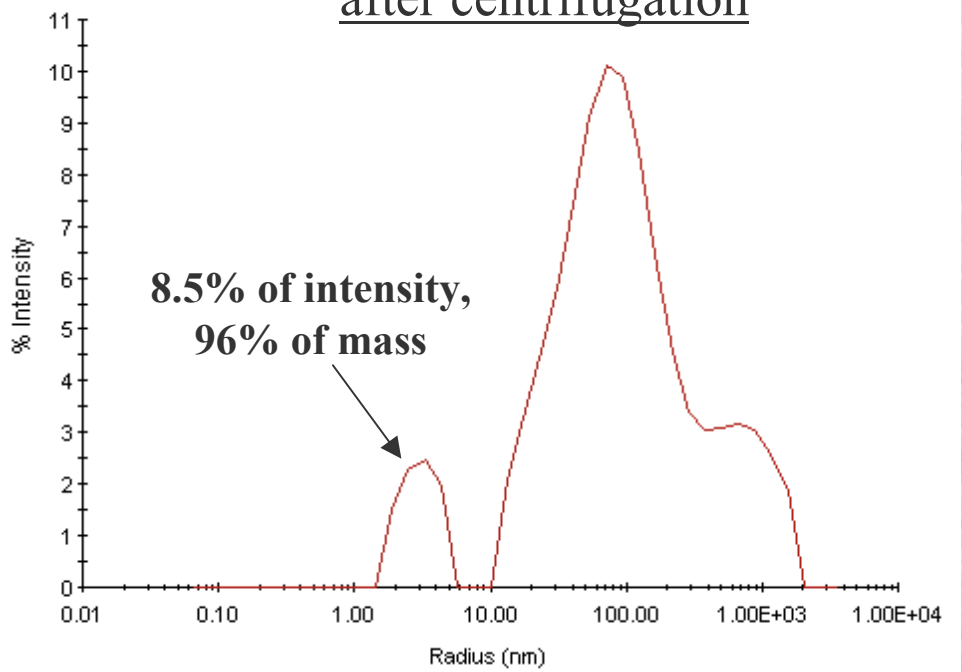
DLS drawback 3: "Blinded by the light"

It can be difficult to detect the main component in the glare from large aggregates; if you lose the main peak, you can't quantitate fractions

as supplied



after centrifugation



Strengths of DLS

1. high sensitivity to large aggregates that may be immunogenic and/or precursors to visible particulates
2. covers an enormous range of size in one analysis (range of mass > 10^9)
3. done at equilibrium; theoretically senses all forms of aggregates
4. batch mode
   - ★ no dilution
   - ★ no change of solvent conditions
   - ★ no loss of species to frit or column matrix

Despite its limitations, DLS can be quite useful for:

1. detecting large aggregates at levels below 0.01%
2. tracking down which process steps generate large aggregates
3. relative ranking of different formulations or processes (which is better)
4. accelerated stability analysis done *in situ* in the DLS instrument

Recommended applications/approaches

1. Use all these orthogonal approaches to test whether your SEC method is missing anything that is significant
2. Use sedimentation velocity and on-line static LS to help develop better SEC methods
3. Trace onset of damage during manufacturing using DLS
4. For products formulated at high protein concentrations, dilute into PBS and run sedimentation velocity to detect long-lived aggregates that may persist *in vivo* ["dilute and shoot" protocol]

With thanks to the people who made the proteins

1. Several clients who allow me to show data for "protein X"
2. Kunio Misono at Cleveland Clinic Foundation (sANPR)
3. former Amgen colleagues