

Adventures in aggregate land: Biotech applications of AUC and complementary methods"

John Philo

V.P. & Director of Biophysical Chemistry





Outline

- Why do we care about aggregates in biopharmaceuticals?
- Review a few basic facts about aggregates and mechanisms of aggregation
- Utility of sedimentation velocity for analysis of long-lived aggregates
- What can other methods like light scattering add to the picture?
- A few words about field-flow fractionation (FFF)



Protein aggregates: What is all the fuss about?

- Aggregates (both large and small) often are a major degradation product
 - Hence they often are the major factor limiting shelf life
- Aggregates in the product may affect its:
 1. manufacturability
 - clogged columns or diafiltration membranes
 2. bioactivity (potency)
 3. serum half-life or absorption rate
 4. **immunogenicity**



Why is there heightened concern about immunogenicity?

- In Europe about 200 patients taking Eprex® (one brand of recombinant erythropoietin, EPO) developed antibodies that cross-reacted and neutralized their own internally-produced EPO
- Consequently those patients make no new red blood cells and require regular transfusions (Pure Red-Cell Aplasia)
- While the published evidence suggests that this was not due to aggregates in Eprex, this incident has raised alarm bells about immunogenicity



Some known cases where aggregates cause immunogenicity

1. Early versions of intravenous immunoglobulin from donor blood (IVIg) had high aggregate levels and caused anaphylaxis
 - similar experience for human serum albumin
2. Aggregate levels in human growth hormone (hGH) correlated with persistence of anti-hGH antibody in patient serum
3. A recent study using interferon- α and transgenic mice confirmed that immunogenicity depends on the type and size of the aggregates

S. Hermeling *et al.* (2006) *J. Pharm. Sci.* 95, 1084-1096.



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

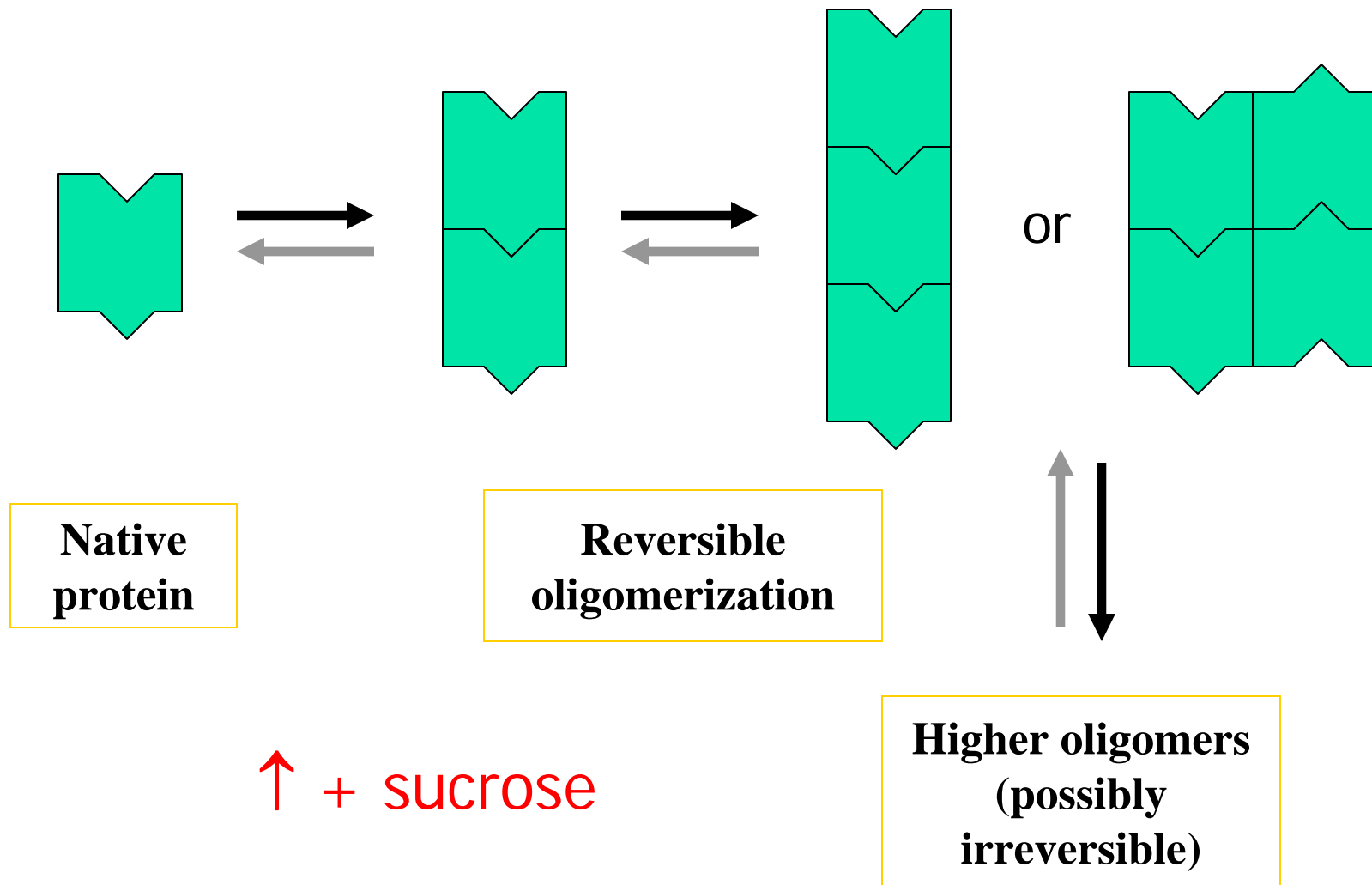
1. rapidly-reversible non-covalent small oligomers (dimer, trimer, tetramer...)
2. irreversible non-covalent oligomers
3. covalent oligomers (*e.g.* disulfides)
4. “large” aggregates (> 10-mer)
 - ★ could be reversible if non-covalent
5. “very large” aggregates (diameter ~50 nm to 3 μm)
 - ★ could be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

“soluble”

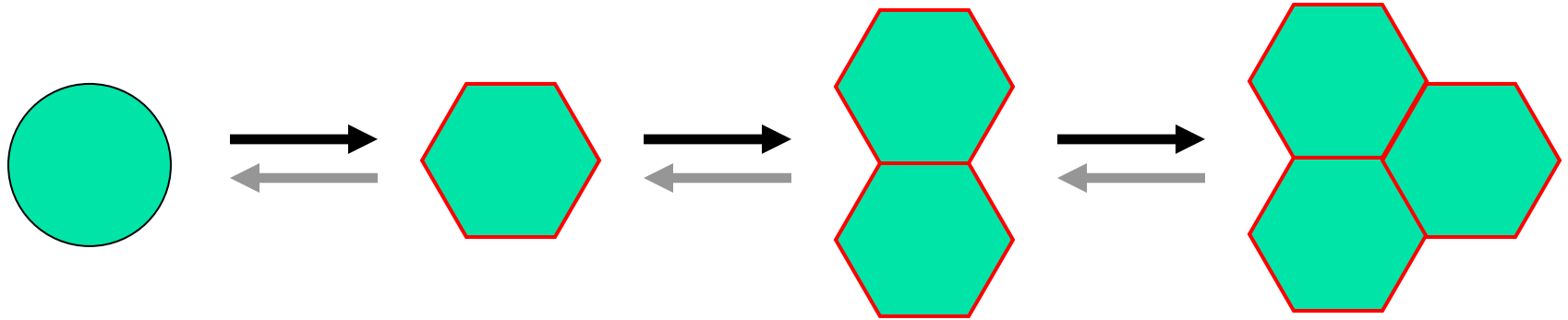
“insoluble”



Aggregation mechanisms (1): reversible association of native protein



Aggregation mechanisms (2): oligomerization following conformational change



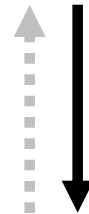
Native protein

Conformational change or partial unfolding

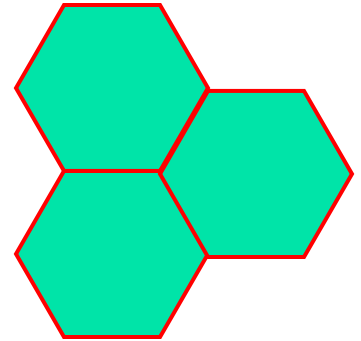
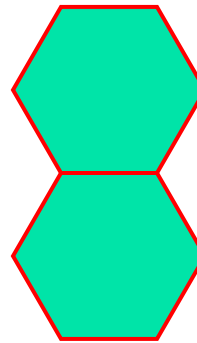
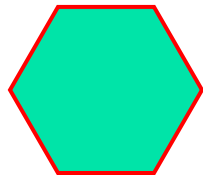
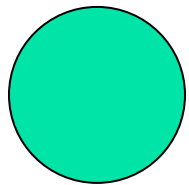
Oligomerization of non-native protein

Higher oligomers (probably irreversible)

↓ + sucrose



Aggregation mechanisms (3): oligomerization driven by covalent modification



**Native
protein**

**Modified
protein
(oxidation,
deamidation,
etc.)**

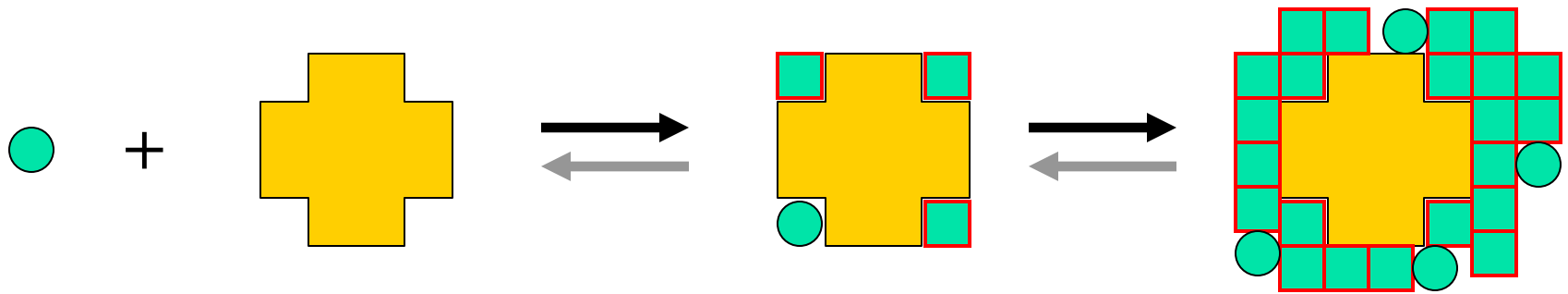
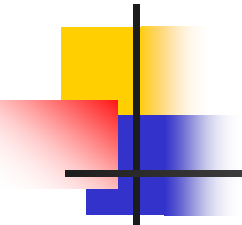
**Oligomerization
of modified
protein**



