Focus on aggregation: causes, impact & characterization

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Outline

- Why do we care about aggregation in biopharmaceuticals?
- Review some basic facts about aggregate sizes and types
- Mechanisms for aggregation
- Utility of sedimentation velocity for analysis of aggregation
- Introduction to classical light scattering used on-line with SEC (SEC-MALLS)
- A few words about field-flow fractionation (FFF)

Protein aggregates: What is all the fuss about?

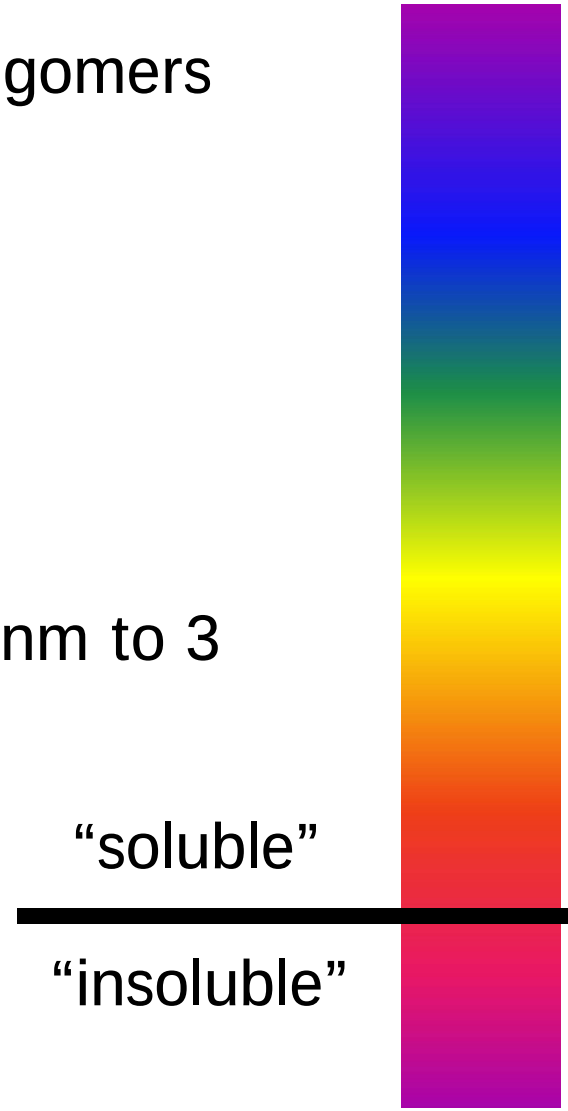
- Aggregates (or partially assembled states for products like VLPs that are supposed to be associated) are often a major degradation product
  - Hence they often are a major factor limiting shelf life
- Aggre[illegible]tates in the pr[illegible]

Size matters...

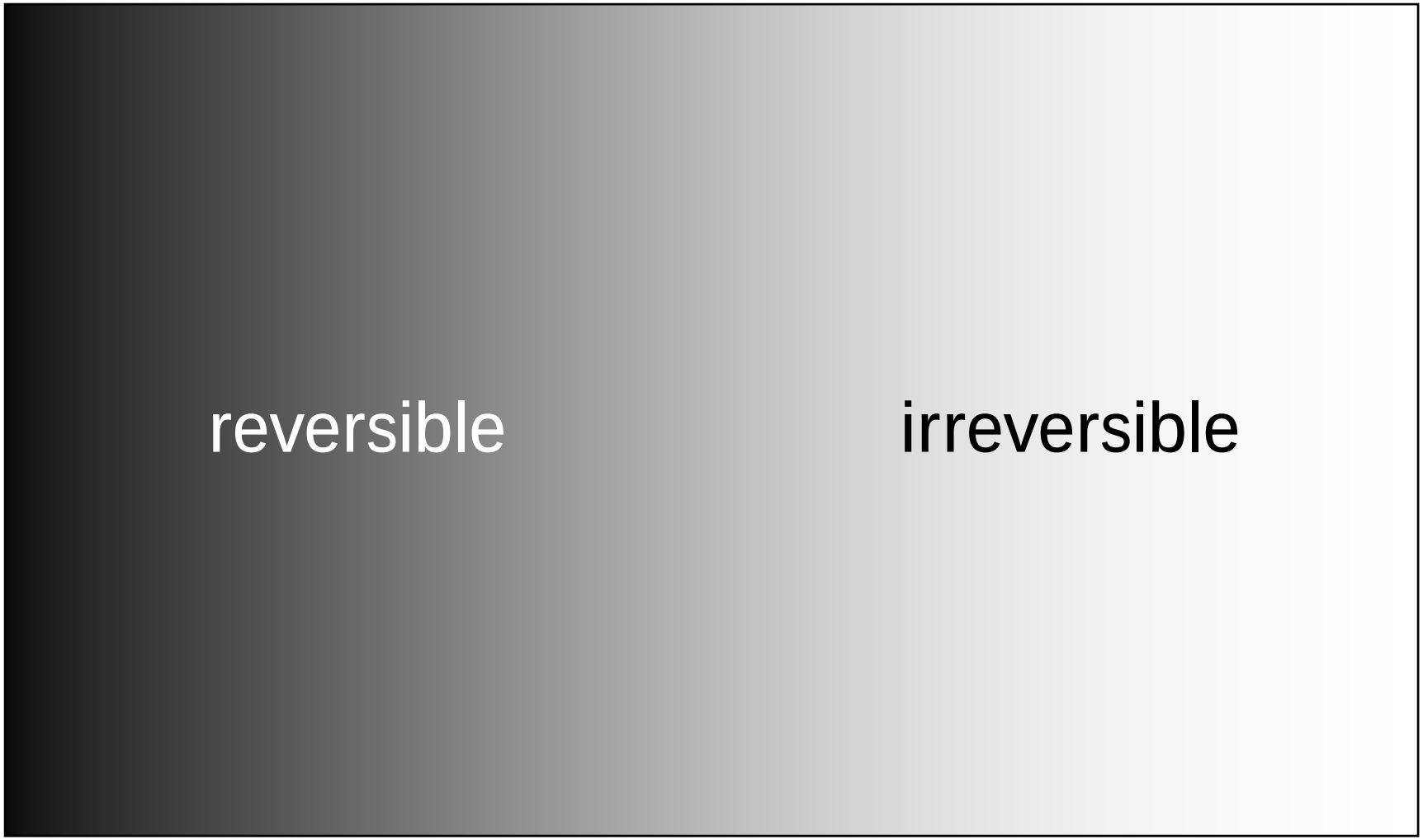
1. manufacturability
   - clogged columns or diafiltration membranes
2. bioactivity (potency)
3. serum half-life or absorption rate
4. **immunogenicity**

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



Reversible *vs.* irreversible aggregates



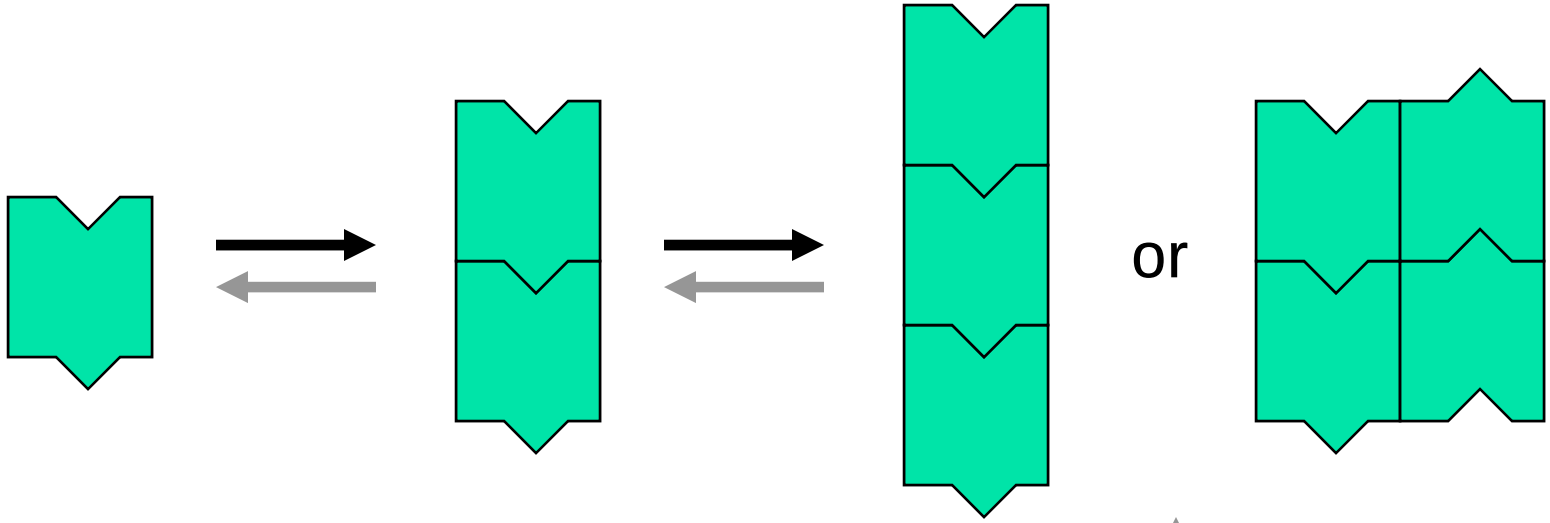
Whether aggregates are “irreversible” or “reversible” depends on the context

