Focus on aggregation: types, causes, characterization, and impact

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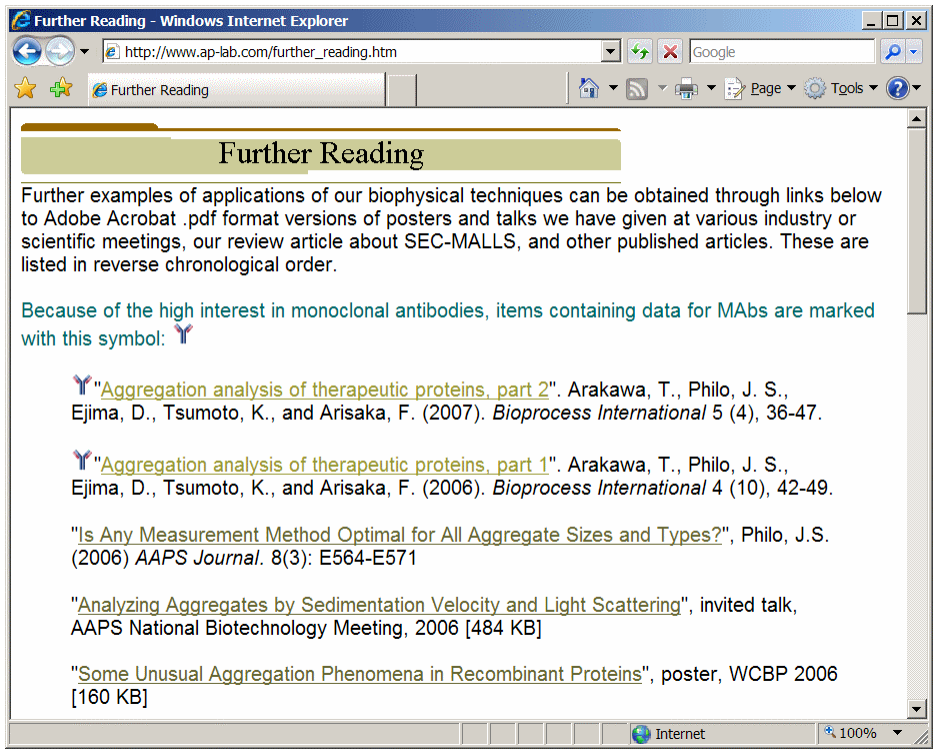
Outline

- Why do we care about aggregates in biopharmaceuticals?
- Review some basic facts about aggregate sizes and types
- Aggregation mechanisms
- Utility of sedimentation velocity for analysis of long-lived aggregates
- A few words about field-flow fractionation (FFF)

Time won't permit talking about light scattering techniques today, but...

- Background and examples 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



Protein aggregates: What is all the fuss about?

- Aggregates (both large and small) often are a major degradation product
  - Hence they often are a 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 made no new red blood cells and require regular transfusions (Pure Red-Cell Aplasia)
- While the manufacturer has published evidence that this immunogenicity was not due to aggregates, 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

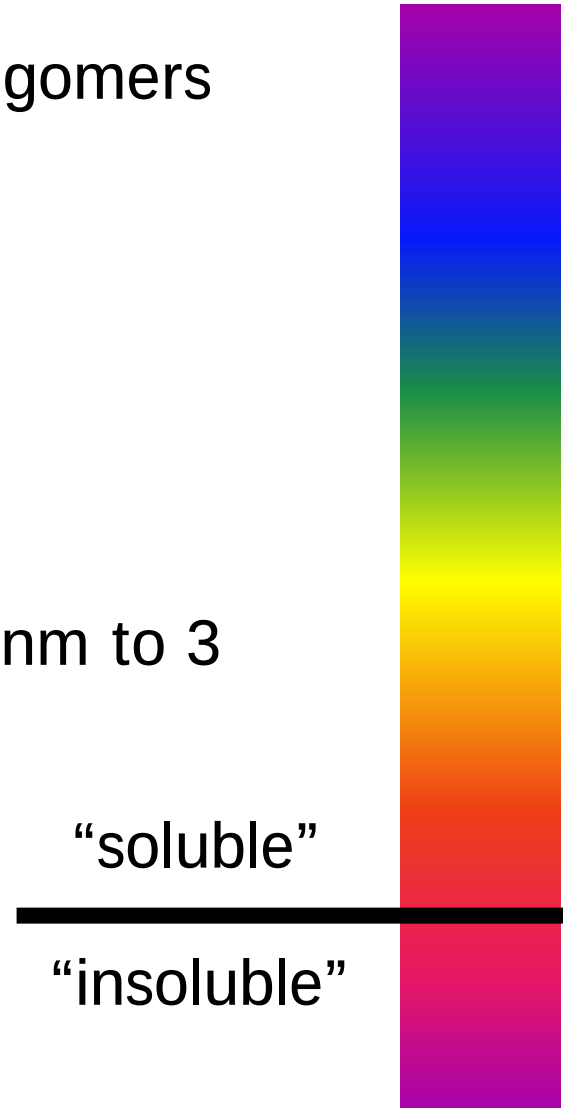
   S. Hermeling *et al.* (2006) *J. Pharm. Sci.* 95, 1084-1096.