**Higher oligomers
(possibly
irreversible)**

↓ ↑ + sucrose

Aggregation mechanisms (4): nucleation controlled aggregation ("seeding")



Native protein

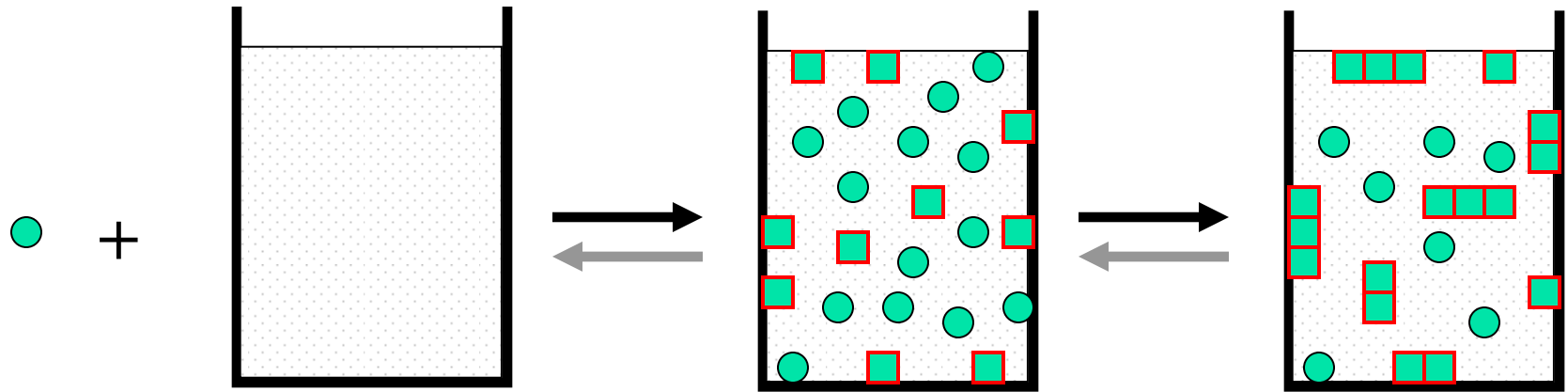
**Critical nucleus
(aggregate of native or modified protein, or a contaminant)**

Addition of protein monomers onto surface of nucleus (often with partial unfolding)

Visible particulates or precipitation

↑ + sucrose

Aggregation mechanisms (5): surface-induced aggregation



Native
protein

Container
surfaces and
air-liquid
interfaces

Adsorption of
protein monomers
onto surfaces
promotes partial
unfolding

Aggregation of
altered protein (as
in mechanism 2)

↑ + sucrose

↓ + detergent



The analytical challenge

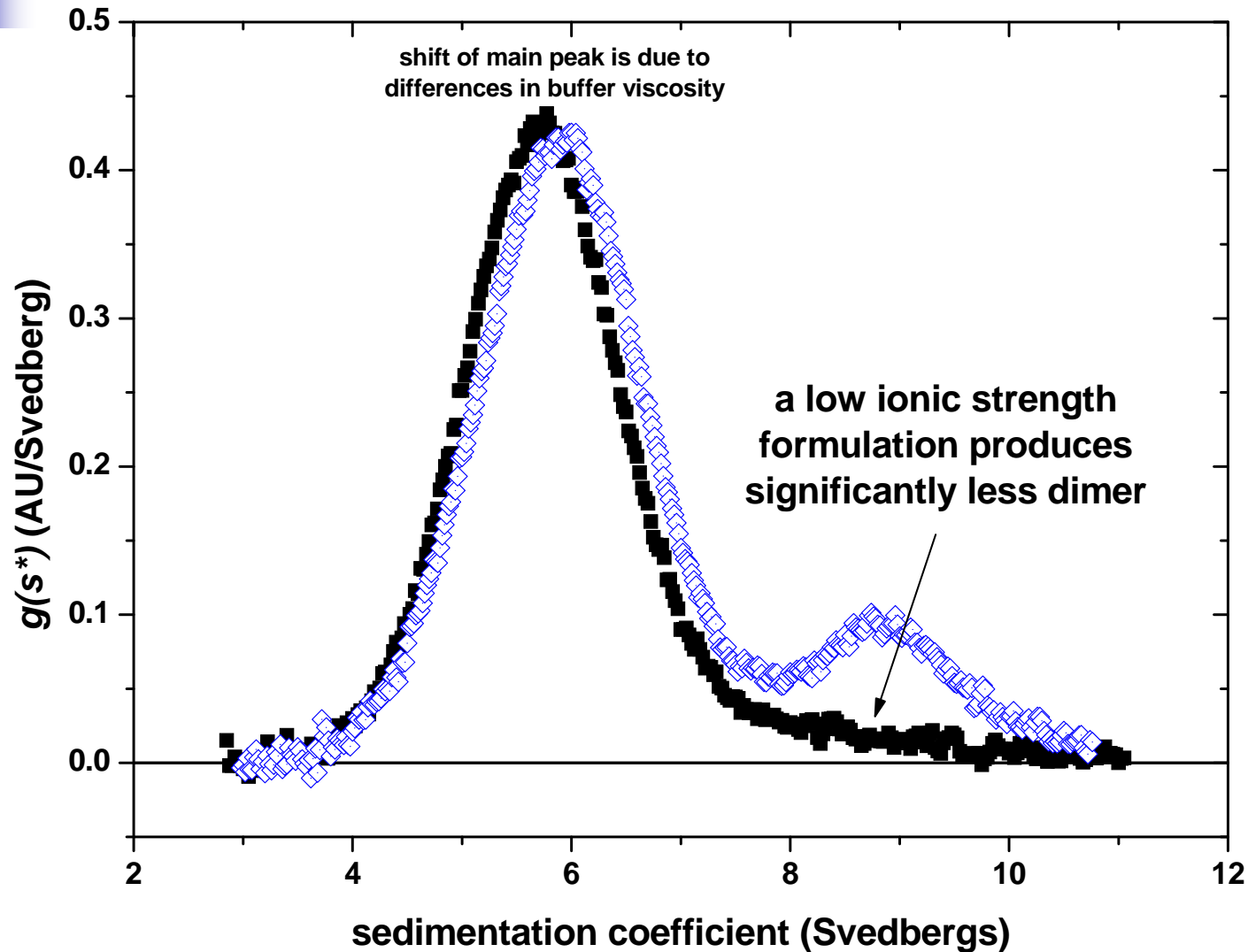
1. Any protein sample may contain aggregates with a wide range of sizes, types, and lifetimes
2. Any one analysis method may not detect all the aggregate sizes or types that are present
3. The measurement itself may perturb the aggregate distribution that was initially present



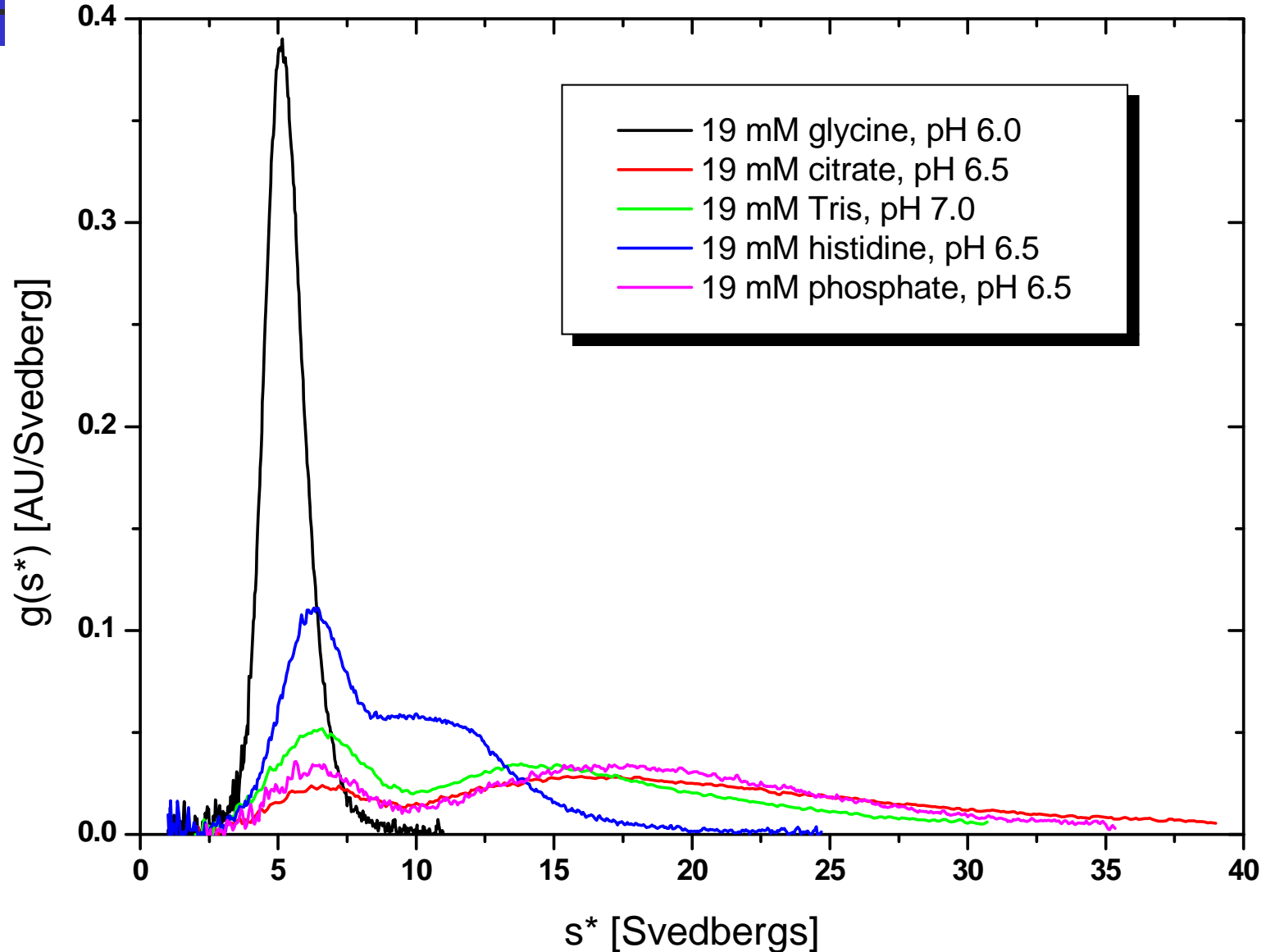
The measurement itself may create or destroy aggregates