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

Aggregates have a spectrum of lifetimes

- rates of non-covalent association and dissociation (half-times) can vary from milliseconds to days
- many common analytical methods will detect only the longer-lived species
- metastable oligomers with dissociation rates of hours to days occur fairly frequently
  - likely true for large structures like VLPs or KLH
  - not uncommon for small proteins, monoclonal antibodies
- it may take hours to days for the size distribution to re-equilibrate after a change in concentration, solvent conditions or temperature

Aggregation mechanisms (1): reversible association of native protein



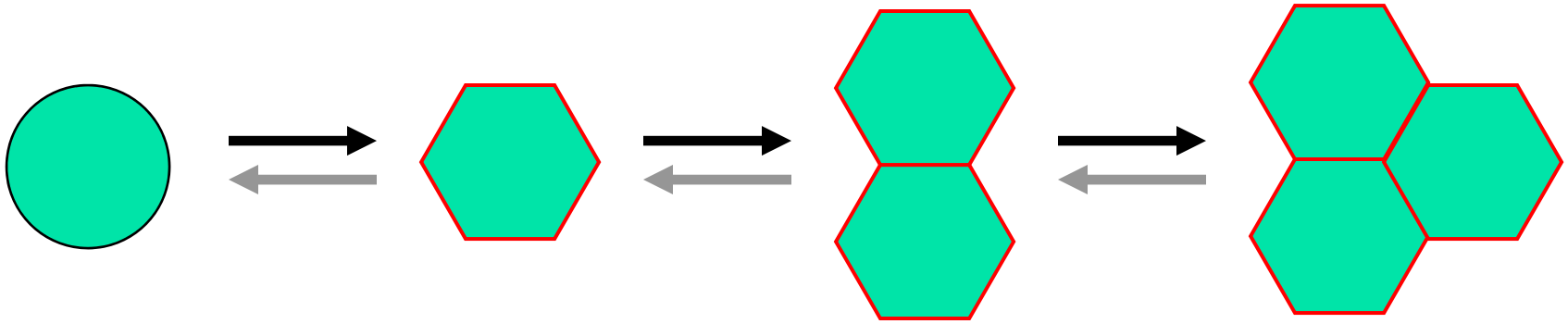
Native protein

Reversible oligomerization

Higher oligomers (possibly irreversible)

↑ + sucrose

Aggregation mechanisms (2): oligomerization following conformational change



Native protein

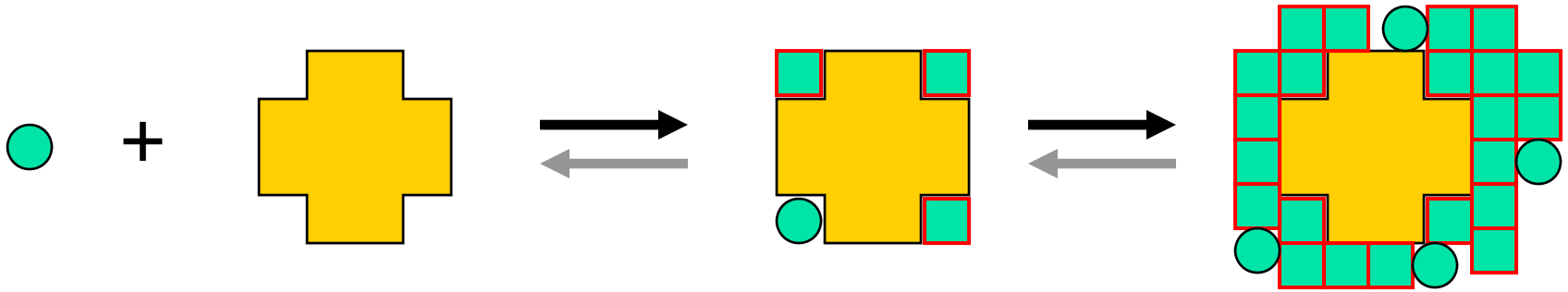
Conformational change or partial unfolding

Oligomerization of non-native protein

Higher oligomers (probably irreversible)

↓ + sucrose

Aggregation mechanisms (3): nucleation controlled aggregation (“seeding”)



Native protein

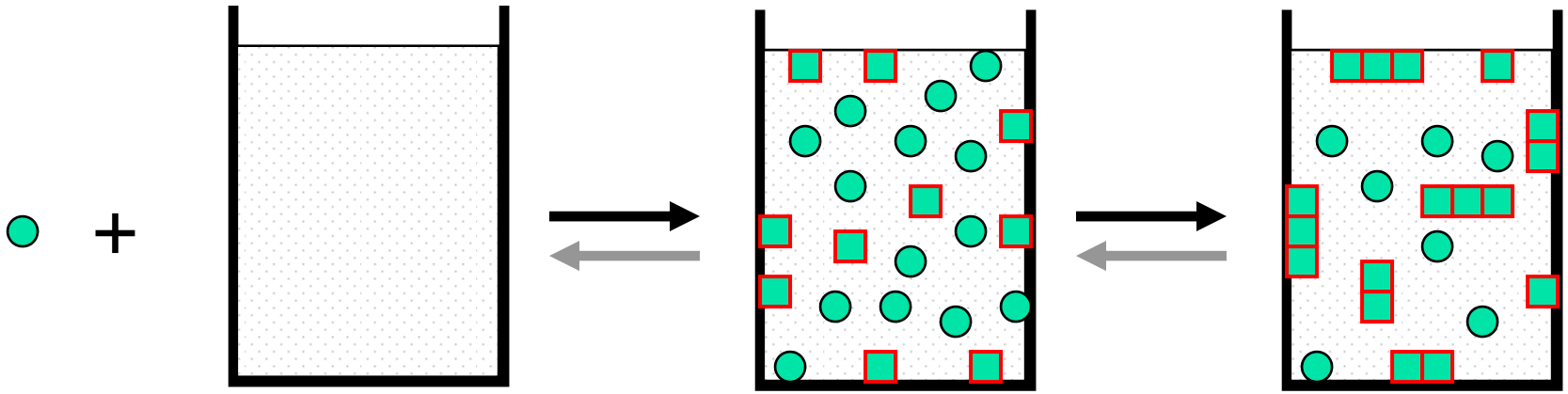
Critical nucleus (aggregate of native or modified protein, or a contaminant)

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

Visible particulates or precipitation

↑ + sucrose

Aggregation mechanisms (4): 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

Our analytical challenge

1. Any 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**	**FFF**
dilution	+++	+	++
change of solvent conditions	+++	-	++
adsorption to surfaces	+++	+	++
physical filtration (*e.g.* column frit)	+++	-	-
physical disruption (*e.g.* shear forces)	++	-	-
creation of new aggregates can be caused by:			
change of solvent conditions	+++	-	++
surface or shear-induced denaturation	++	-	+
concentration on surface	-	-	++

Regulatory concerns about analytical methods for aggregation/assembly

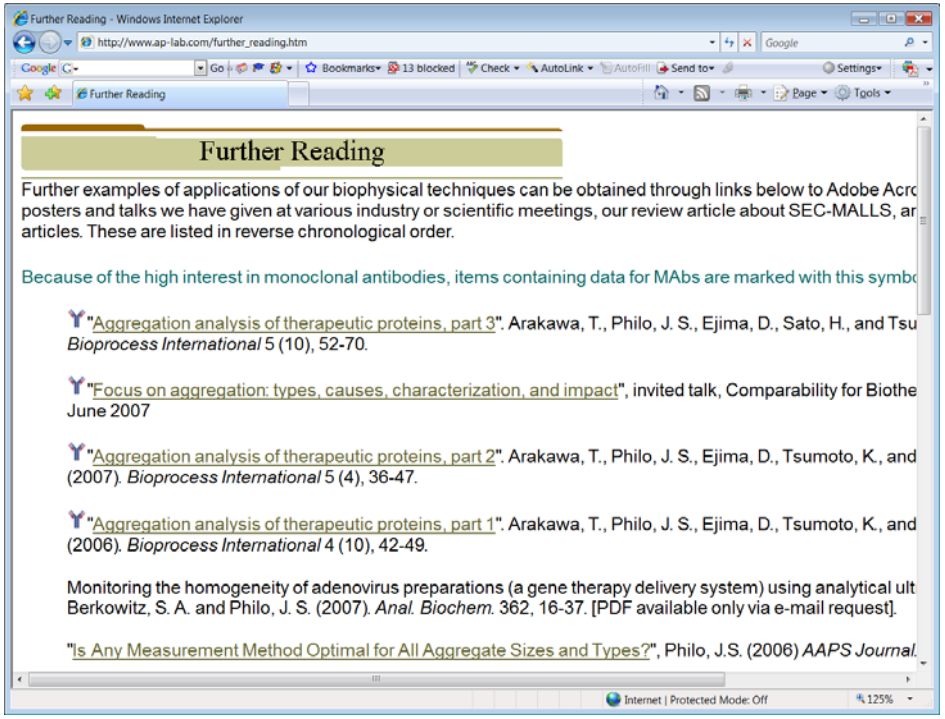
- Adverse events and other problems with certain protein therapeutics have heightened awareness of the limitations of common analytical methods
- For protein therapeutics they will now nearly always ask for cross-validation of sizing protocols by orthogonal methods
- May see some spill-over over these concerns to vaccine products

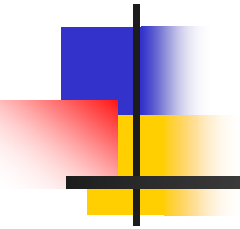
Alternatives to SEC for ‘native’ sizing

- analytical ultracentrifugation (AUC)
  - sedimentation velocity (primarily)
  - sedimentation equilibrium (occasionally)
- light scattering
  - flow mode classical scattering used after SEC (SEC-MALLS) ← has been validated
  - batch mode dynamic light scattering (DLS)
  - batch mode classical scattering ← has been validated
- field-flow fractionation (FFF)
  - usually used with MALLS to measure true MW

Time won't permit talking about dynamic light scattering or in detail about other methods today, but...