AAPS Protein Aggregation and Immunogenicity Focus Group

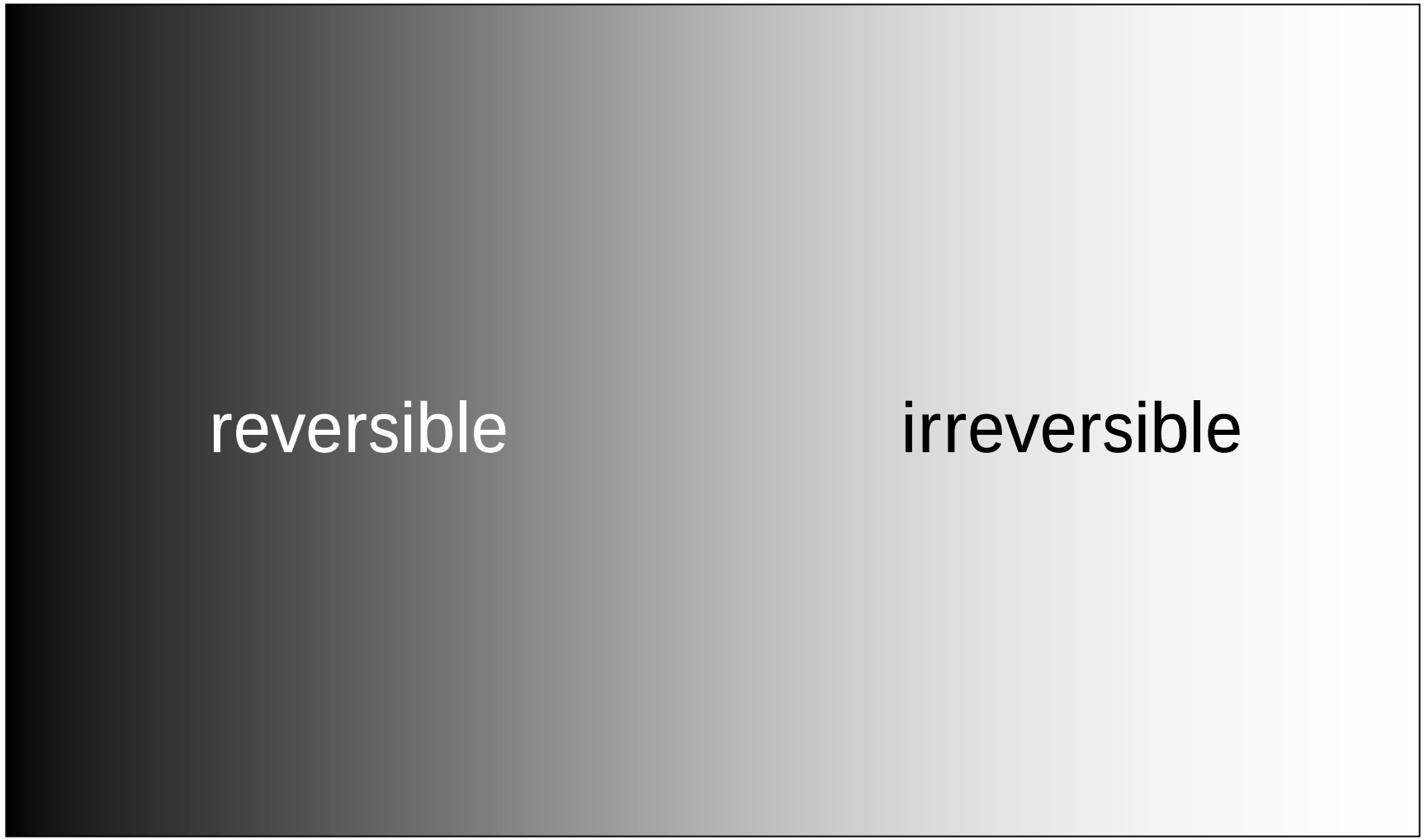
- Sponsored a workshop September 2006 in Colorado to summarize current state-of-the-art and remaining challenges
  - talks, posters, summaries available at http://www.aapspharmaceutica.com/inside/Focus_Groups/ProteinAgg/index.asp
- Goal is to form an industry consortium to sponsor (pay for) new studies to better define how immunogenicity varies with aggregate type and size
- Please join us!

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



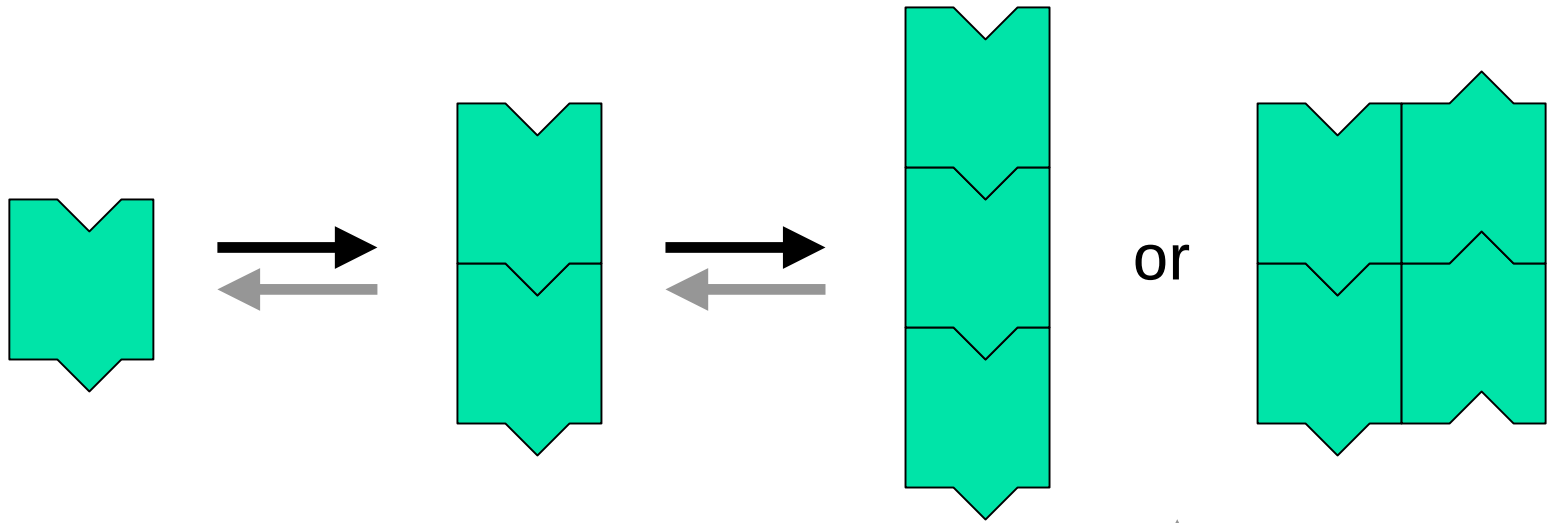
Whether aggregates are “irreversible” or “reversible” depends on the context

- 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
- metastable oligomers with dissociation rates of hours to days occur fairly frequently
  - ★ for an antibody example see J.M.R. Moore *et al.* (1999) *Biochemistry* 38: 13960-13967
  - ★ see also Philo, J.S. (2006) *AAPS Journal* 8(3): E564-E571
- many common analytical methods will detect only the longer-lived species
- it may take hours to days for a protein to re-equilibrate its association after a change in concentration, solvent conditions or temperature