dissociation or loss of aggregates can be caused by:	SEC	SV	FFFF
dilution	+++	+	+++
change of solvent conditions	+++	-	++
adsorption to surfaces	+++	+	++
physical filtration (<i>e.g.</i> column frit)	+++	-	-
physical disruption (<i>e.g.</i> shear forces)	++	-	-
creation of new aggregates can be caused by:			
change of solvent conditions	+++	-	++
surface or shear-induced denaturation	++	-	+
concentration on surface	-	-	++

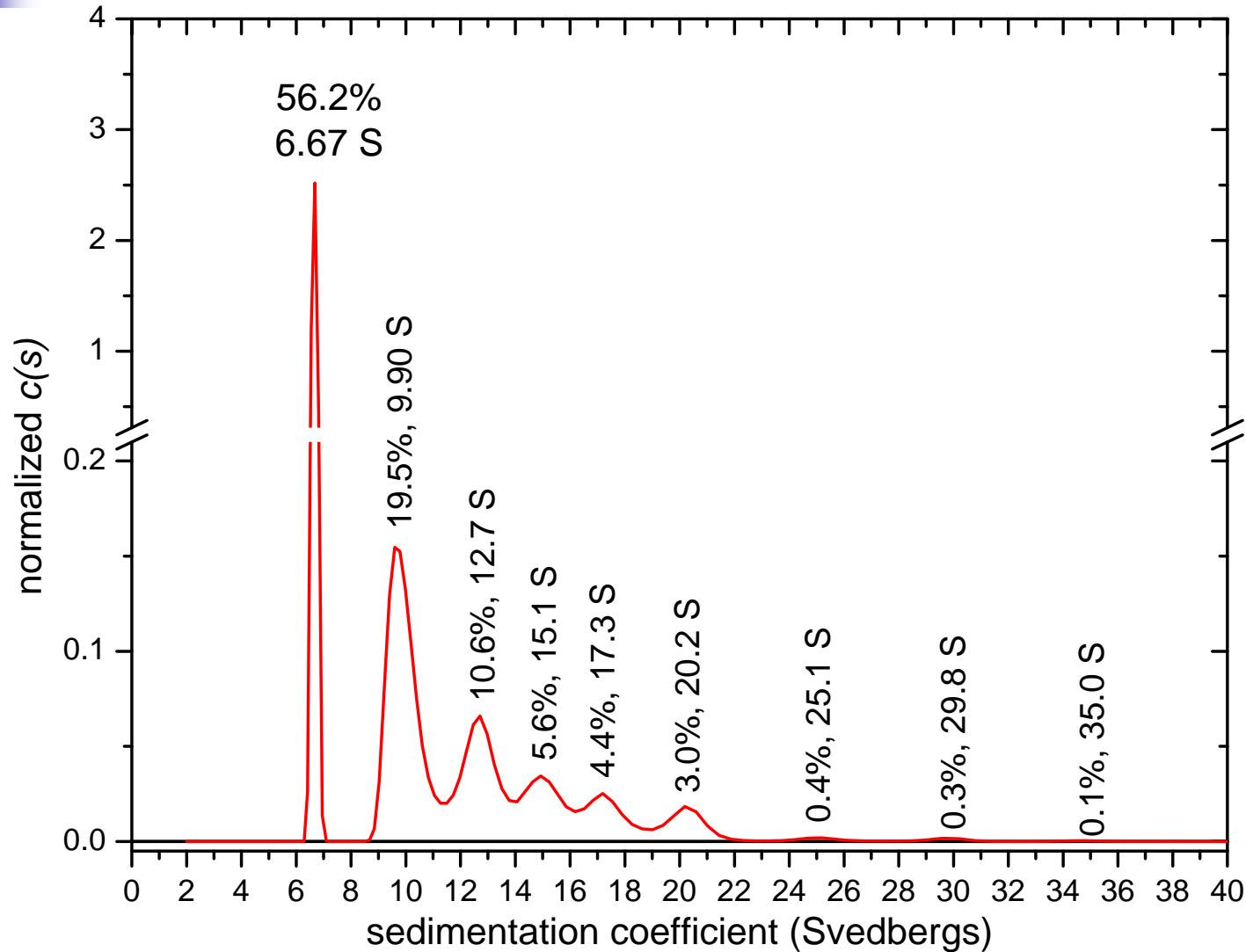
Velocity analysis of two different formulations of an antibody, each analyzed in its own formulation buffer, reveals differences in aggregation



Analysis of antibody samples after accelerated stability (heat stress) studies in various buffers



This is an accelerated stability sample of a monoclonal antibody analyzed using the high-resolution $c(s)$ method

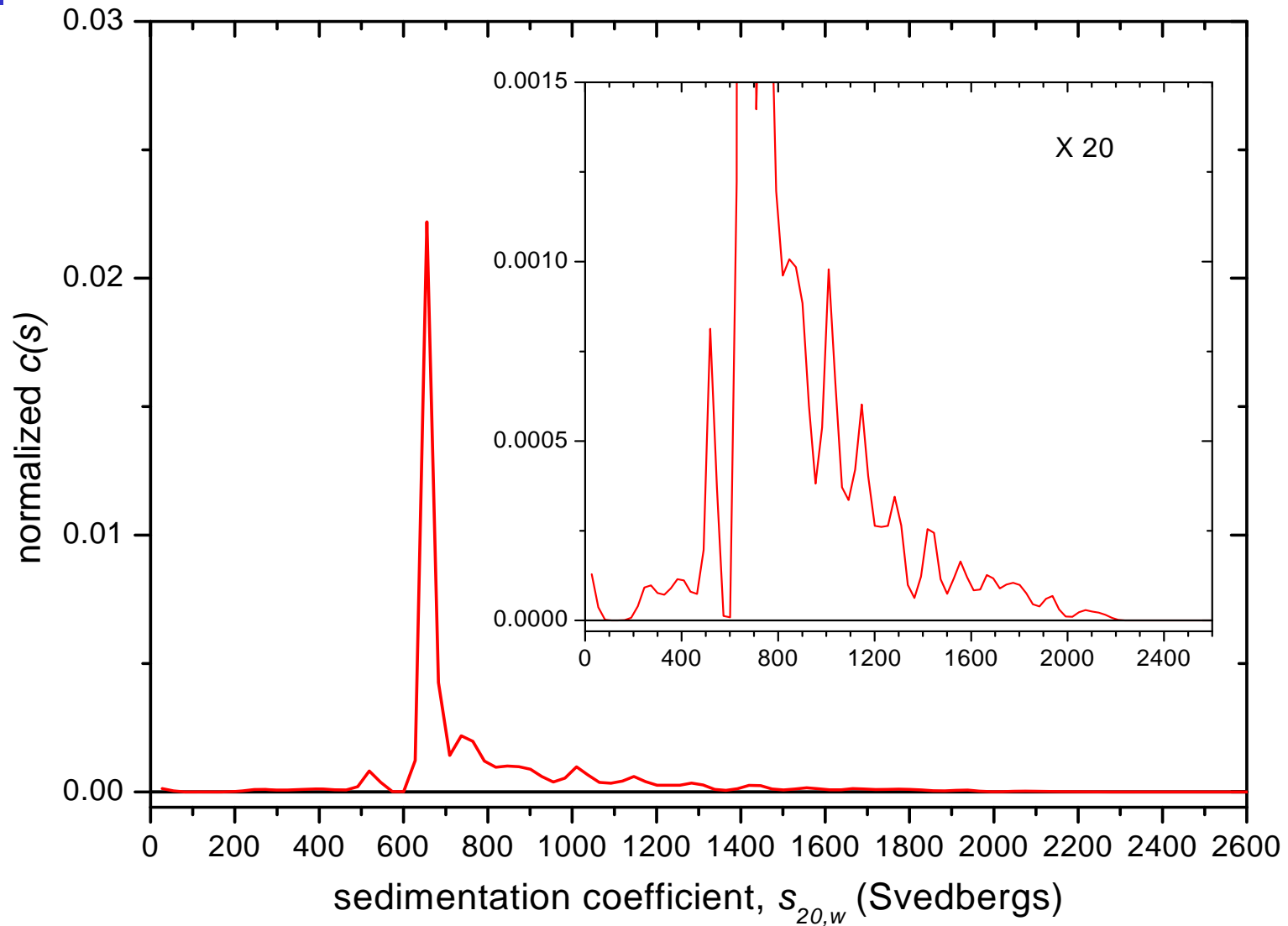




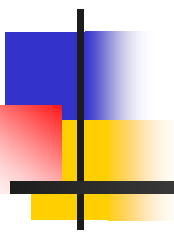
Often a significant fraction of the aggregates never elute from the SEC column

Sample	Total Aggregates by SV	Total Aggregates by SEC	Total Aggregates by SEC with Arginine
MAb1 stressed	43.8%	38.5%	42.6%
MAb2 stressed	52.6%	26.5%	43.4%

