

# Analyzing Aggregates by Sedimentation Velocity and Light Scattering



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# Outline

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- Quickly review some basic facts about aggregate sizes and types
- Basic principles and application examples for 3 methods
  1. sedimentation velocity
  2. classical light scattering used with SEC
  3. batch-mode dynamic light scattering



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

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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  $\mu\text{m}$ )
  - ★ could be reversible if non-covalent
6. visible particulates
  - ★ probably irreversible





# Aggregates have a spectrum of lifetimes

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- 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*, in press
- 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



# Our analytical challenge

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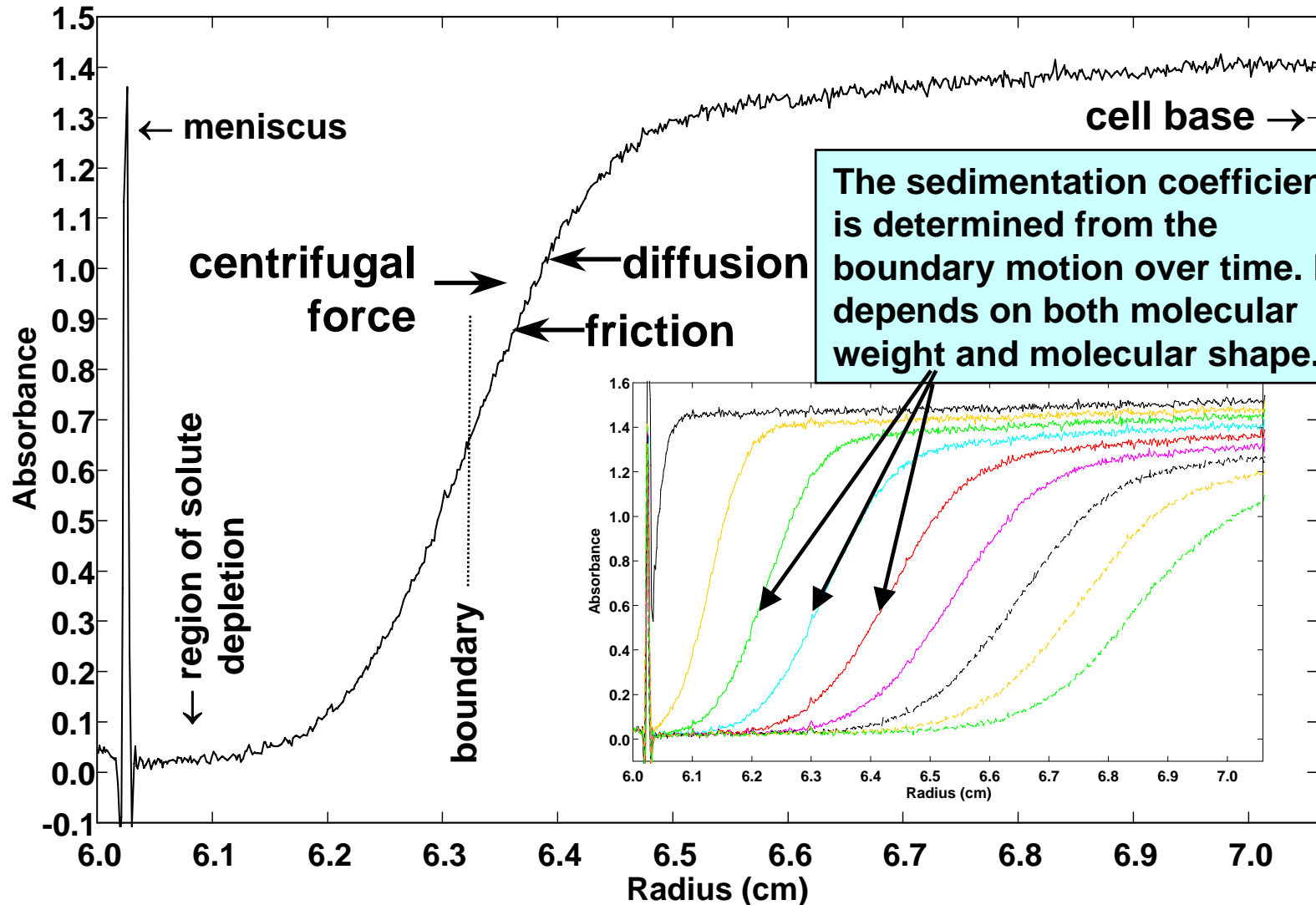
1. Any protein sample may contain aggregates with a wide range of sizes, types, and lifetimes
2. Any one analysis method may not detect all the aggregate sizes or types that are present
3. The measurement itself may perturb the aggregate distribution that was initially present
  - dilution may dissociate reversible aggregates
  - change of solvent conditions may dissociate or increase aggregates
  - adsorption or filtration effects may remove aggregates