Aggregation mechanisms (1): reversible association of native protein



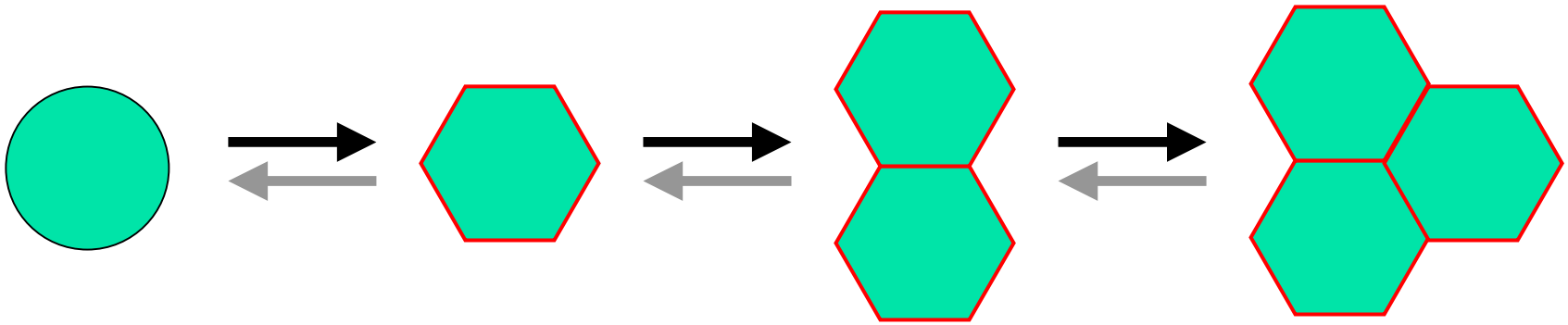
Native protein

Reversible oligomerization

Higher oligomers (possibly irreversible)

↑ + sucrose

Aggregation mechanisms (2): oligomerization following conformational change



Native protein

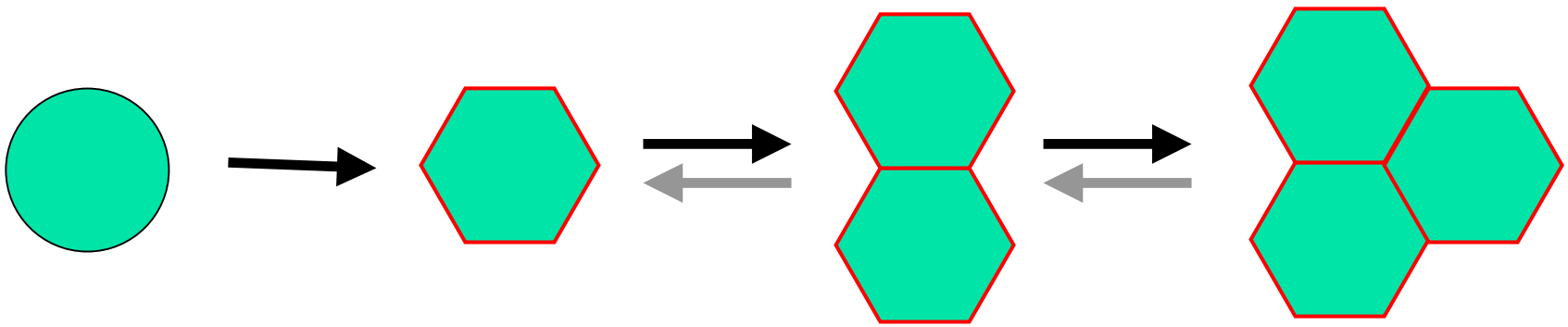
Conformational change or partial unfolding

Oligomerization of non-native protein

↓ + sucrose

Higher oligomers (probably irreversible)

Aggregation mechanisms (3): oligomerization driven by covalent modification



Native protein

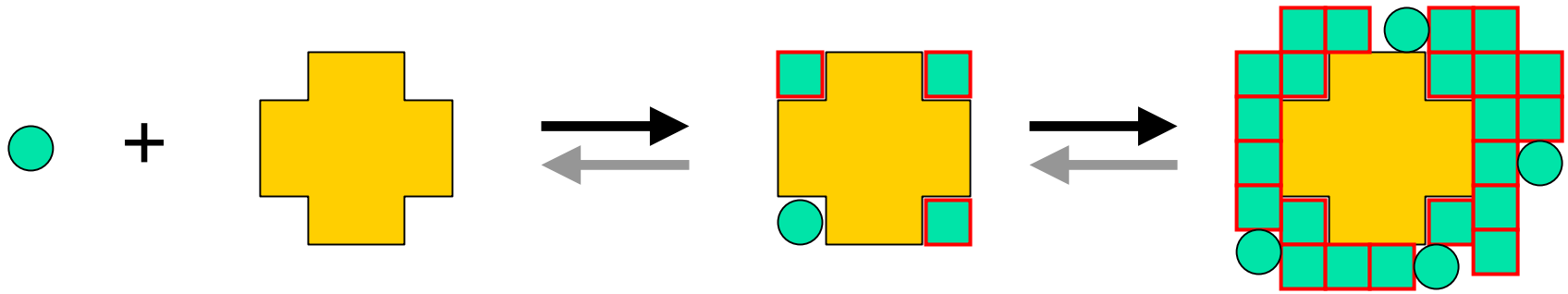
Modified protein (oxidation, deamidation, *etc.*)

Oligomerization of modified protein

Higher oligomers (possibly irreversible)

↓↑ + sucrose

Aggregation mechanisms (4): nucleation controlled aggregation (“seeding”)