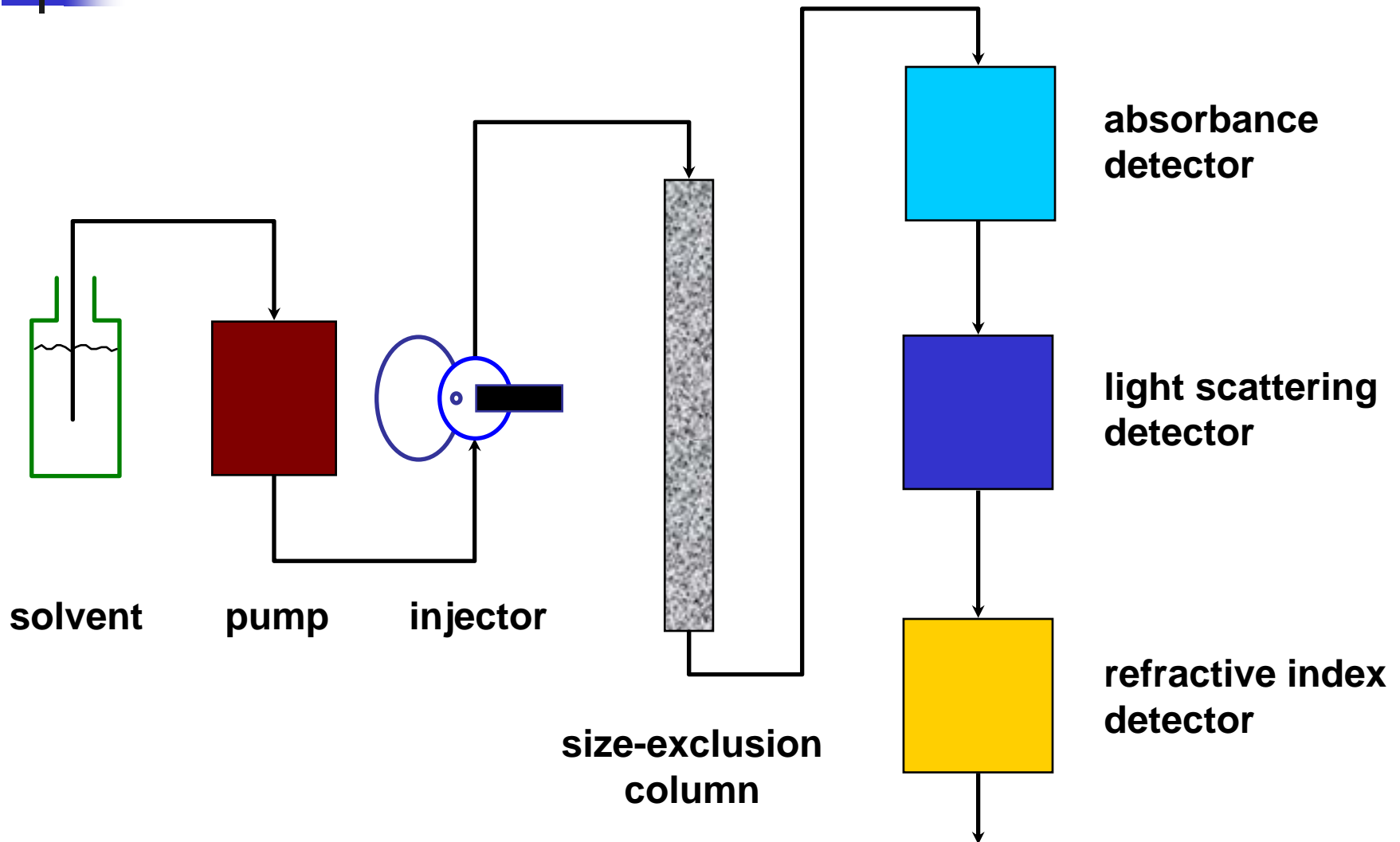
Heterogeneity and aggregation in an adenovirus gene therapy vector



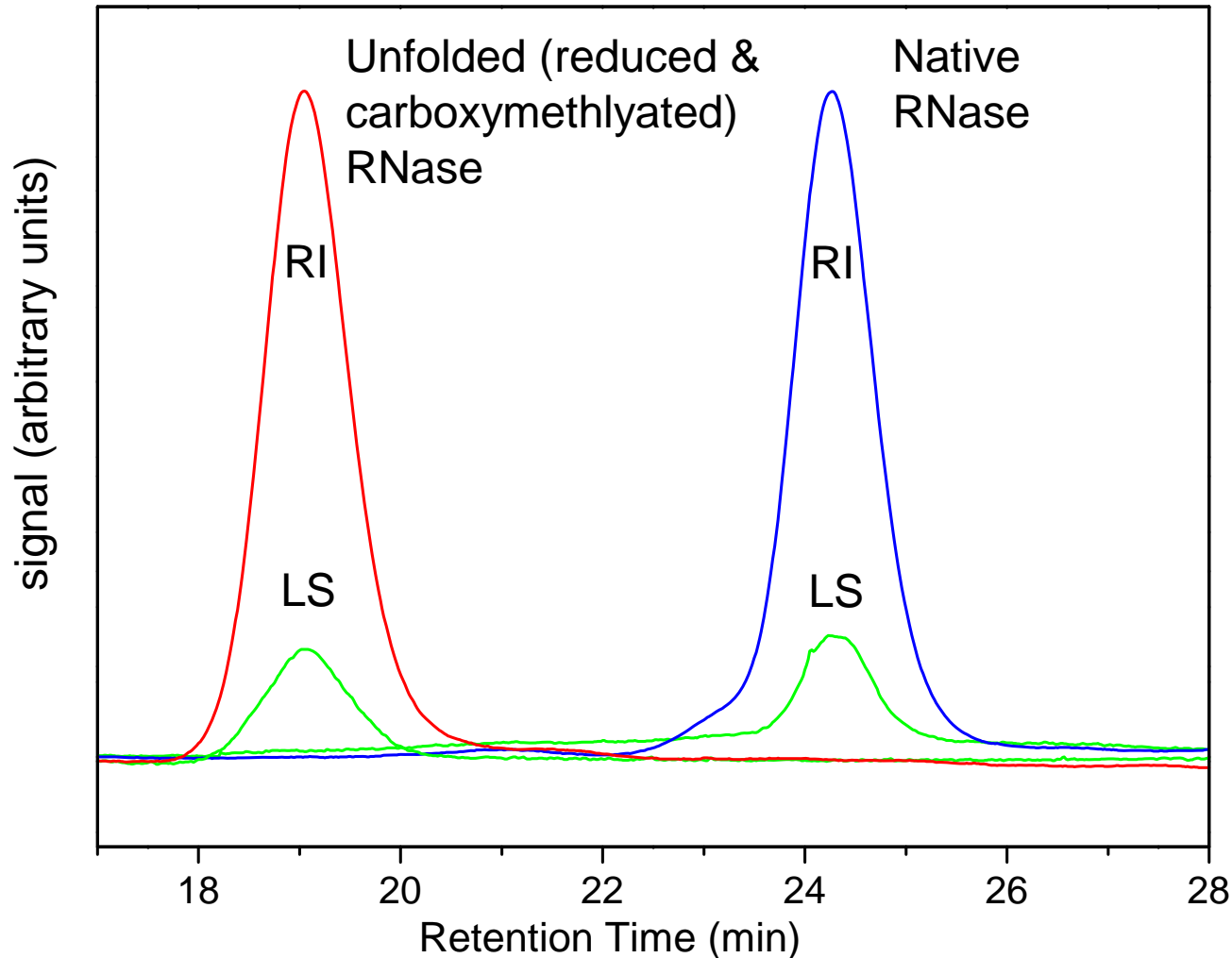
Methods other than AUC can be helpful and complementary: SEC-MALLS shows an “aggregate” isn’t an aggregate



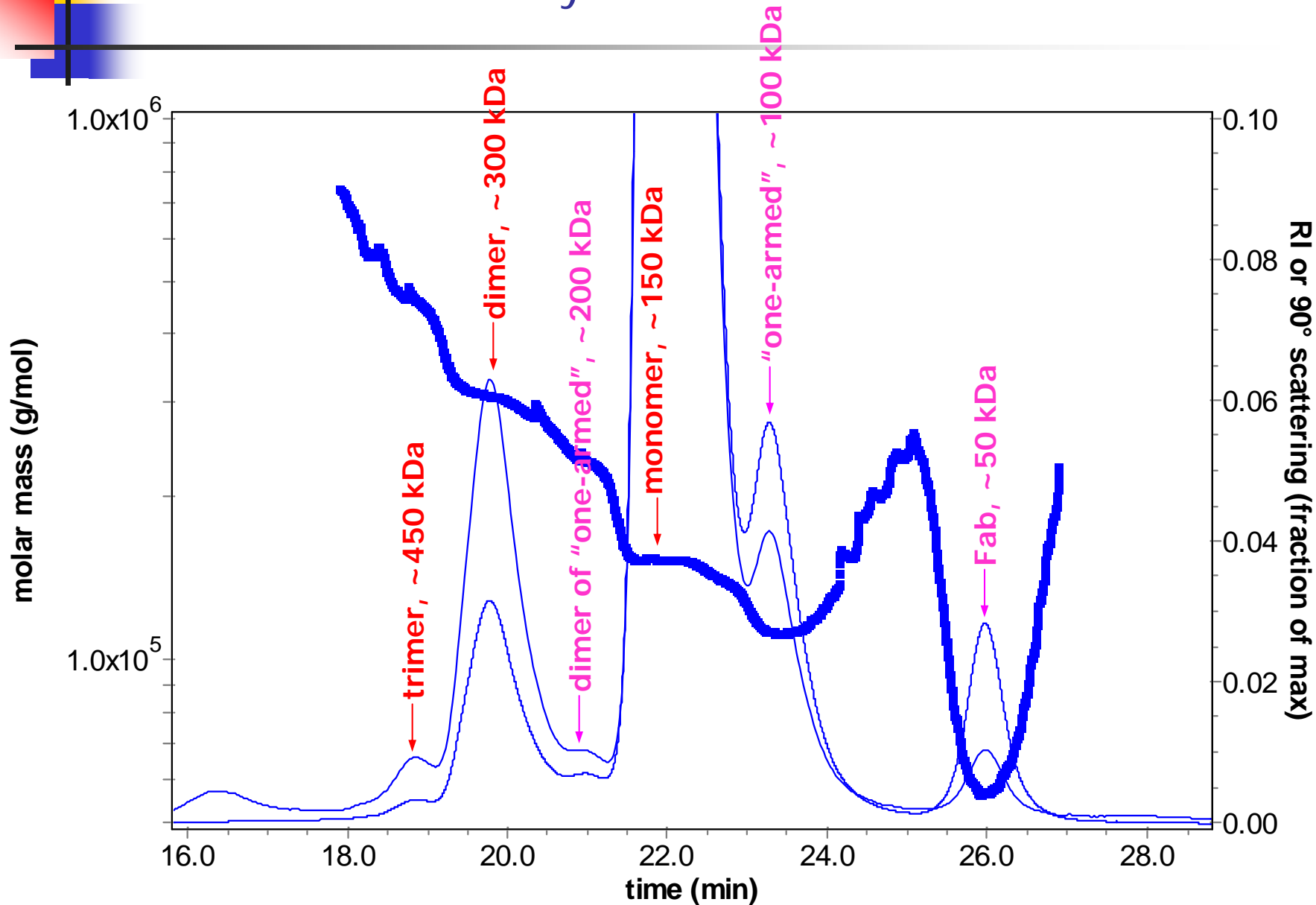
Typical setup for size-exclusion chromatography with on-line light scattering detection (SEC-MALLS)