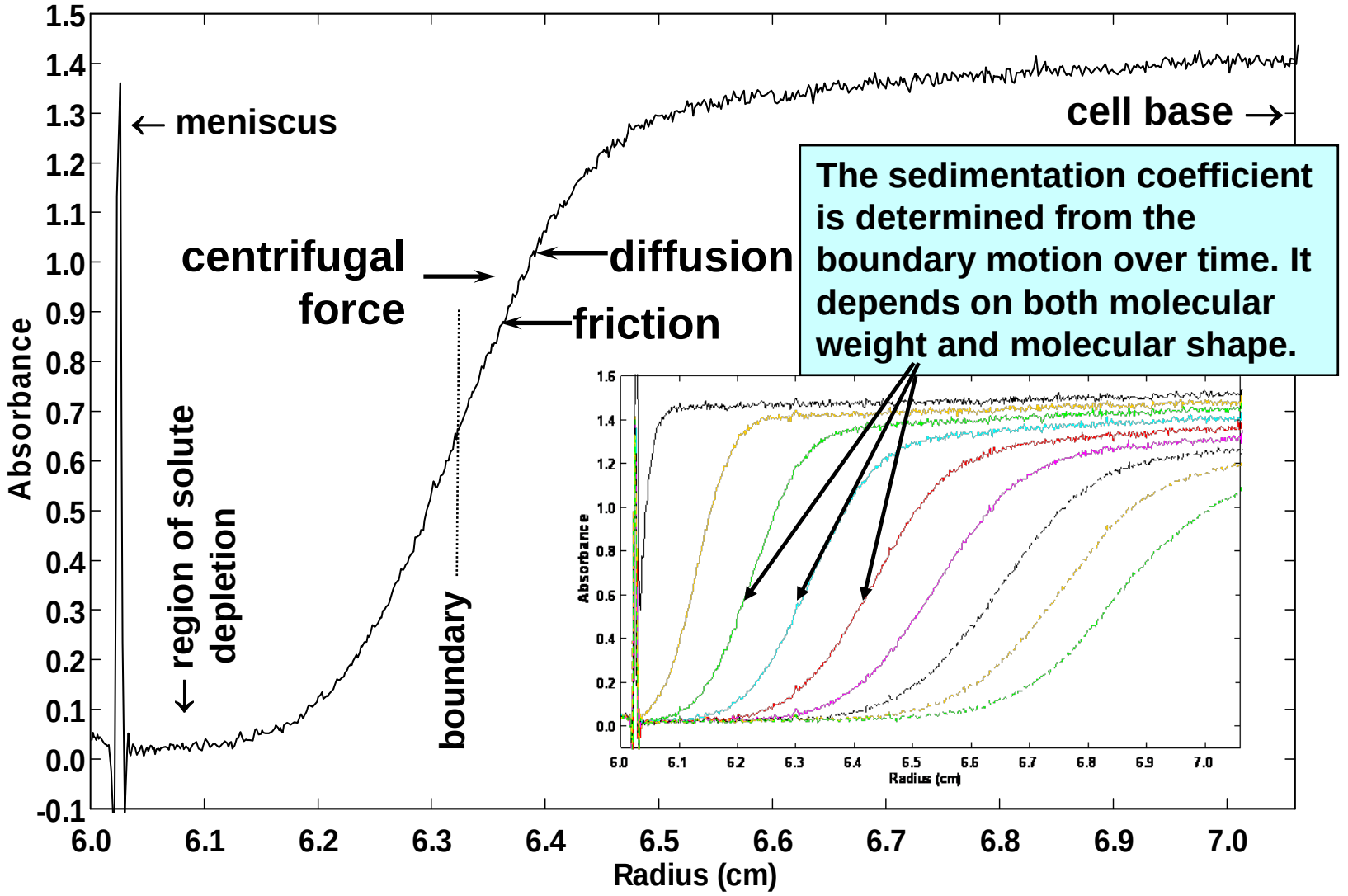
- Background and examples for DLS, SV, and SEC-MALLS can be found on the APL web site, www.ap-lab.com
- Many articles, talks, and posters on aggregation and comparability studies can be downloaded from our 'Further Reading' page