Native protein

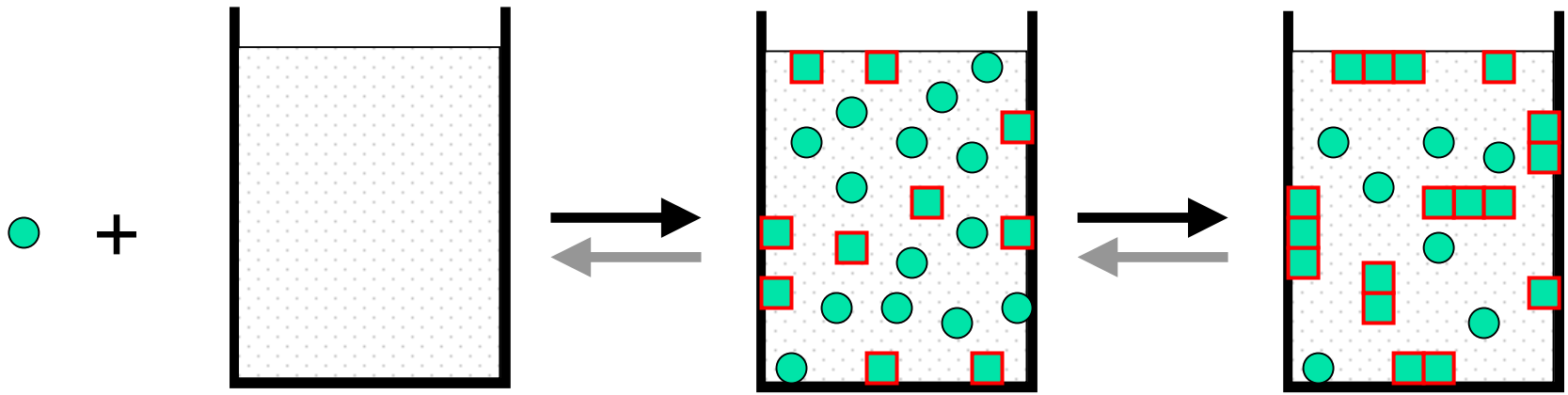
Critical nucleus (aggregate of native or modified protein, or a contaminant)

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

Our 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**	**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 aggregates

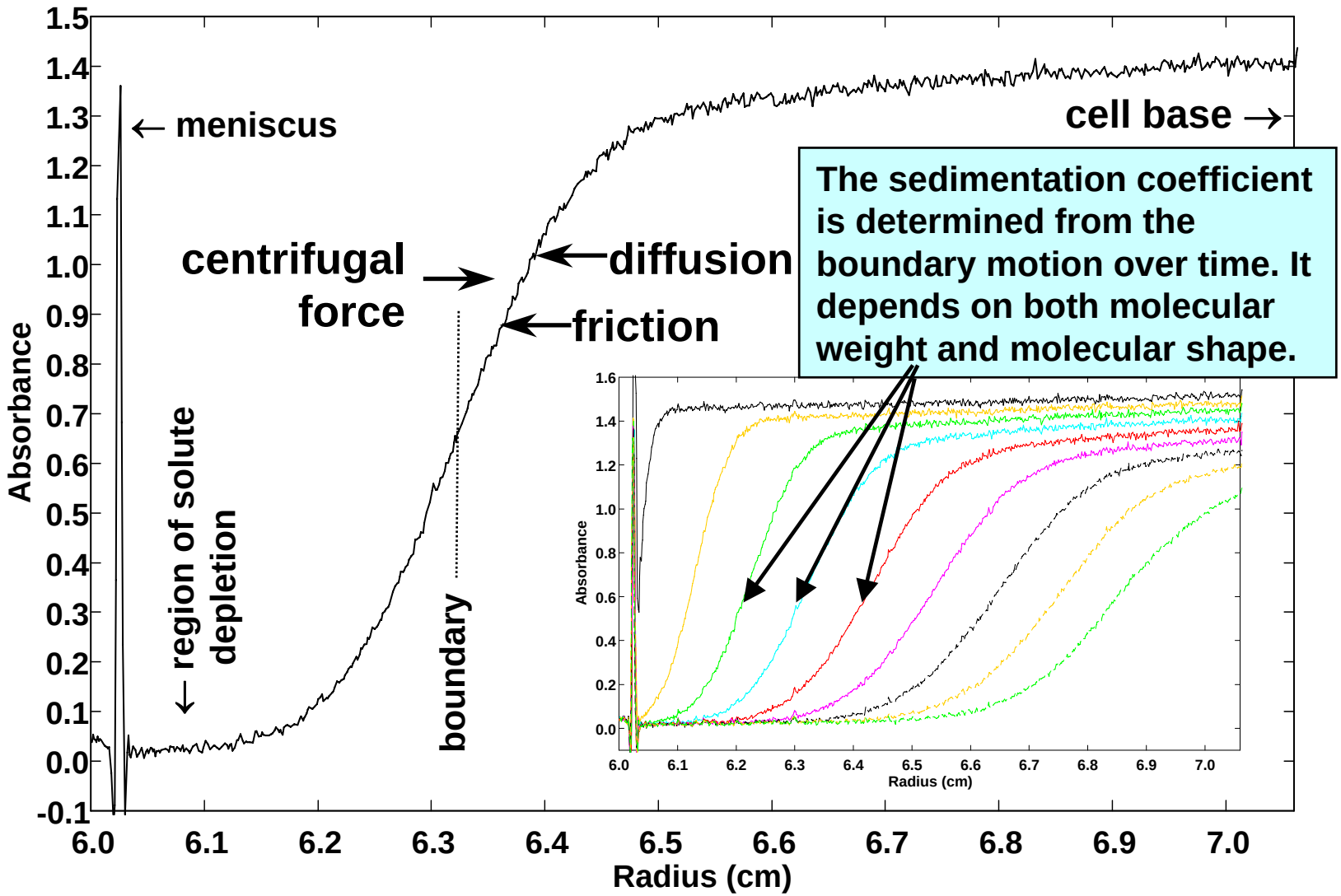
- Although SEC is usually the primary method of aggregate analysis the regulatory agencies are well aware that SEC columns can act as filters and that the SEC mobile phase can change the distribution of non-covalent aggregates
  - but SEC is often the only qualified method that can be validated for lot release
- Therefore by phase 3 (and sometimes earlier) they will now nearly always ask for cross-validation of SEC methods by orthogonal methods