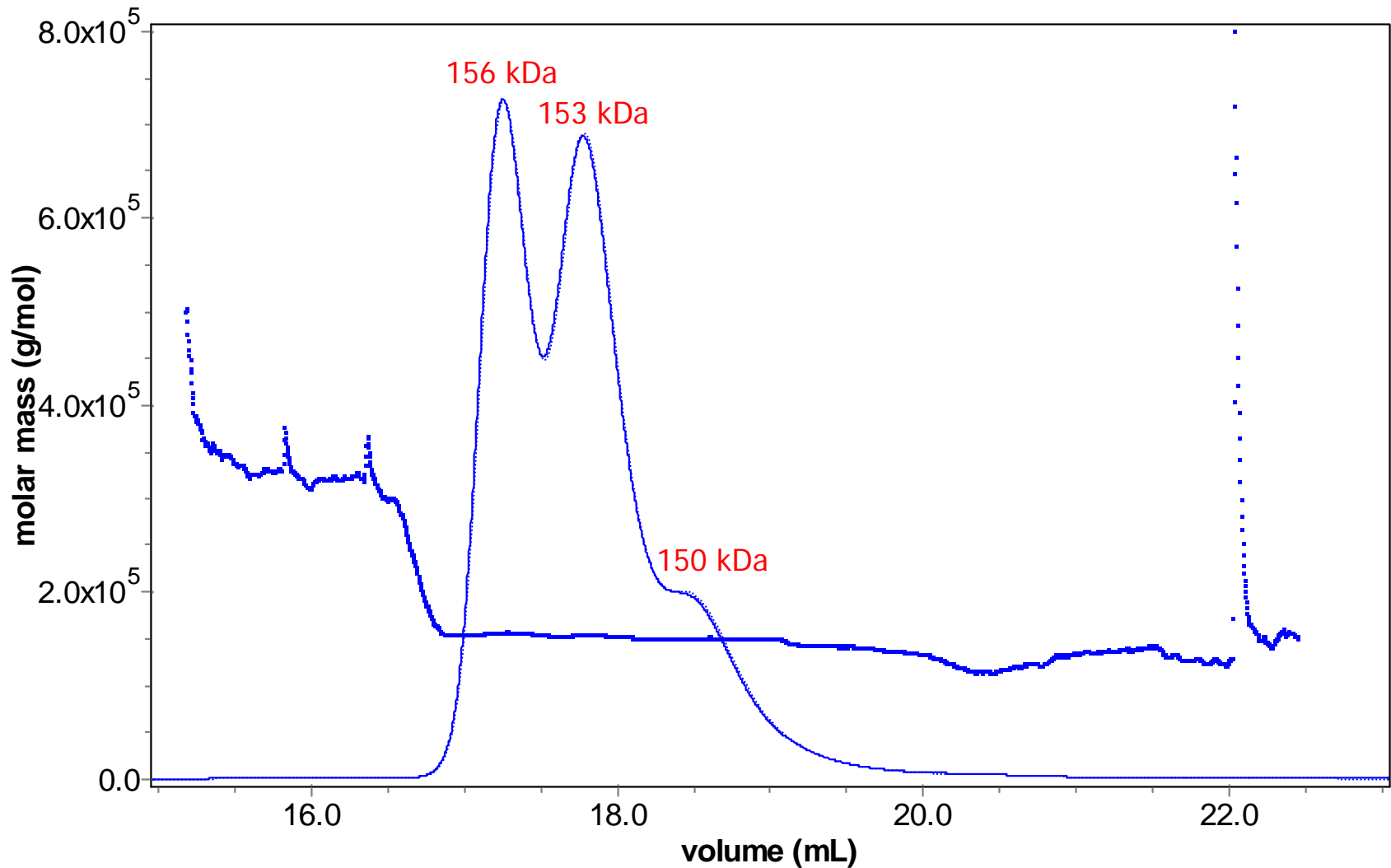
Demonstrating that scattering is independent of elution position and molecular conformation: the ratio of LS to RI signals is the same whether the protein is folded or unfolded



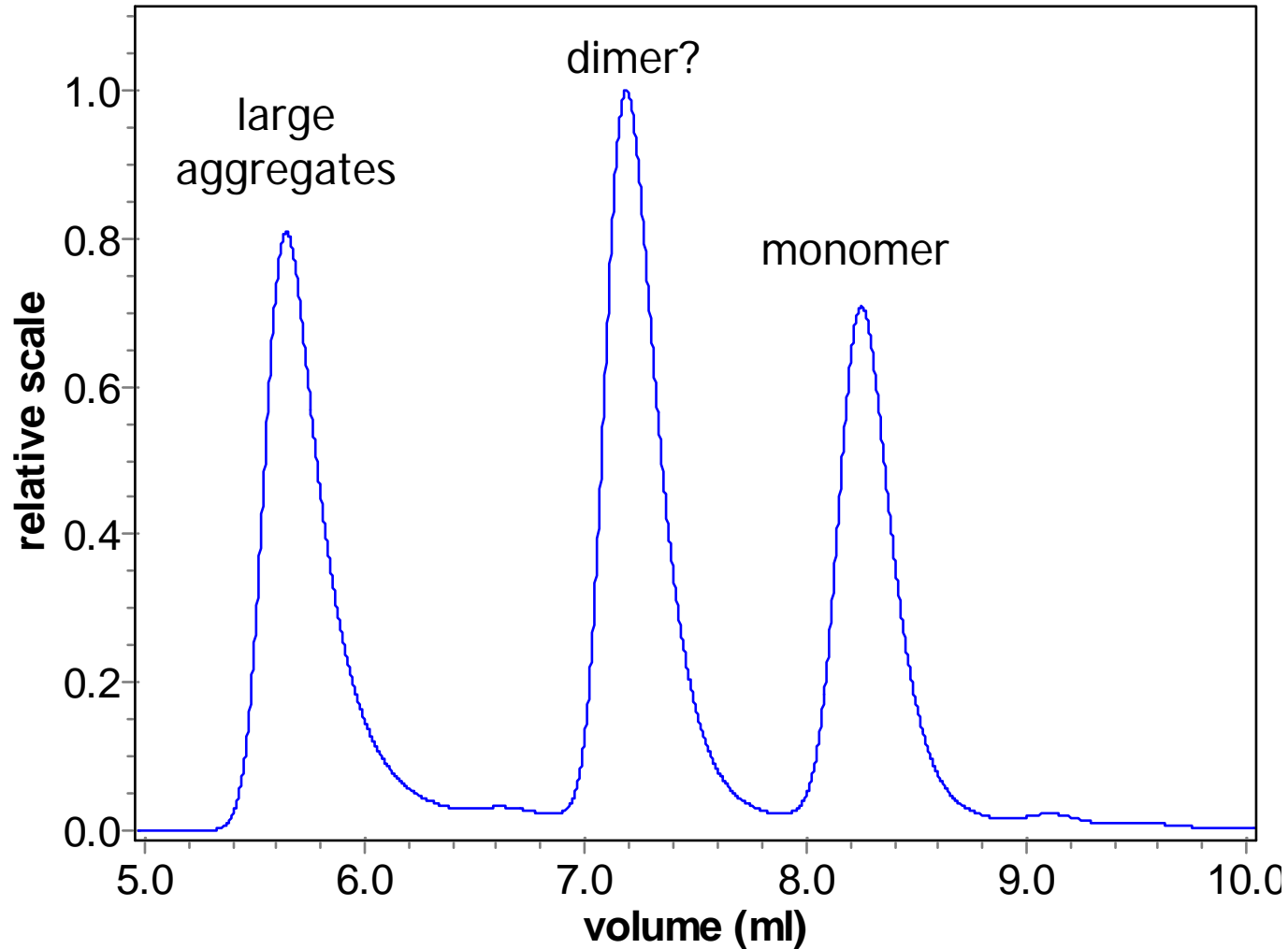
Identifying minor components in a heat-stressed monoclonal antibody via SEC-MALLS