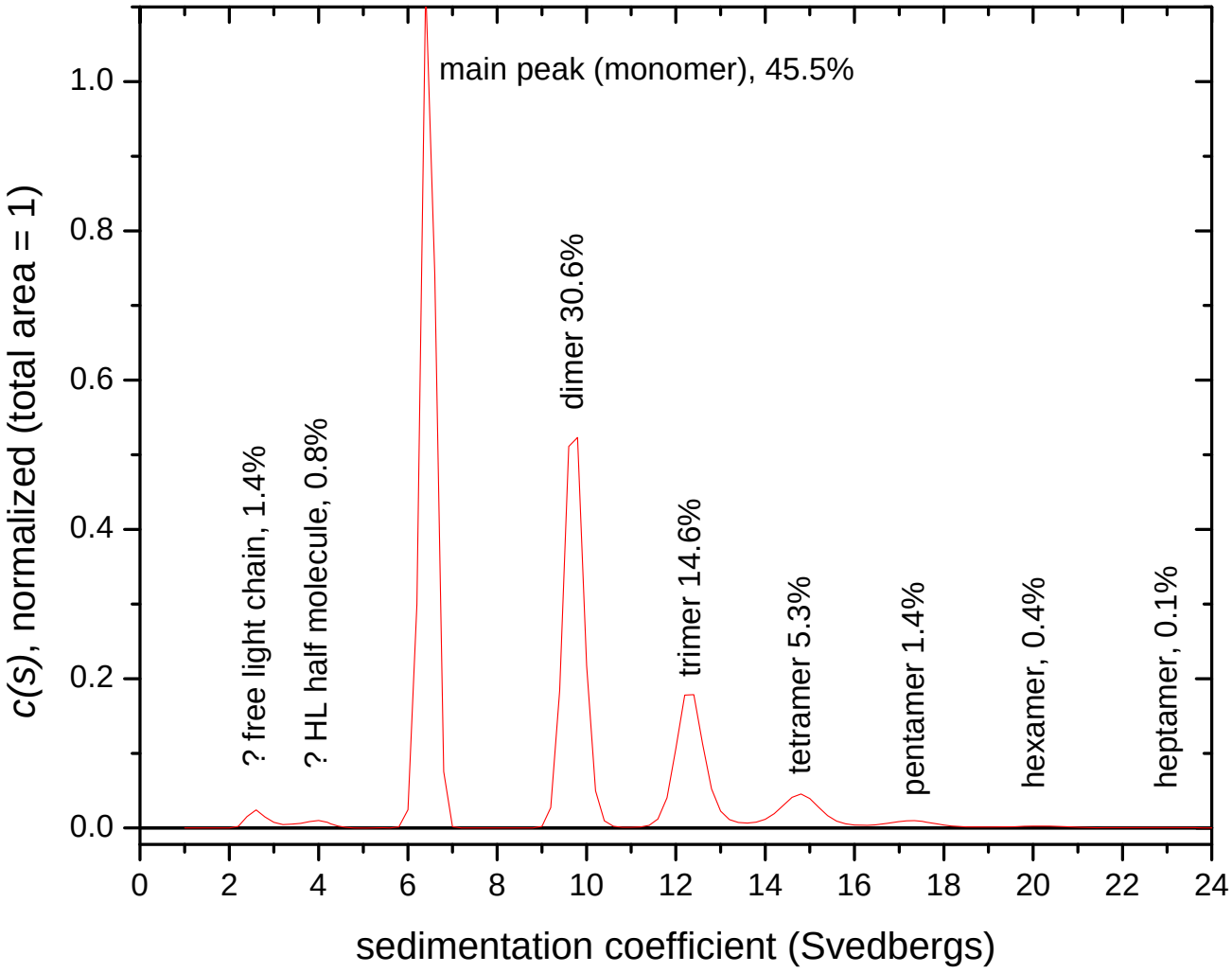


Sedimentation velocity

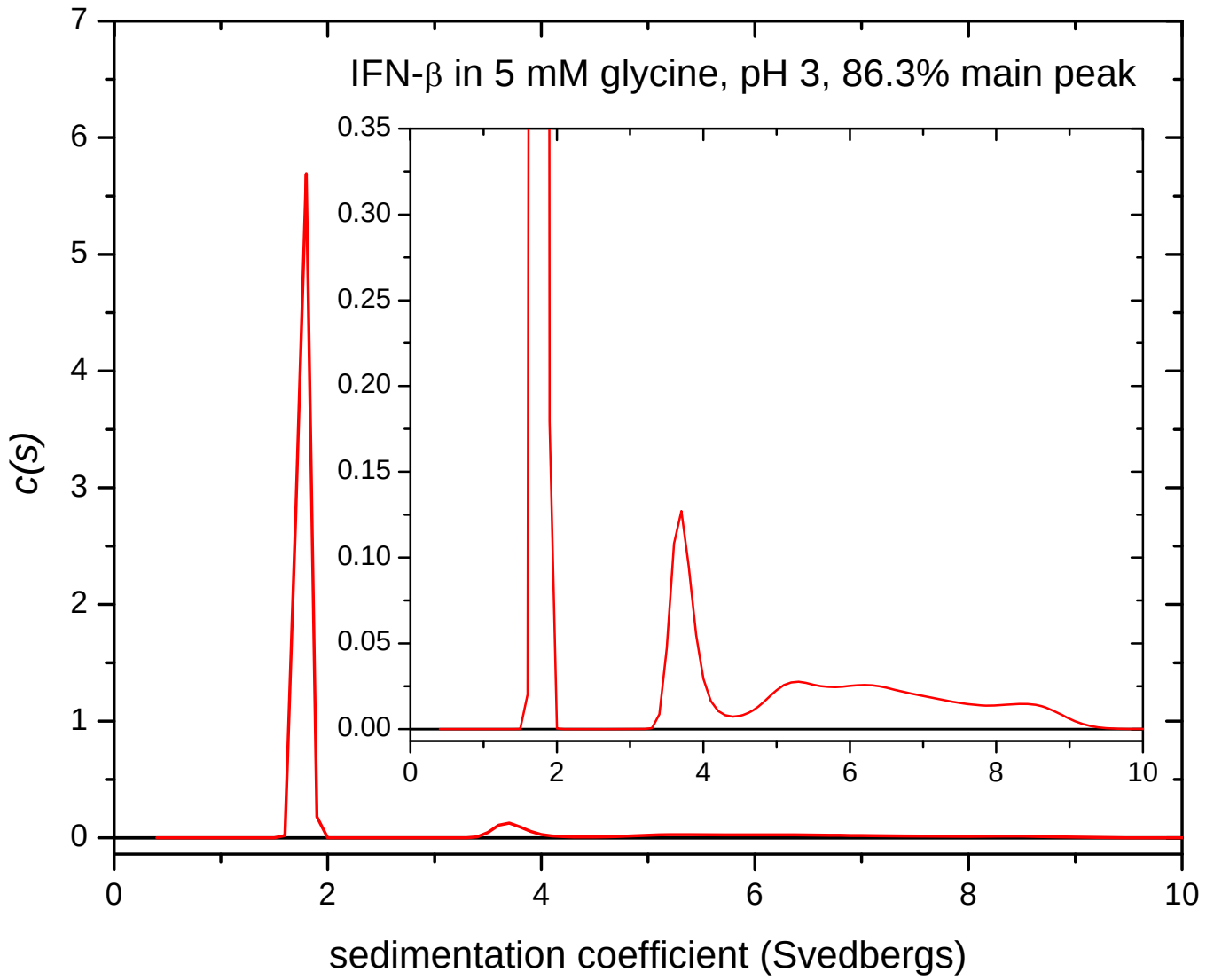
The fundamentals of sedimentation velocity



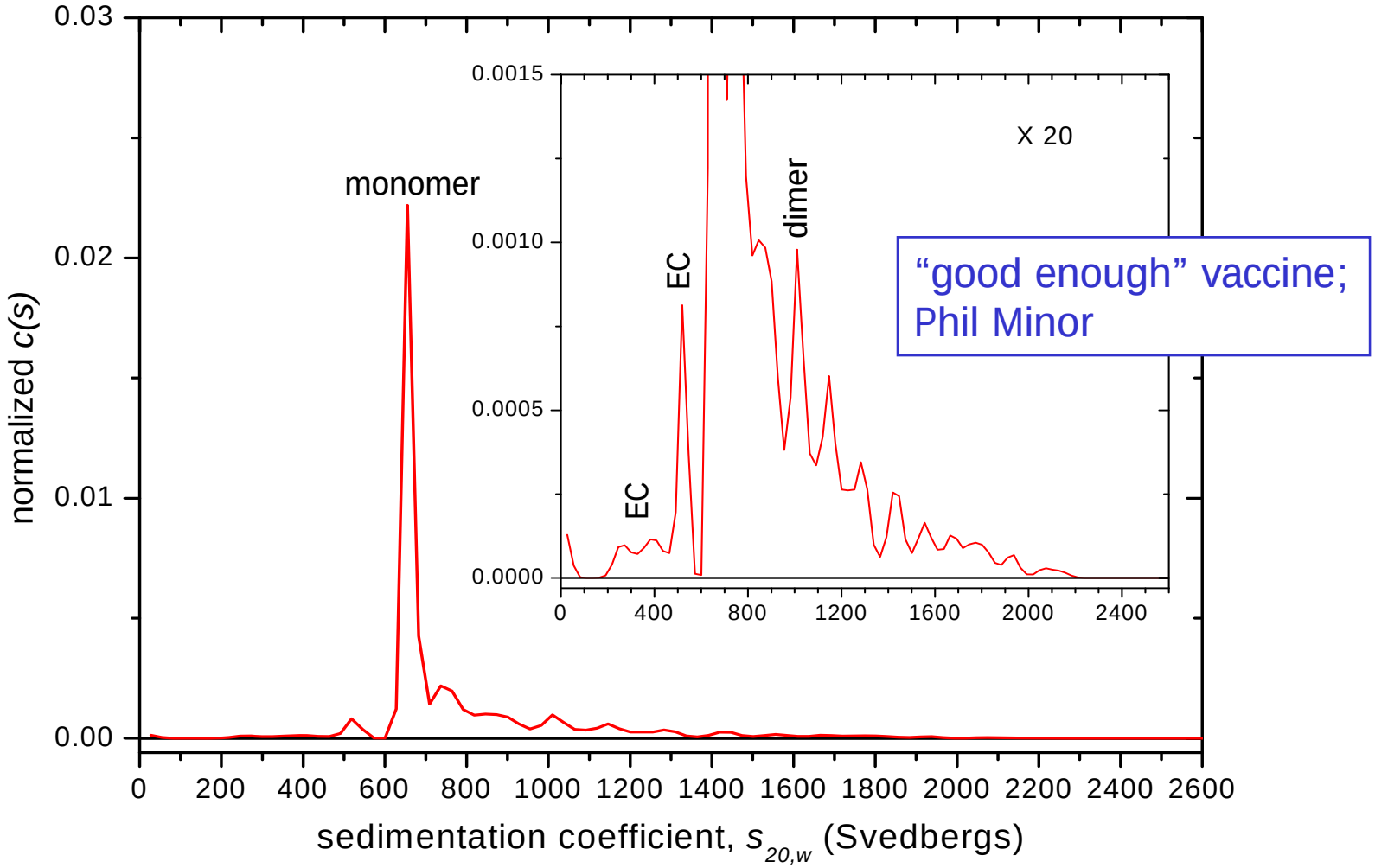
High resolution analysis of a highly stressed antibody sample resolves 6 aggregate peaks plus 2 fragments



This interferon-β sample is 13.7% non-covalent aggregate; by the standard SEC method it would be pure monomer