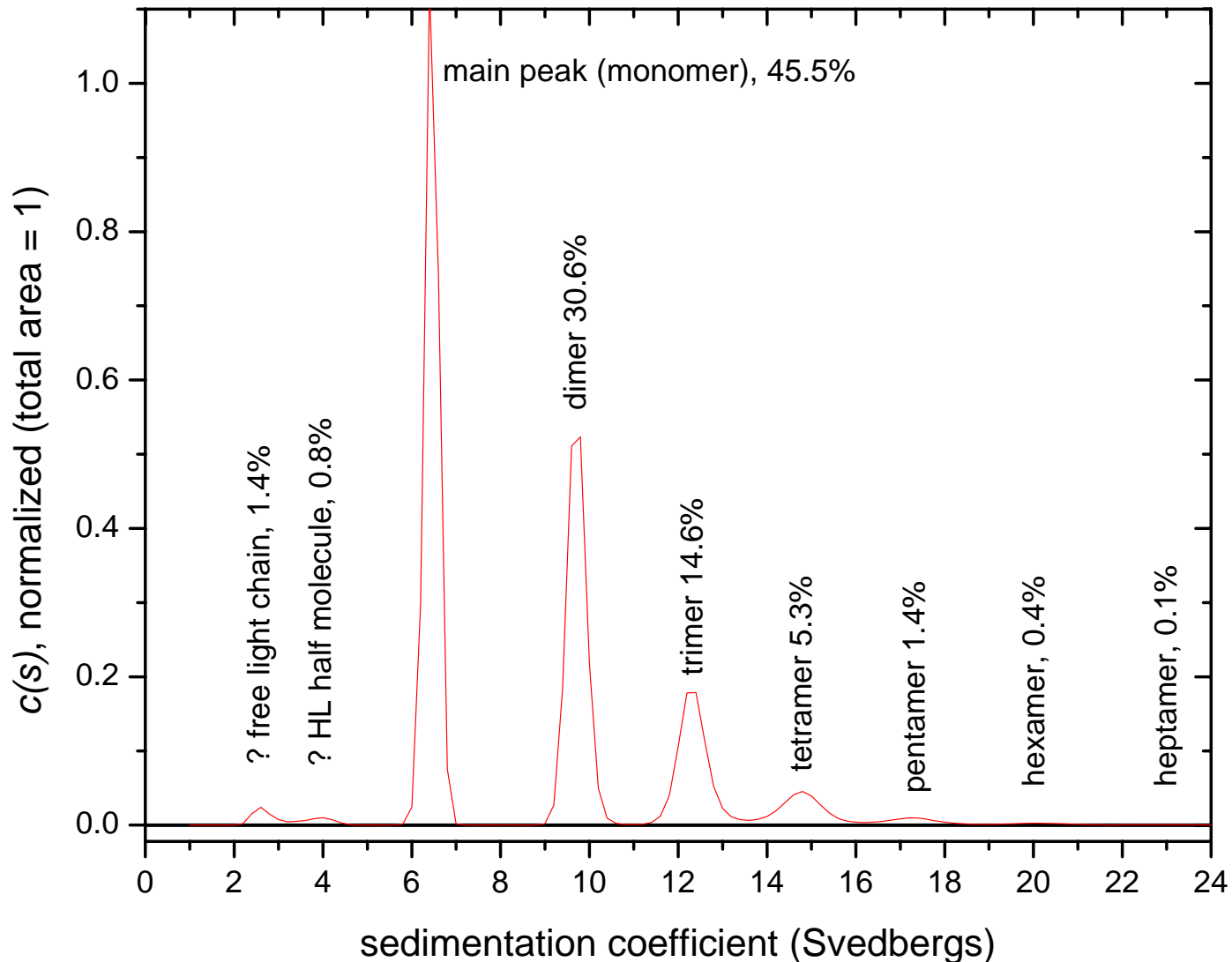
# Sedimentation velocity

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# The fundamentals of sedimentation velocity