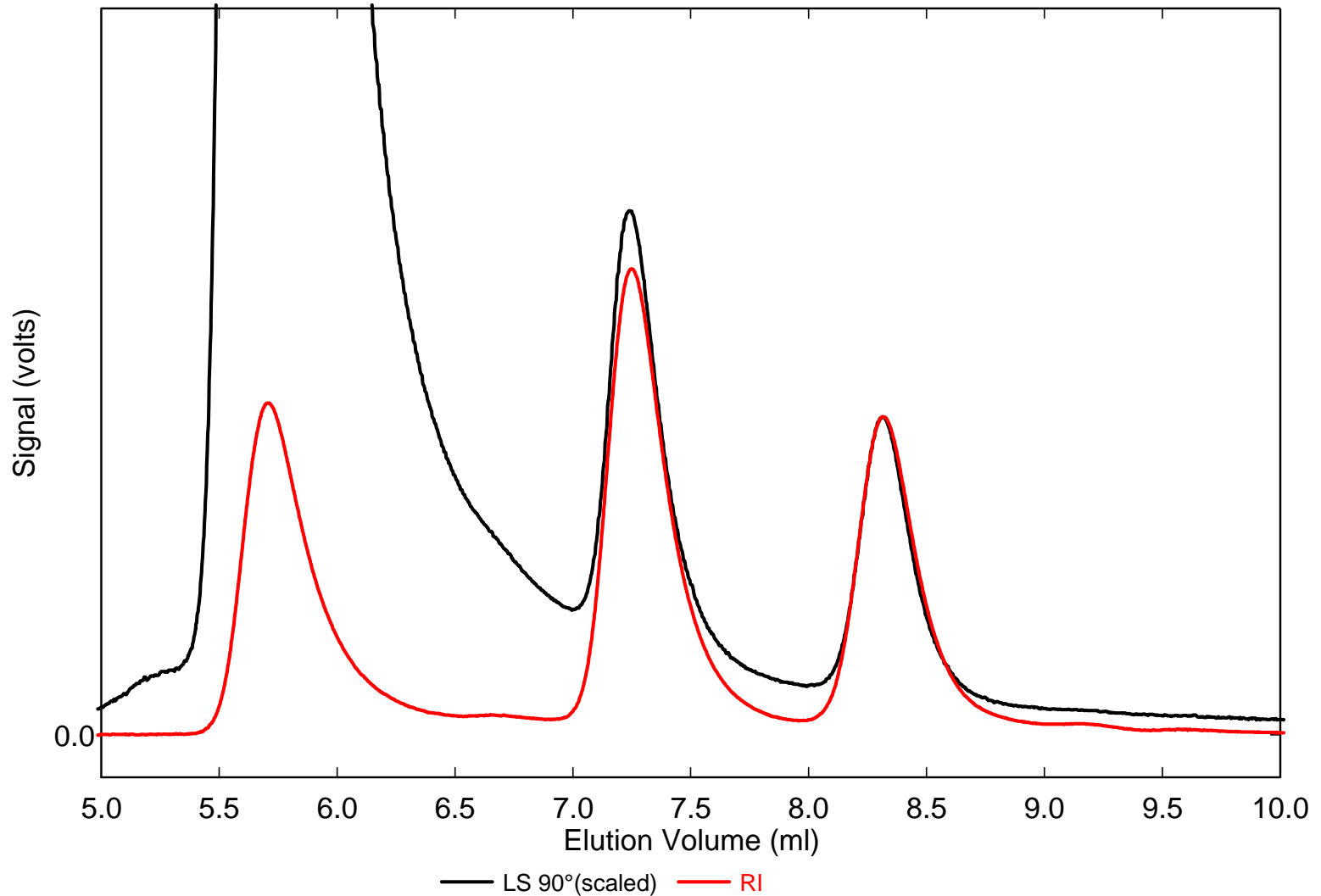
Another antibody has a very unusual elution profile with 3 peaks for monomer



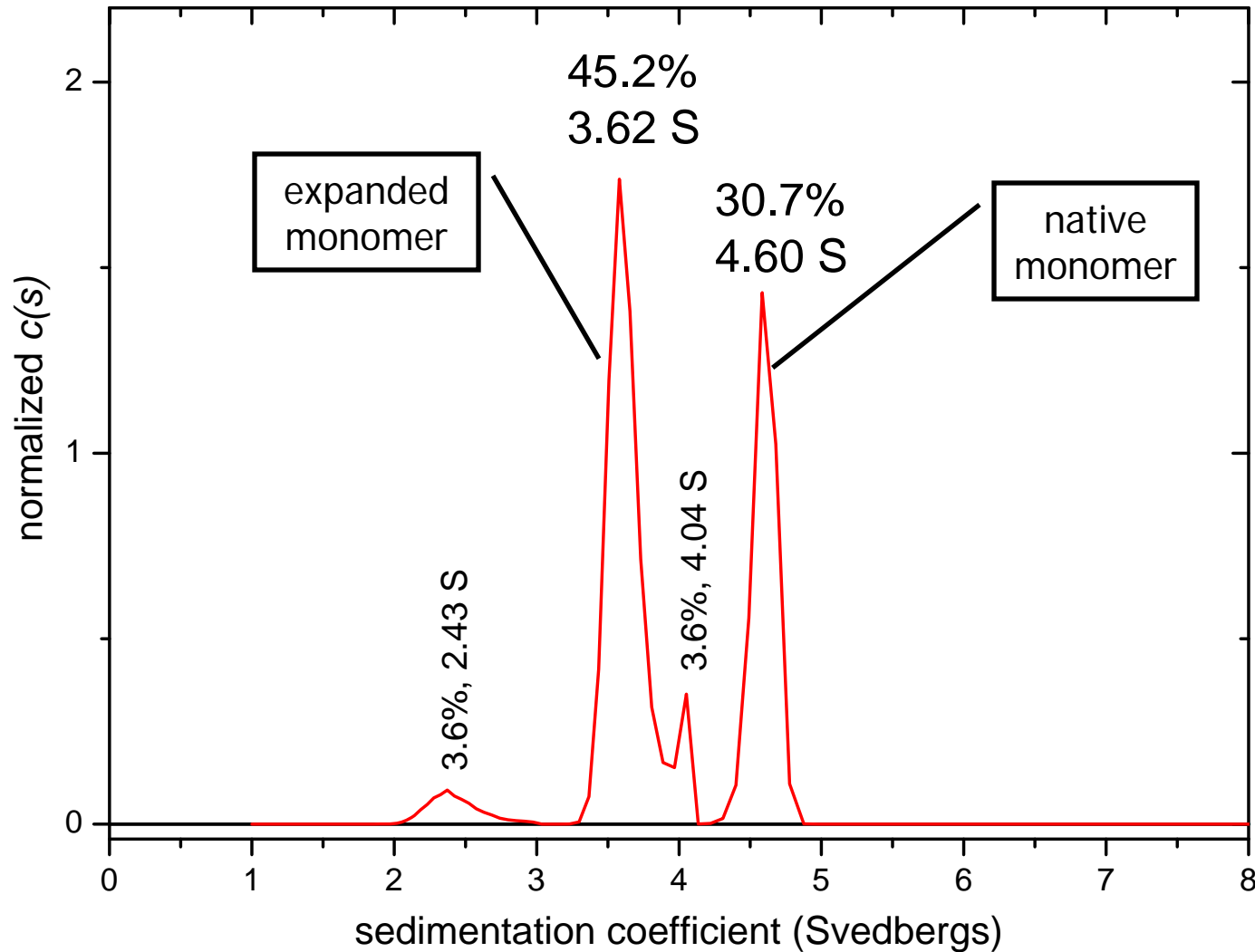
This highly stressed sample of a VaxGen test antigen showed high levels of an SEC peak eluting near the position expected for a dimer



However SEC-MALLS immediately shows that alleged aggregate is actually an altered form of monomer!



Sedimentation velocity confirms formation of an expanded monomer that sediments slowly

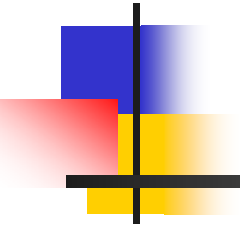




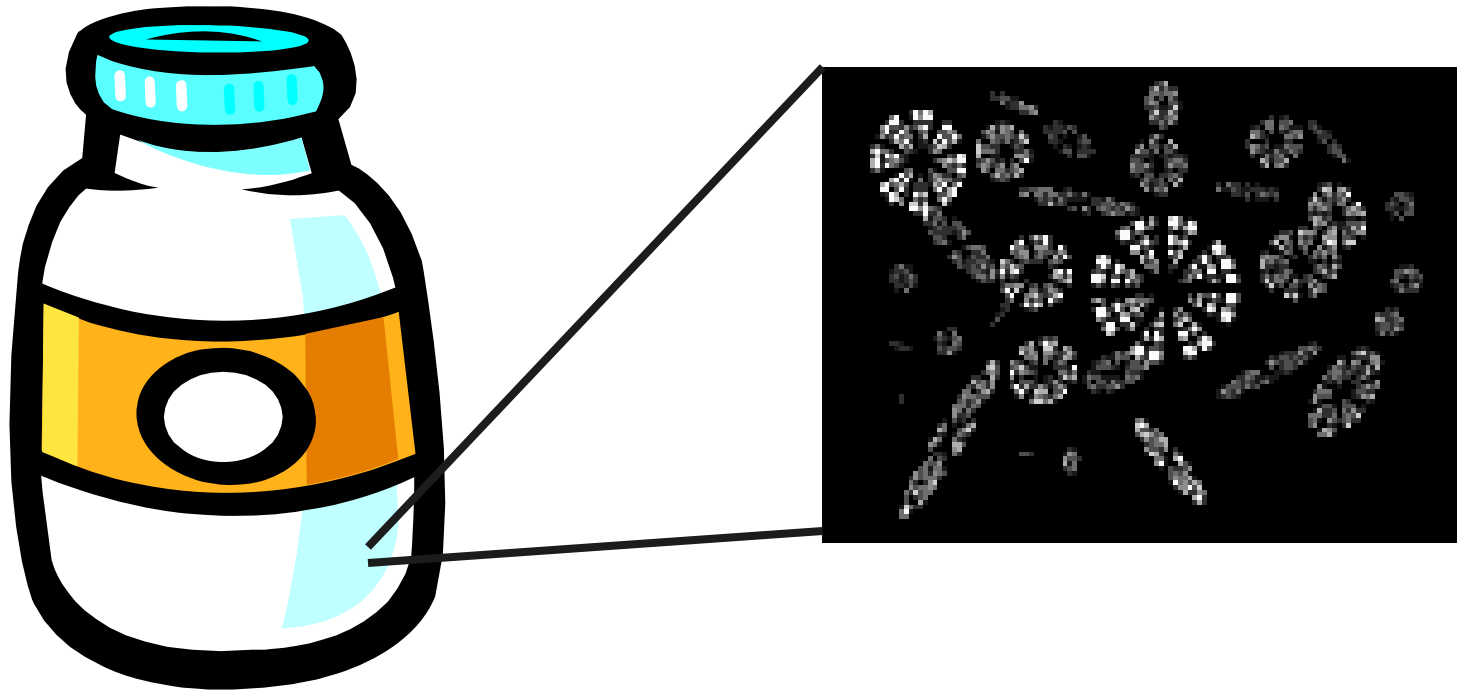
SEC-MALLS is also very useful for protein-protein or protein-DNA interaction studies

- Establishing stoichiometry is a key part of these studies
 - no point in extensive data fitting with the wrong model
- SEC-MALLS can easily determine the stoichiometry of the complex in many cases
 - typically if the K_d is 1 μM or less the complex will not dissociate on the column

Visible particulates and precipitation problems

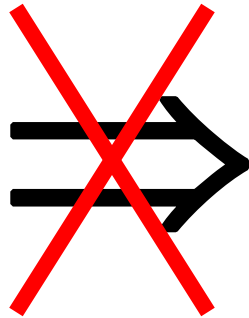


One particularly vexing type of aggregation is “snow”
(a.k.a. “floaters” or “white amorphous material” [WAM])



- may only appear after many months
- often a nucleation-controlled reaction
- often $\leq 0.01\%$ of total protein

When this happens our valuable therapeutic protein can only be used for...