Methods typically used to cross-check SEC

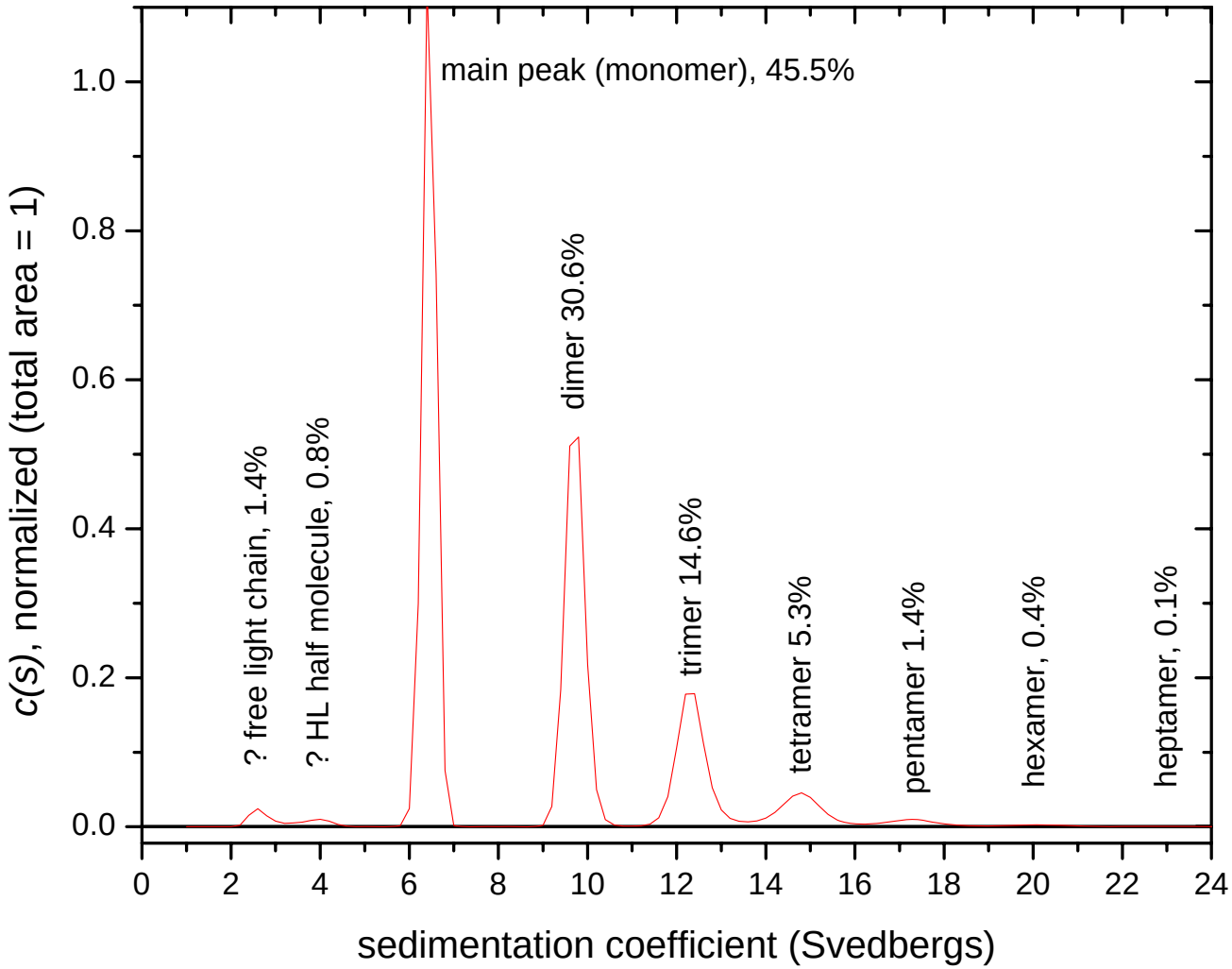
- analytical ultracentrifugation (AUC)
  - sedimentation velocity (primarily)
  - sedimentation equilibrium (occasionally)
- light scattering
  - flow mode classical scattering used after SEC (SEC-MALLS) ← has been validated
  - dynamic light scattering (DLS)
  - batch mode classical scattering ← has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

Sedimentation velocity

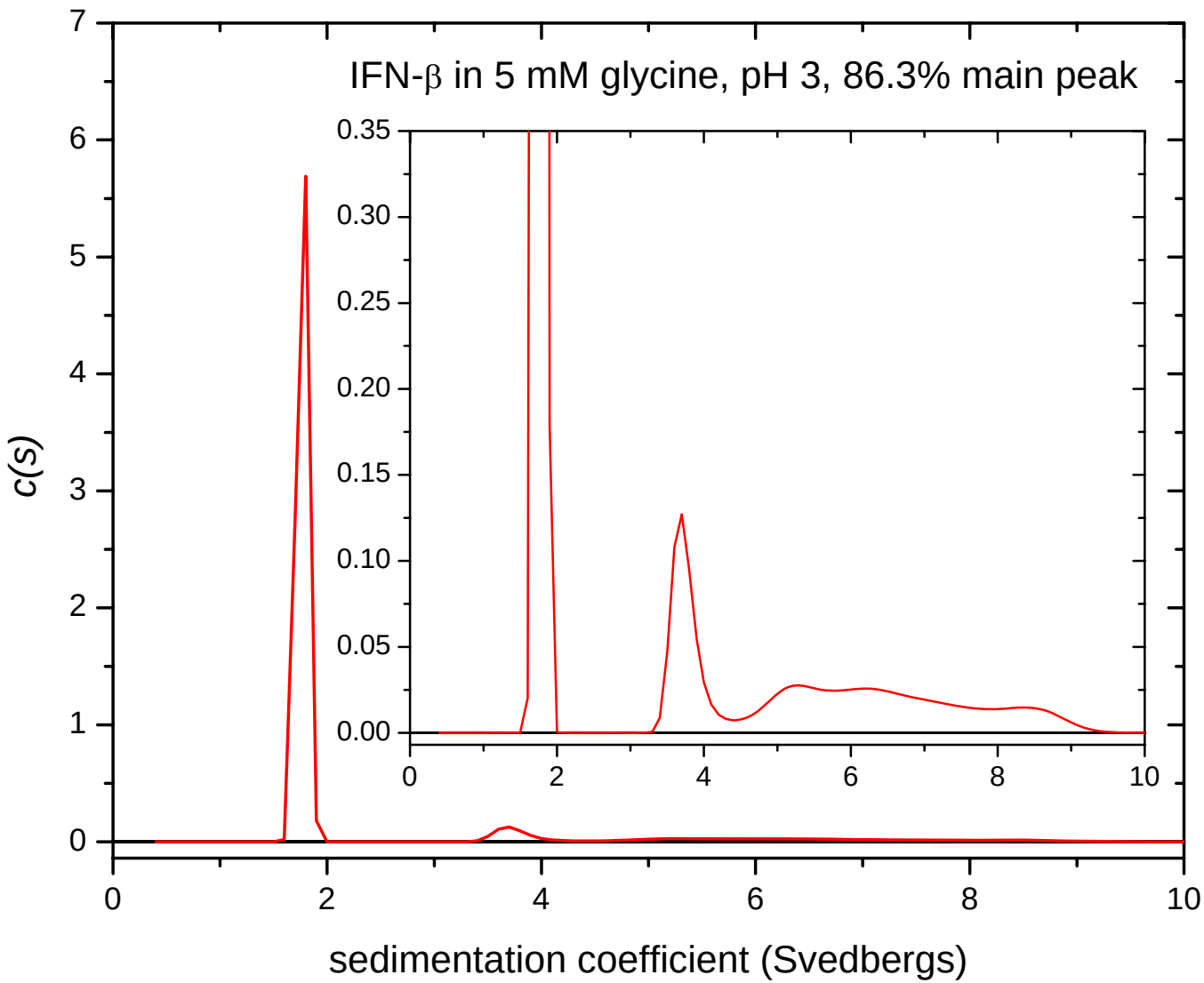
The fundamentals of sedimentation velocity