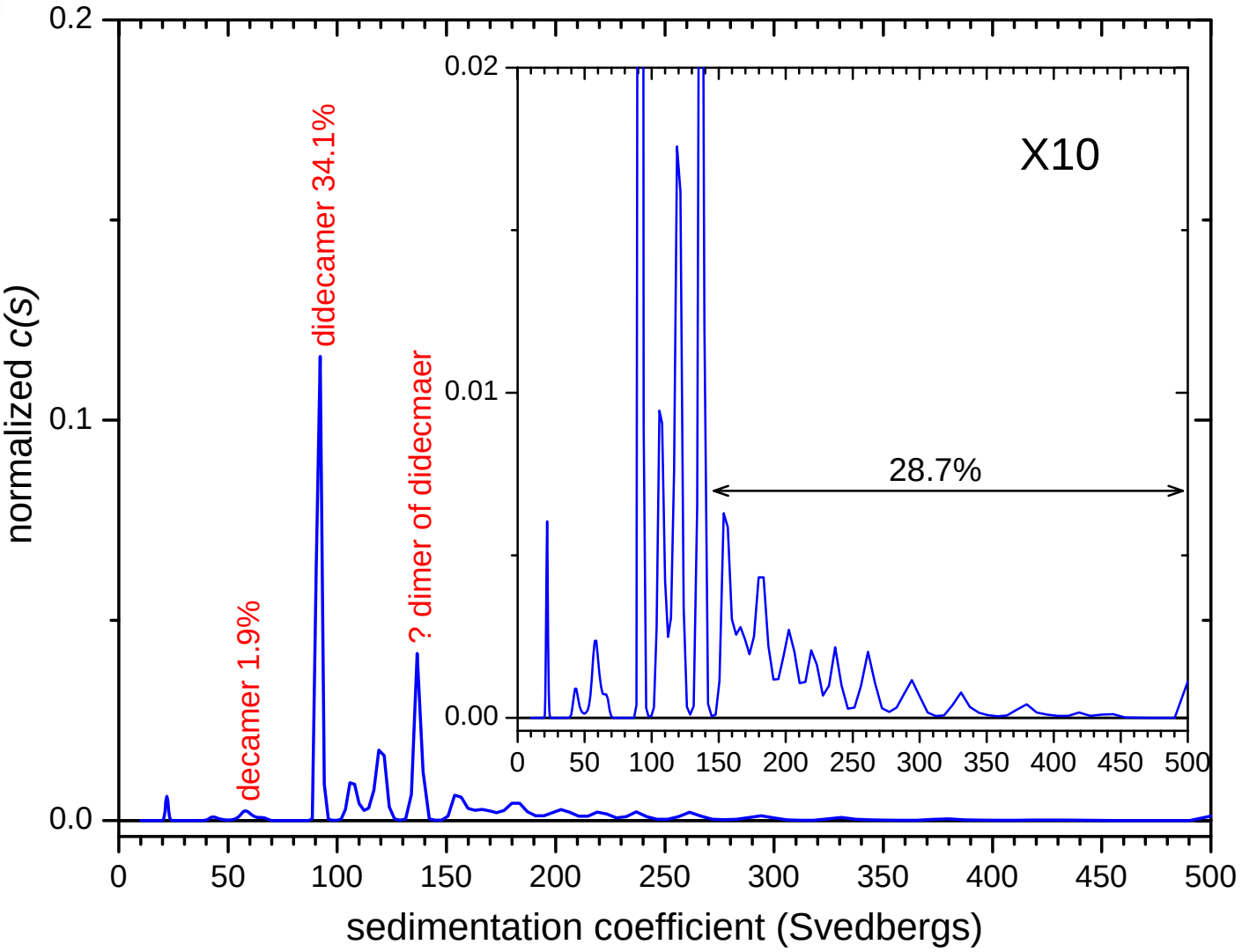
Detection of viral aggregates and empty capsids in an adenoviral gene therapy vector



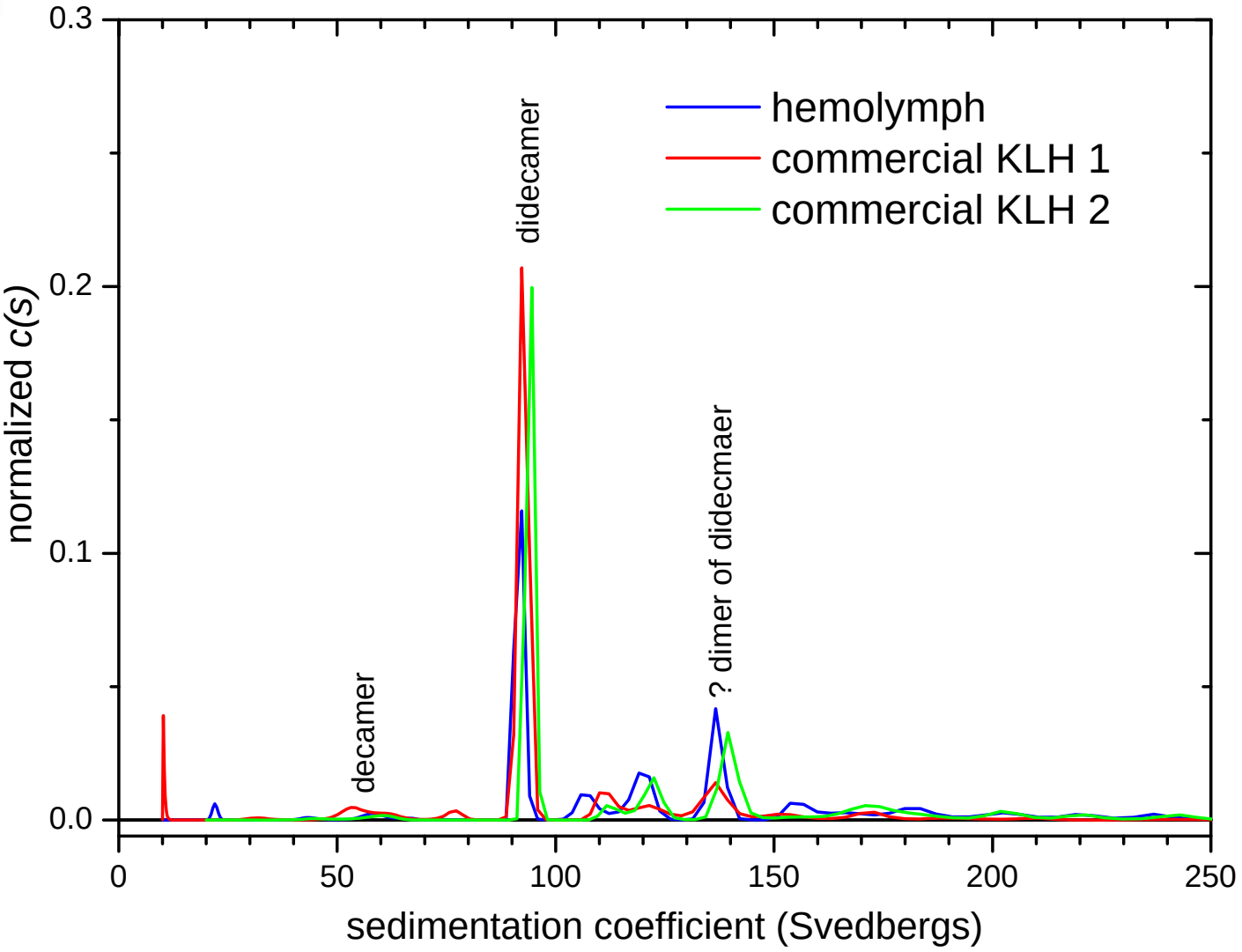
"good enough" vaccine; Phil Minor

Berkowitz, S. A. and Philo, J. S. (2007). Monitoring the homogeneity of adenovirus preparations (a gene therapy delivery system) using analytical ultracentrifugation. *Anal. Biochem*. 362, 16-37.

KLH size distribution in fresh, unpurified hemolymph from a single animal (monitored at 340 nm so only KLH is seen)