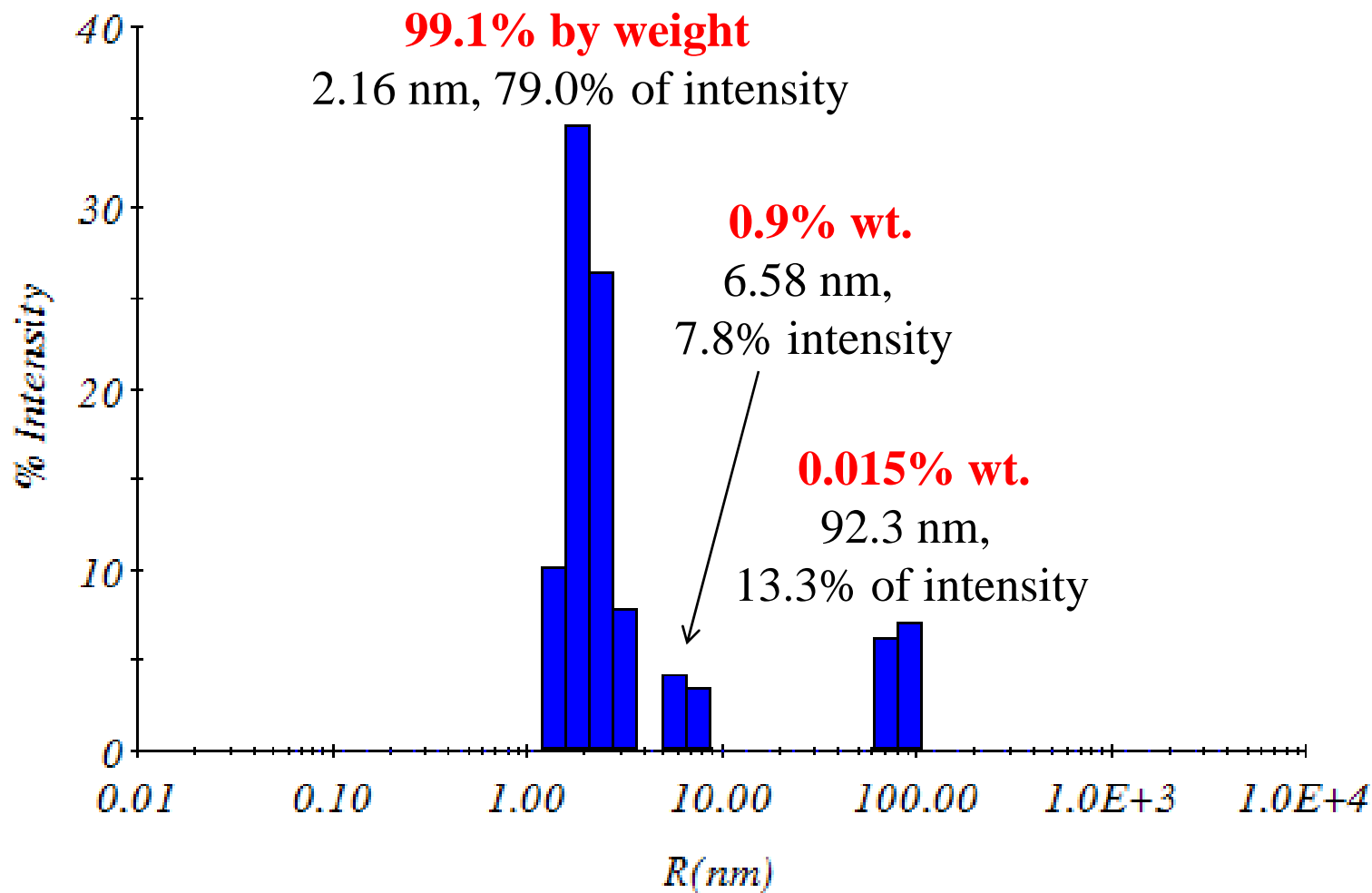
Dynamic scattering is one of the few tools that may be able to detect the precursors that eventually form 'snow'



Dynamic light scattering: the basic idea

1. In dynamic scattering we measure the fluctuations in scattering intensity (~ 100 ns to 30 ms)
2. The time scale of those fluctuations depends on the diffusion coefficient of the macromolecule, which in turn depends on its size
3. As in classical LS, the scattering intensity 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 3 peaks





Two key weaknesses of DLS

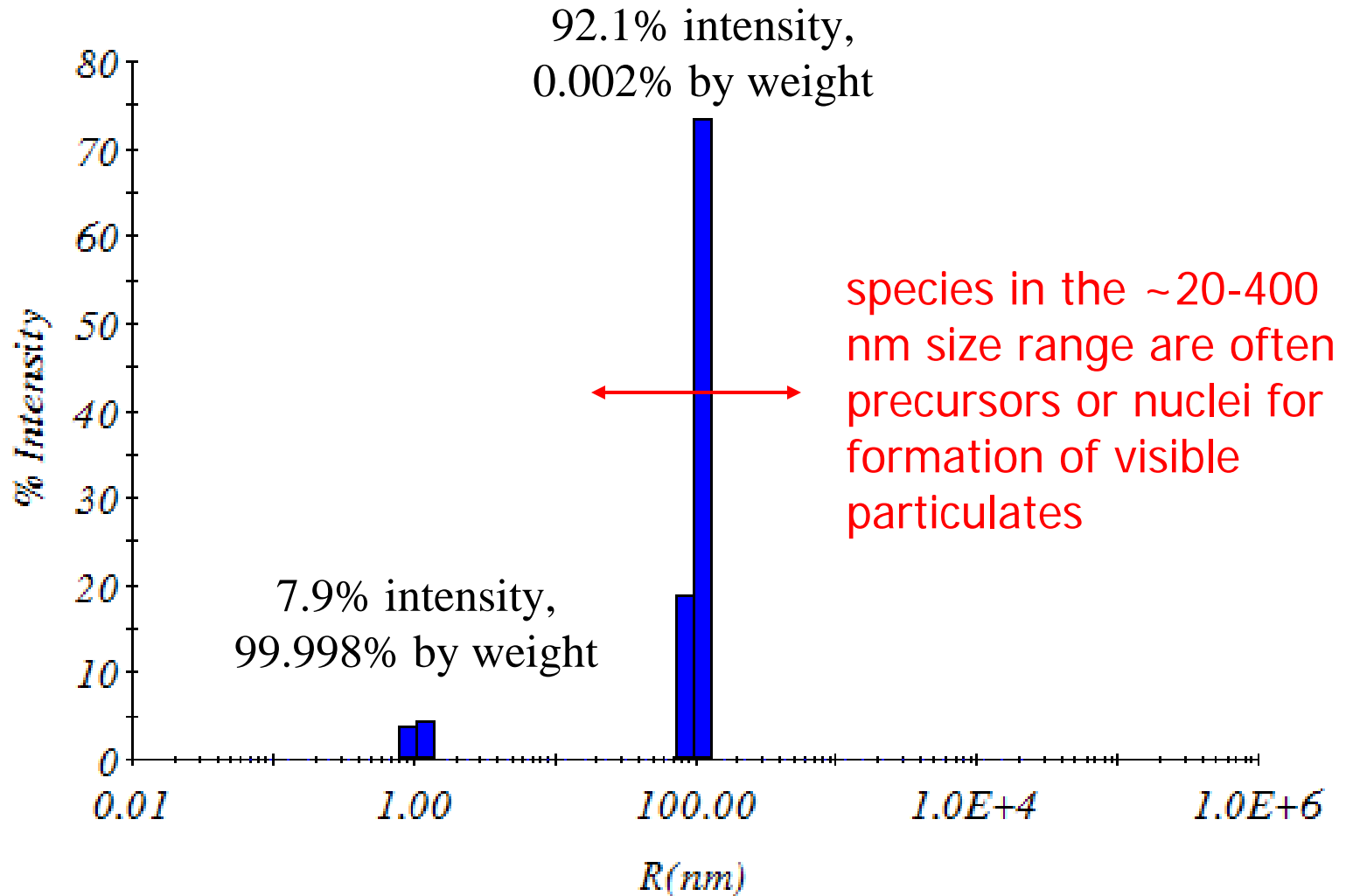
1. Low resolution

- two species are not resolved as separate peaks unless their radii differ ~ 2 -fold (~ 8 -fold in mass)
- consequently DLS is generally not useful to detect or quantify small oligomers (dimer-octamer)

2. Poor quantitation of weight fractions

- Usually at best the reproducibility of weight fractions is only \pm a factor of 2
- There is no universally-accepted standard algorithm to calculate weight fractions; different methods can give quite divergent results

Here is an example for a small peptide that forms visible thread-like particles





In my hands DLS has been the most effective tool for detecting precursors of visible particulates