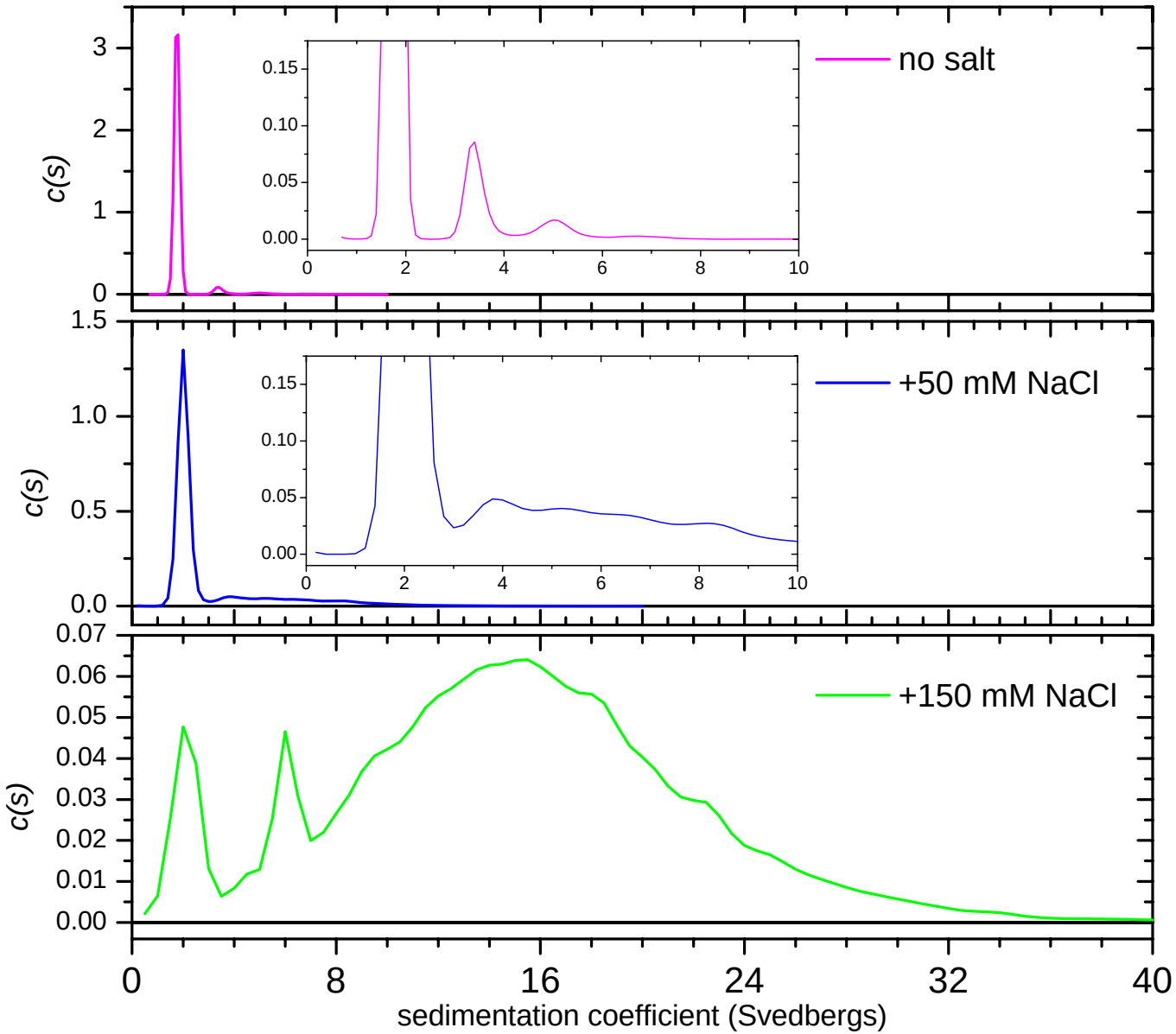
High resolution analysis of a highly stressed antibody sample resolves 6 aggregate peaks plus 2 fragments