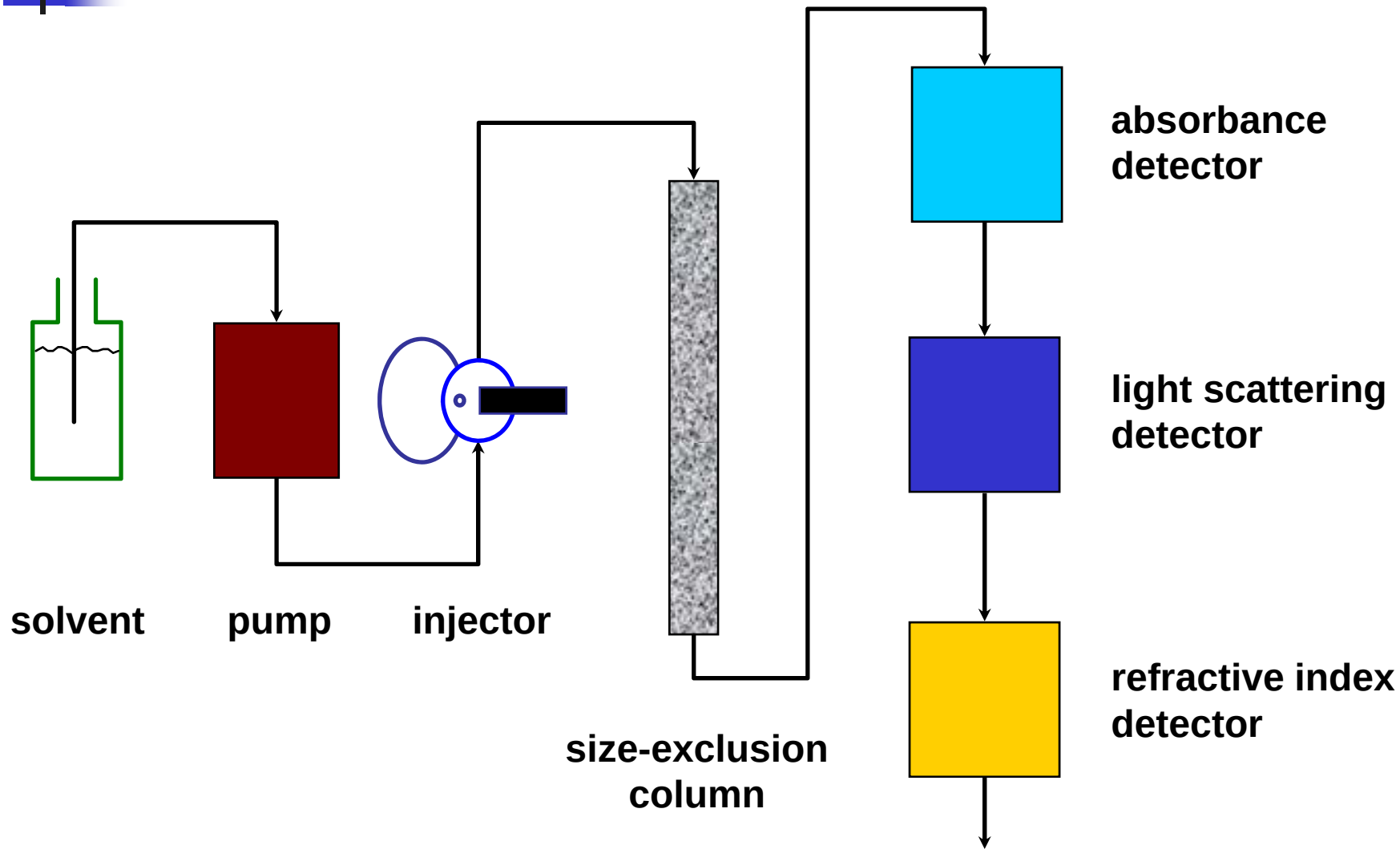


Size distributions for commercial KLH preparations differ from each other and only partially resemble that *in vivo*



Multi-angle classical laser light scattering used on-line with SEC (SEC-MALLS)

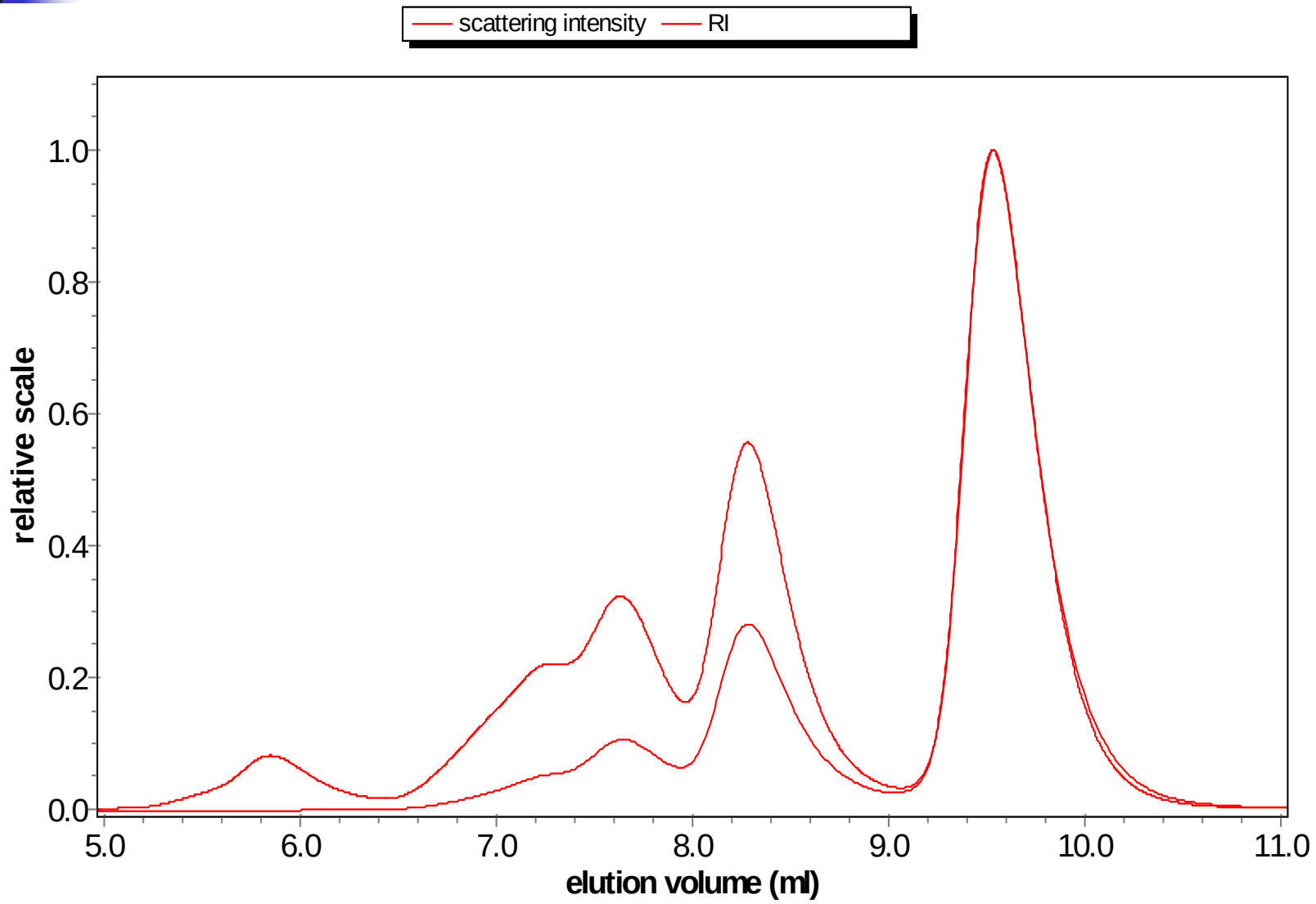
Typical setup for size-exclusion chromatography with on-line light scattering detection



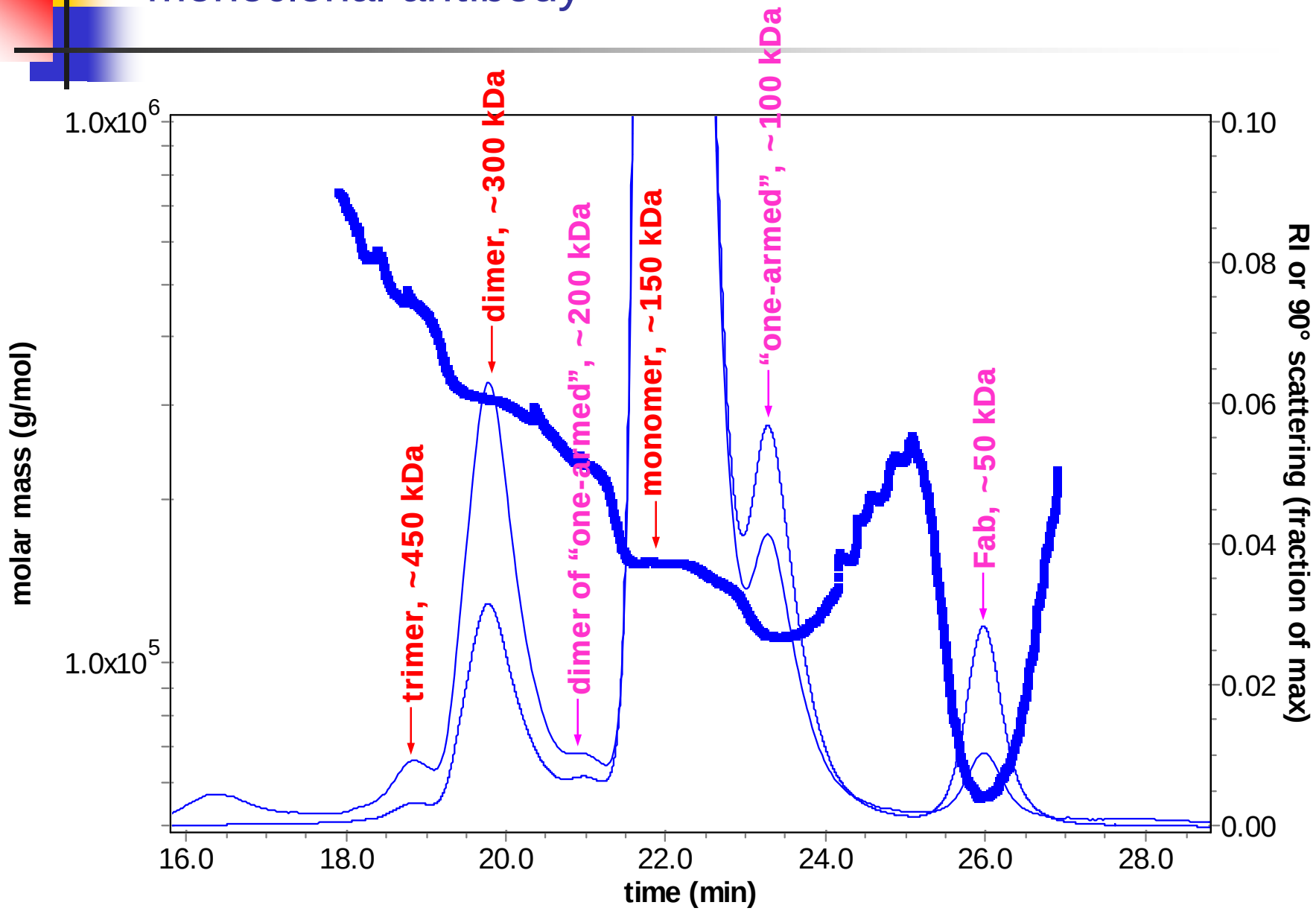
Getting molecular mass from static light scattering: the basic idea