1. Useful for qualitative assessment of different formulations, 'good' *vs.* 'bad' lots
2. Useful to track where in the manufacturing process damage to the protein is occurring
 - in one case tracked to specific pump
 - in another case tracked to viral filtration step
3. Useful to detect contaminant particles that can serve as nuclei onto which protein aggregates (heterogeneous nucleation)
 - silicones
 - glass particles from vials
 - vacuum pump oil from lyophilizers



Field-flow fractionation (FFF)

Principles of cross-flow FFF

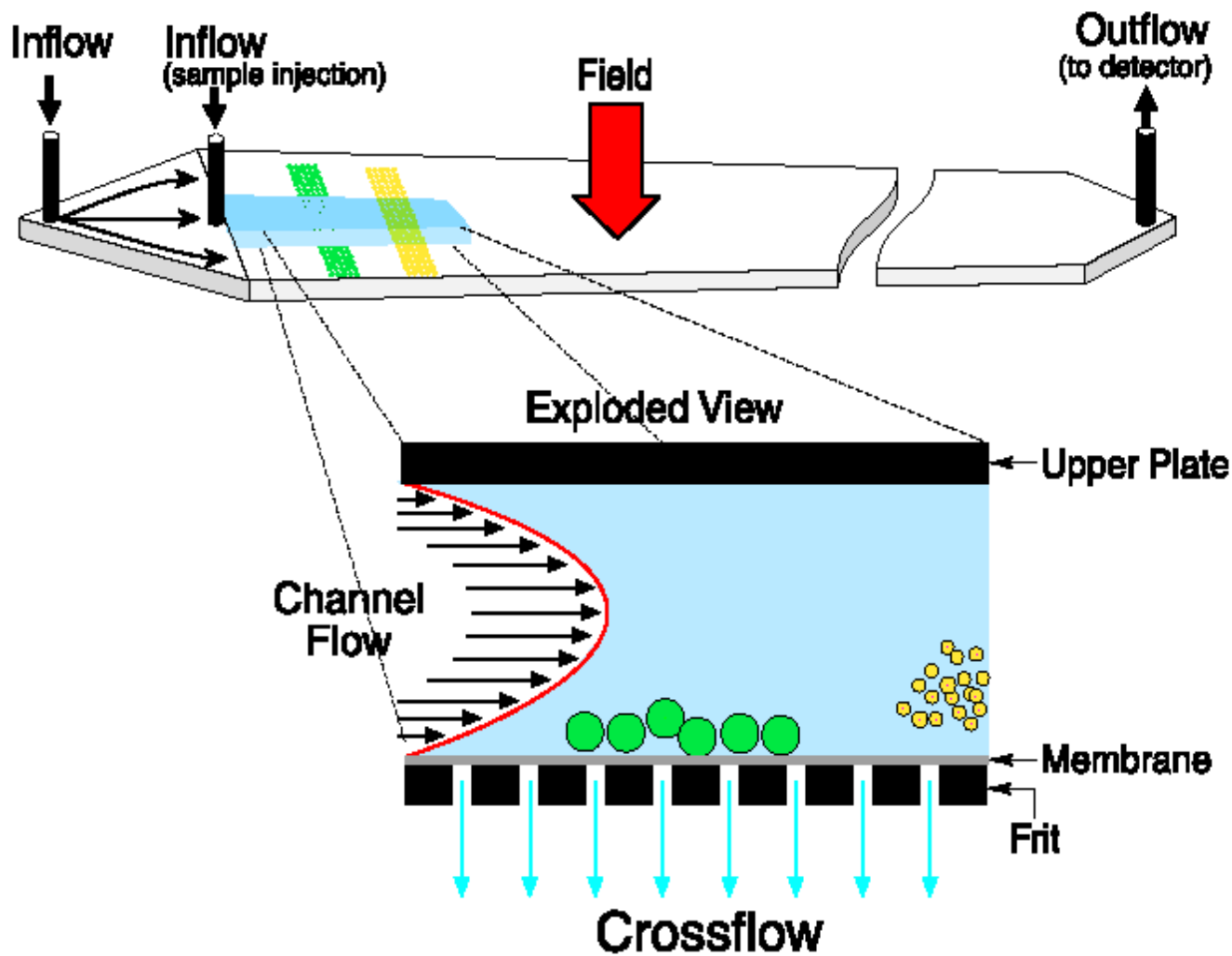
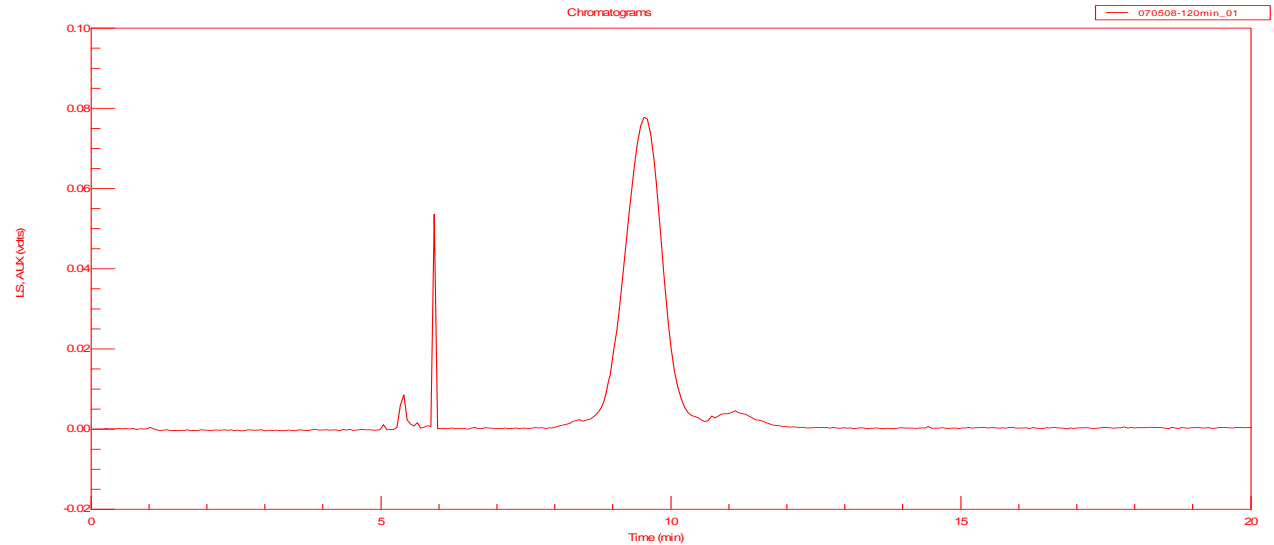


figure courtesy Wyatt Technology

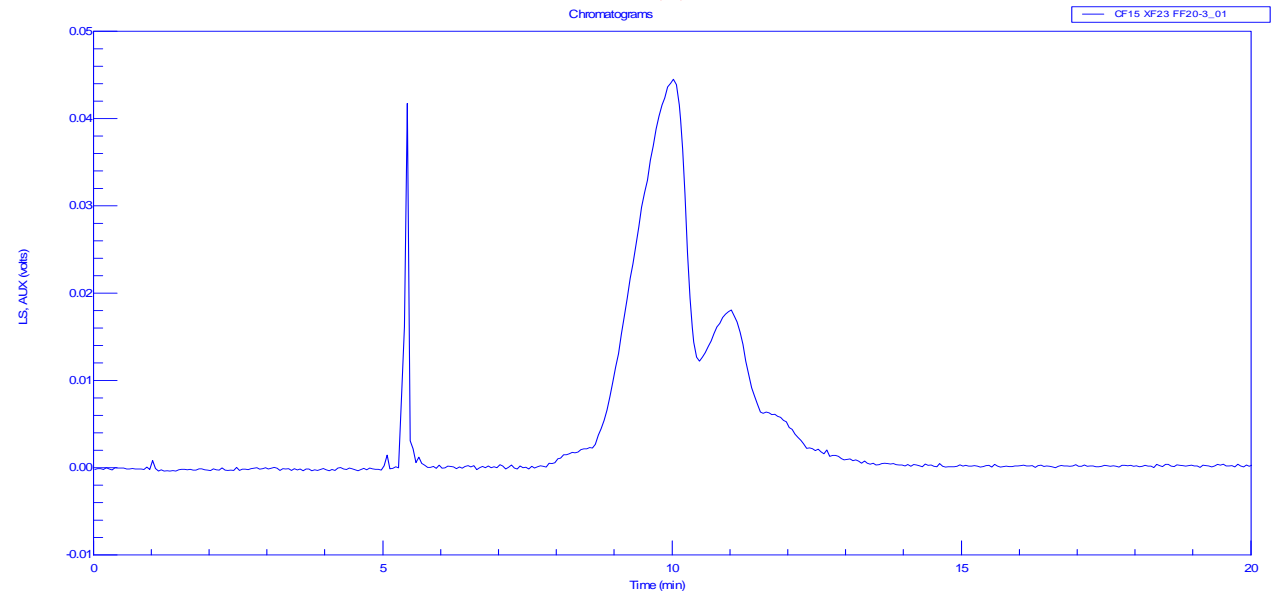
FFF of an acid-exposed monoclonal antibody

[courtesy D. Ejima (Ajinomoto) and K. Tsumoto (U. Tokyo)]

FFF using 0.1 M
citrate, pH 2.9



FFF after titration
to neutral pH, elute
using 0.1 M
phosphate, pH 6.8





Advantages & drawbacks of FFF

- main advantages

1. much less surface area for absorption of sticky aggregates than SEC columns
2. can separate a much wider range of aggregate sizes than SEC

- drawbacks

1. some proteins stick to all the available membranes
2. many parameters need to be optimized during method development



Conclusions

1. Sedimentation velocity can be quite useful for measuring distributions of long-lived aggregates
2. SV is the primary tool we use at APL to cross-check SEC methods (and help improve them)
3. No one method really covers the entire spectrum of aggregate types and sizes, so other methods like light scattering or FFF can also play important roles
4. Our ability to characterize aggregates unfortunately far exceeds our knowledge of how specific aggregate types affect product safety or efficacy