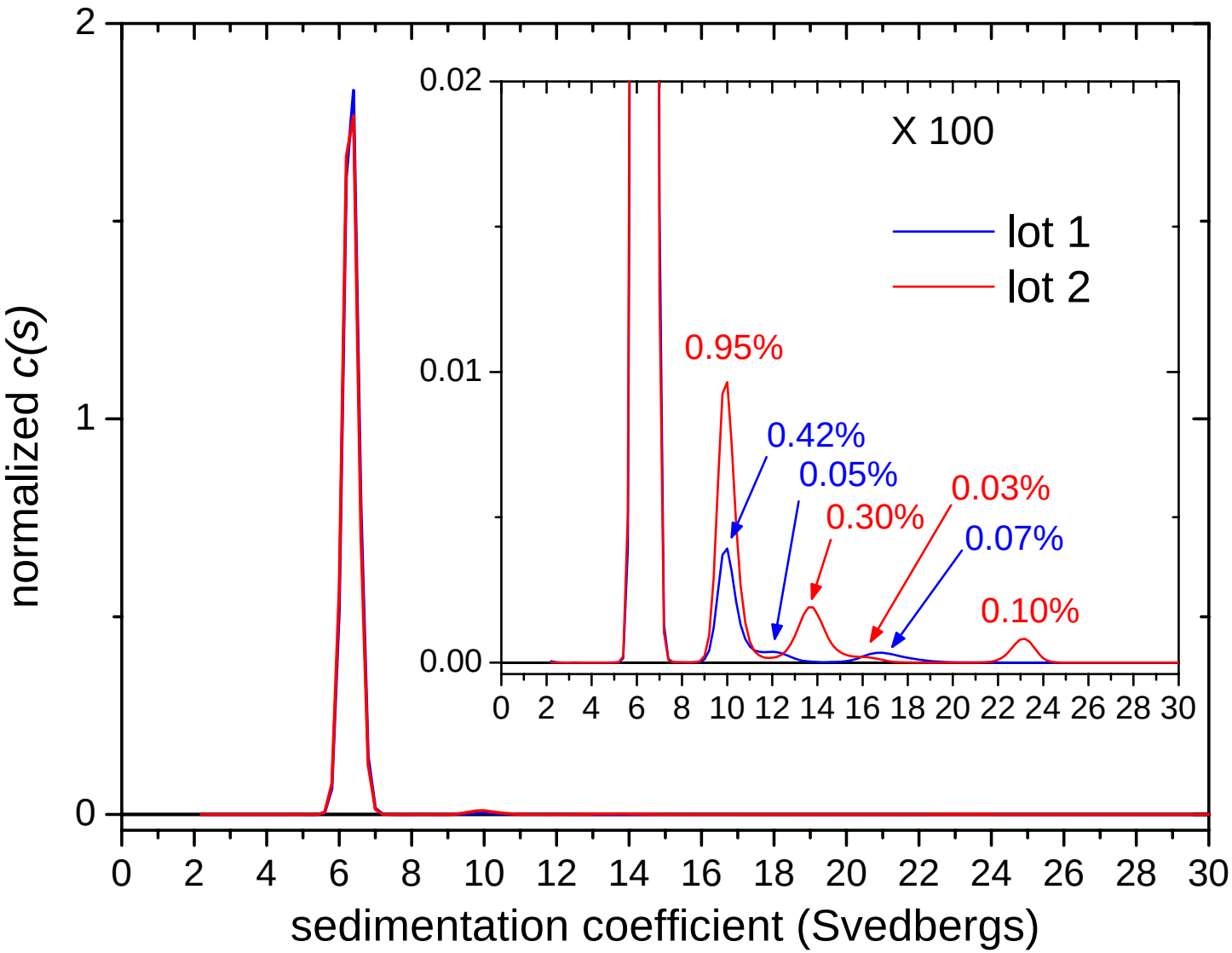
This interferon-β sample is 13.7% non-covalent aggregate; by the standard SEC method it would be pure monomer



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



SV is very useful for comparability studies, giving comparability of conformation as well as aggregation



The peril: *c(s)* distributions are often misunderstood

1. the effective resolution goes down as the fraction of minor peaks goes down
2. the resolution you can achieve for a 150 kDa antibody is much greater than for a 20 kDa cytokine
3. in general it is not possible to uniquely assign a stoichiometry to each aggregate peak
4. the nature of the noise (variability) is very different than in chromatography
5. for reversibly associating proteins the peaks probably do not represent individual molecular species

Field-flow fractionation (FFF)