- from theory, the amount of light scattered by the protein at some angle will be proportional to the product $c \times M \times (dn/dc)^2$
  - *dn/dc* is the "refractive increment" (difference in refractive index relative to solvent); its value is nearly identical for all non-conjugated proteins
- if 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 elution position

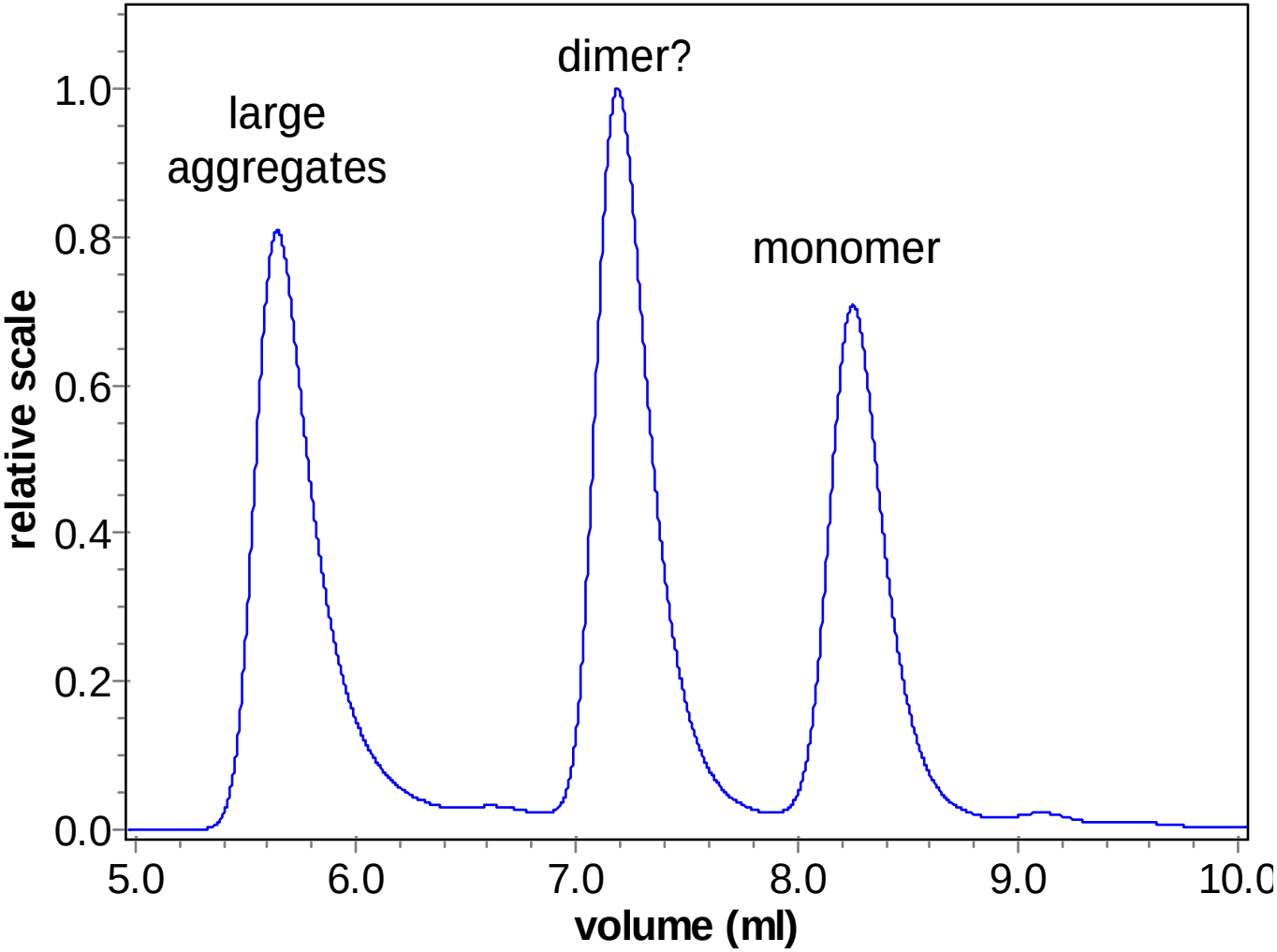
An example for an Fc-fusion protein: the aggregate signals are much stronger in 90° scattering than in the RI chromatogram



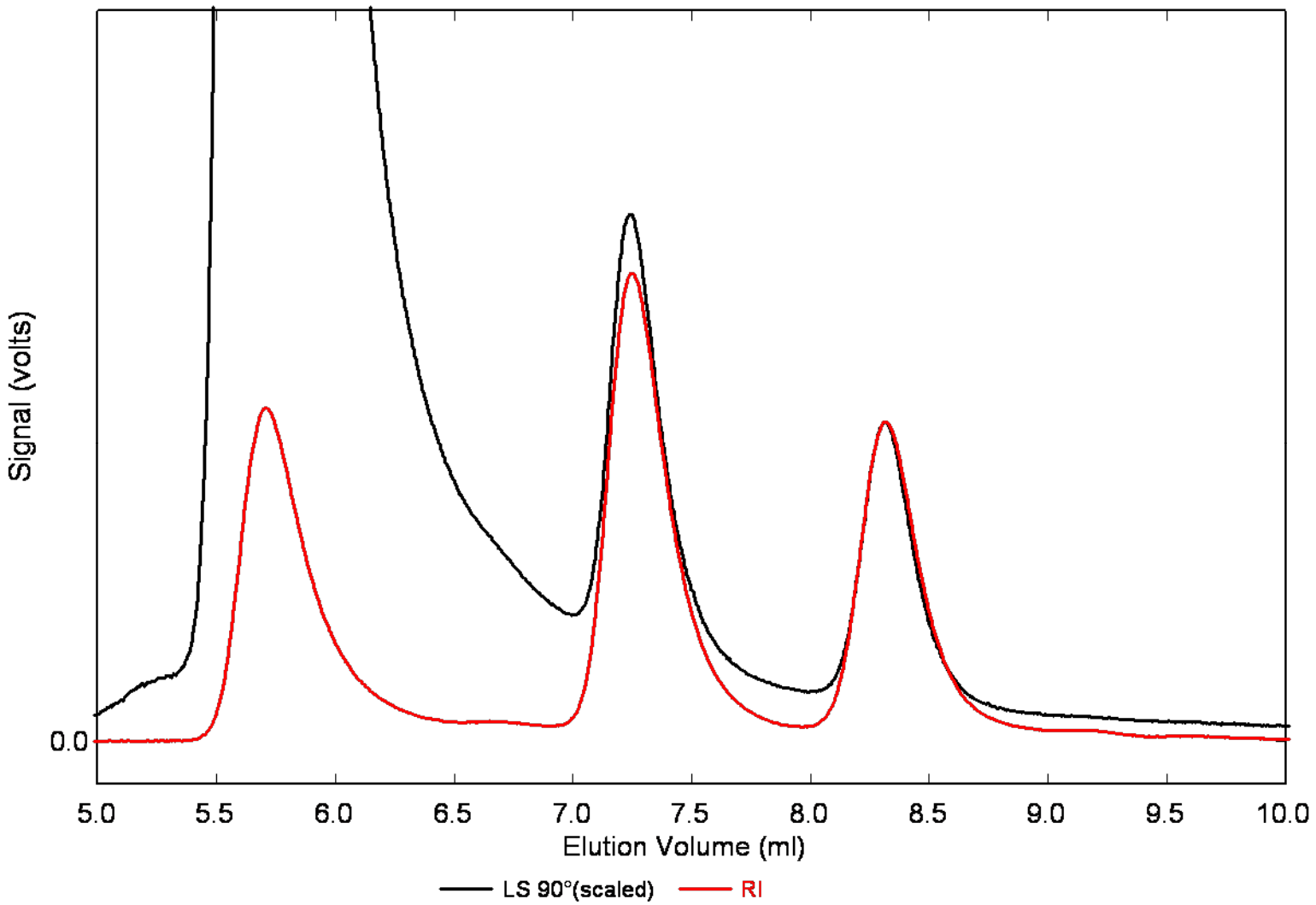
Identifying minor components in a heat-stressed monoclonal antibody



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



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



Field-flow fractionation (FFF)