Principles of cross-flow FFF

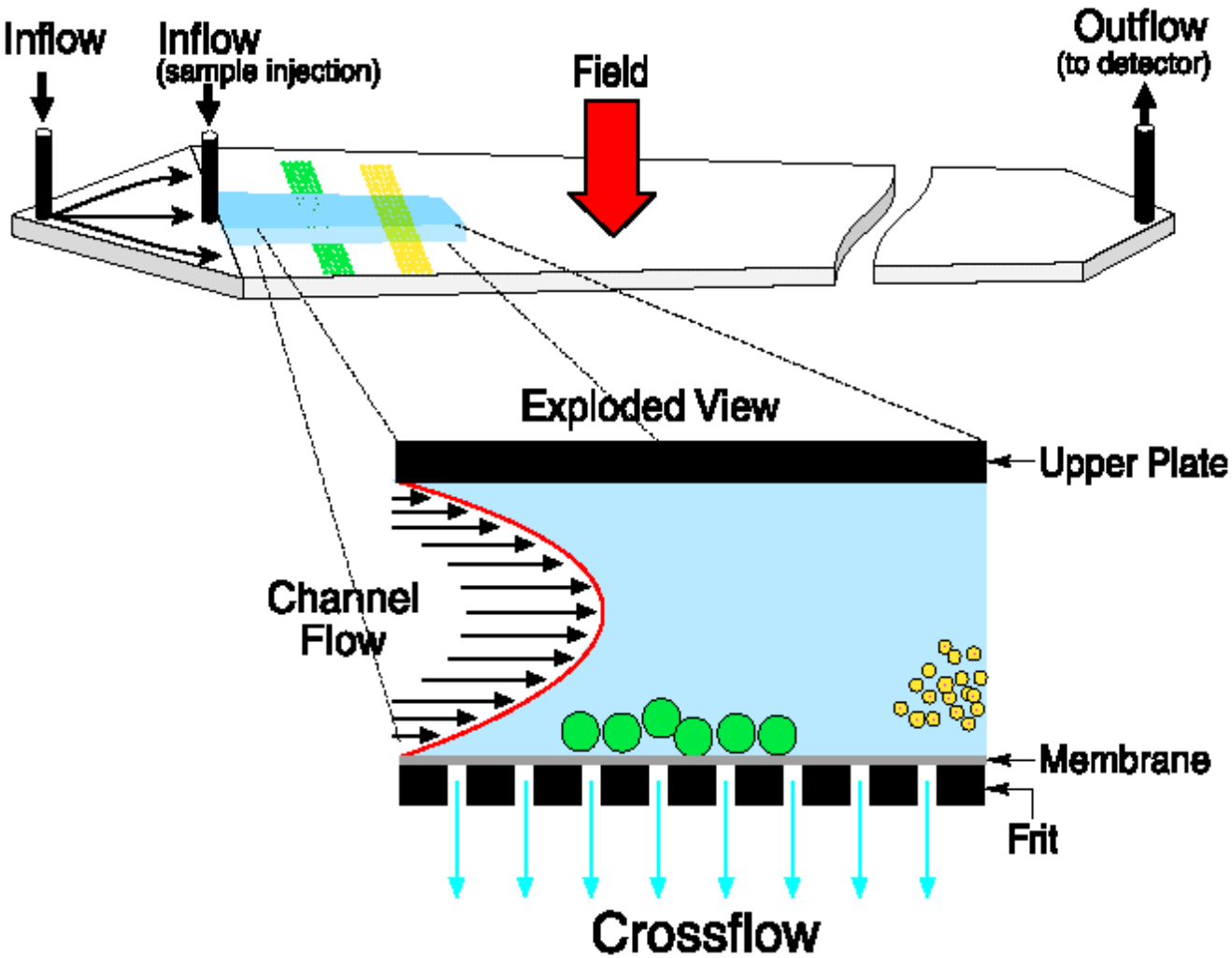
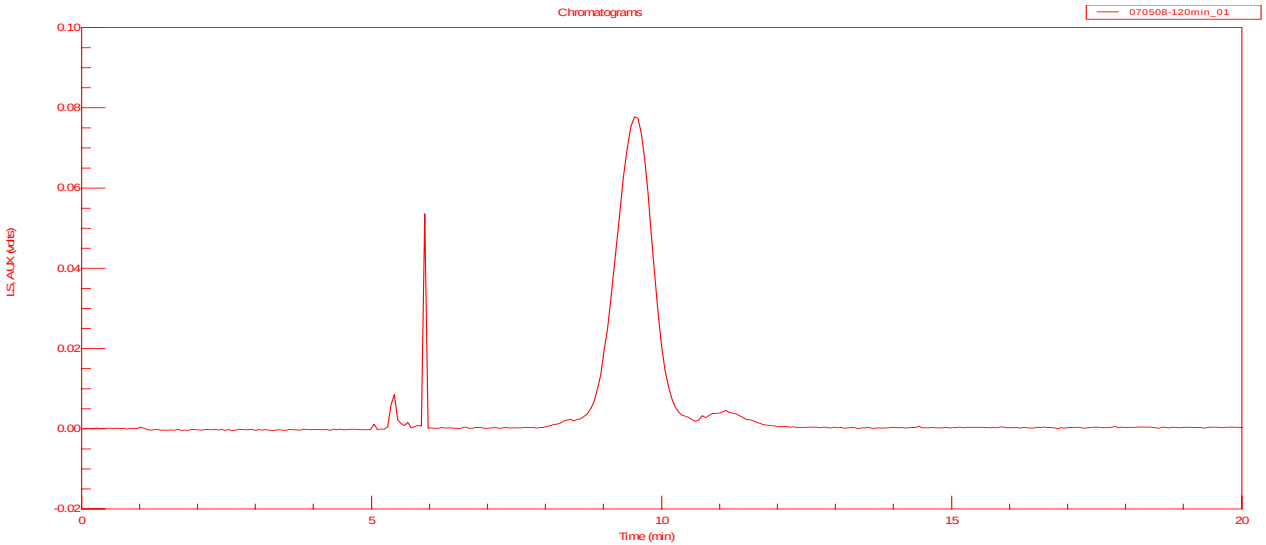


figure courtesy Wyatt Technology

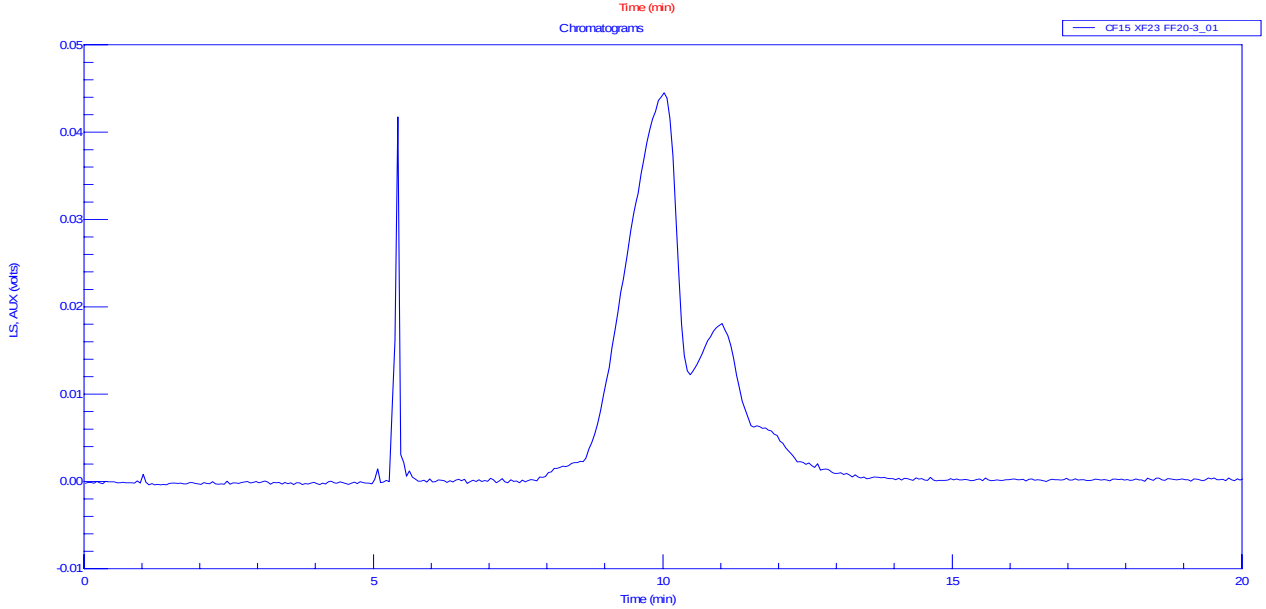
FFF of acid-exposed IgG (2 hr at pH 2.9, 5 °C)

(courtesy K. Tsumoto and D. Ejima)

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
  3. high 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. Sedimentation velocity has many advantageous properties
   - it is the primary tool we use at APL to cross-check SEC methods (and help improve them)
   - it suffers from low throughput and requires a very highly trained operator
4. Our ability to characterize aggregates unfortunately far exceeds our knowledge of how specific aggregate types affect product safety or efficacy