Principles of cross-flow FFF

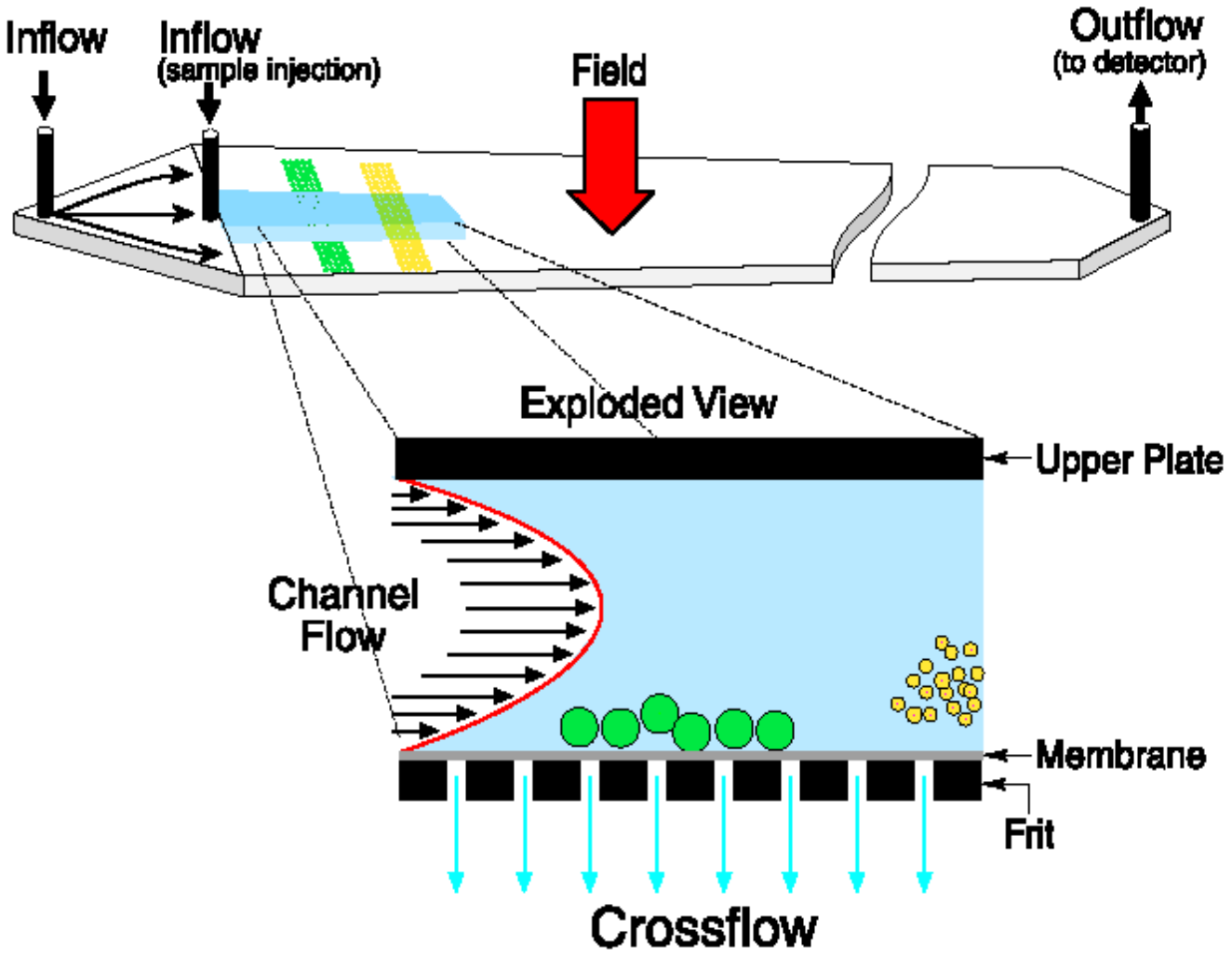
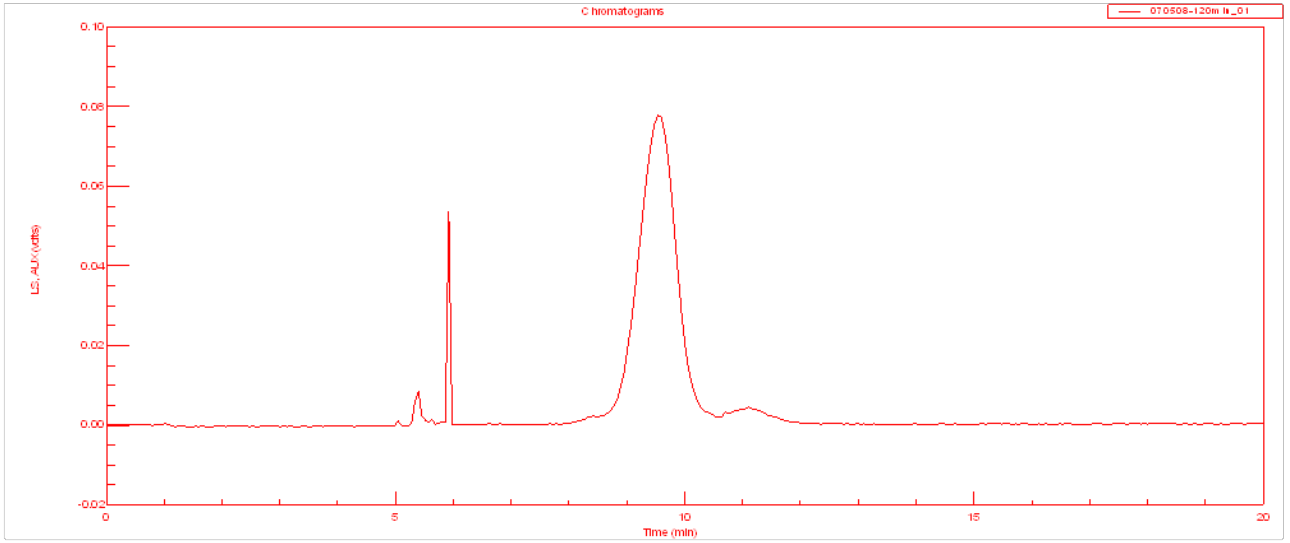


figure courtesy Wyatt Technology

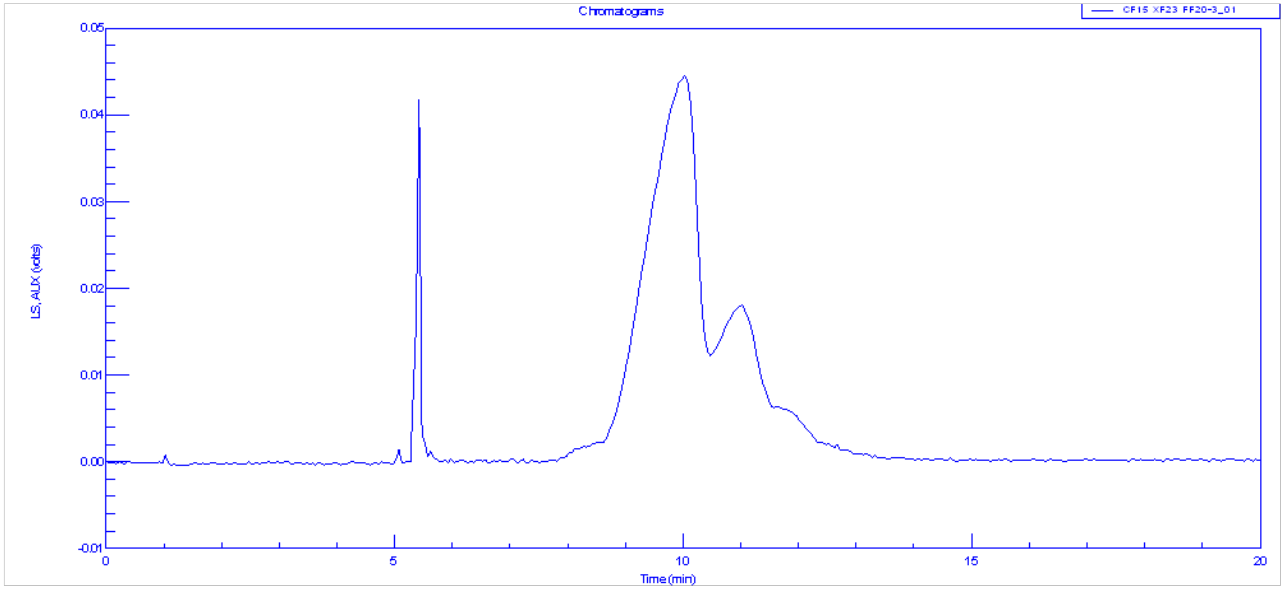
FFF of an acid-exposed monoclonal antibody

[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 sizes than SEC
- drawbacks
  1. some proteins stick to all the available membranes
  2. many parameters need to be optimized during method development
  3. dilution may dissociate reversible aggregates

Summary

1. Aggregation is a complex phenomenon!
2. No single analytical method is optimal for all types and sizes of aggregates
3. Alternative sizing methods tend to be complex, require highly skilled personnel, and are not generally usable for QC
4. Our ability to characterize aggregates or improperly associated species unfortunately far exceeds our knowledge of how such species affect product safety or efficacy