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



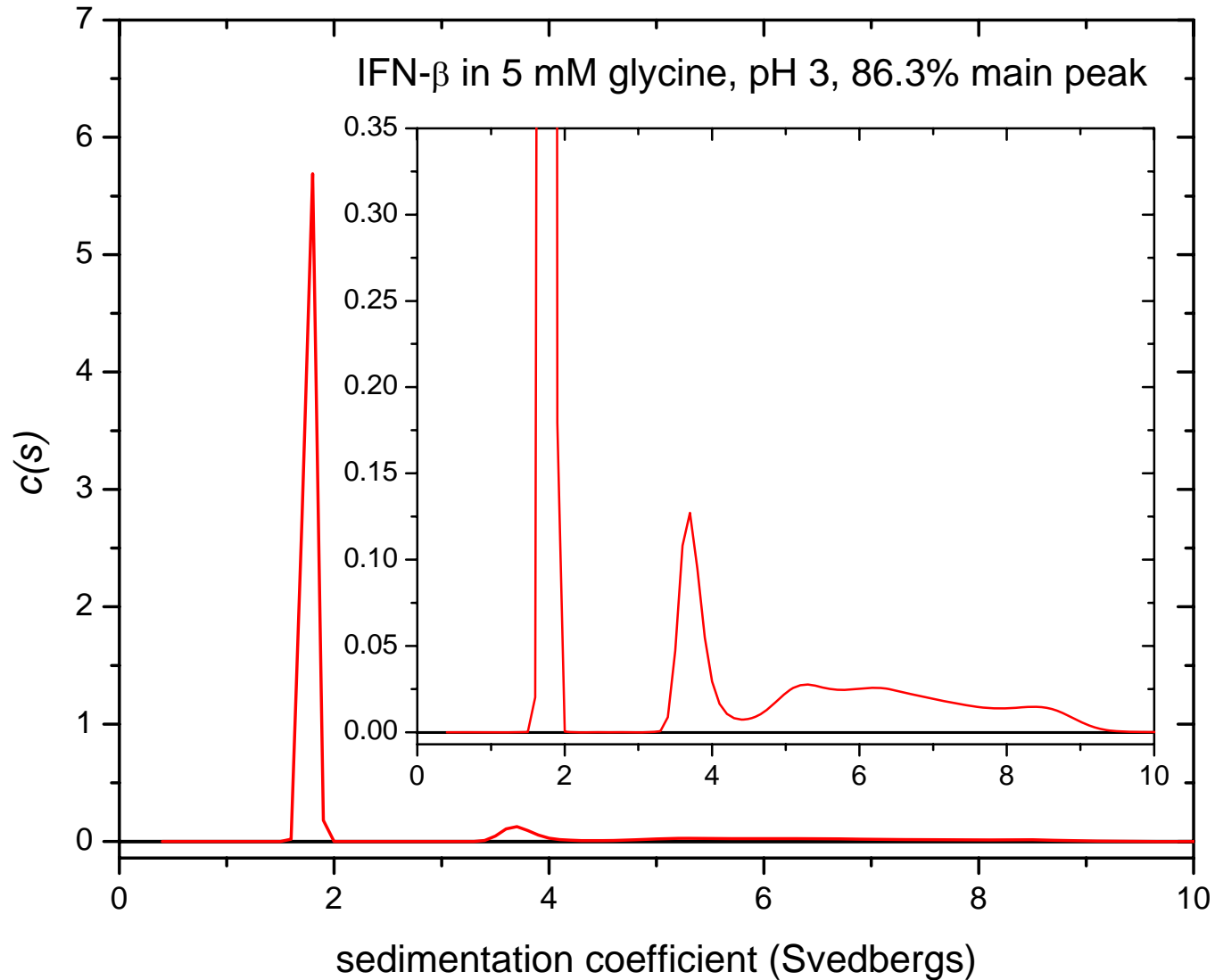


## The peril: $c(s)$ distributions are also often misunderstood

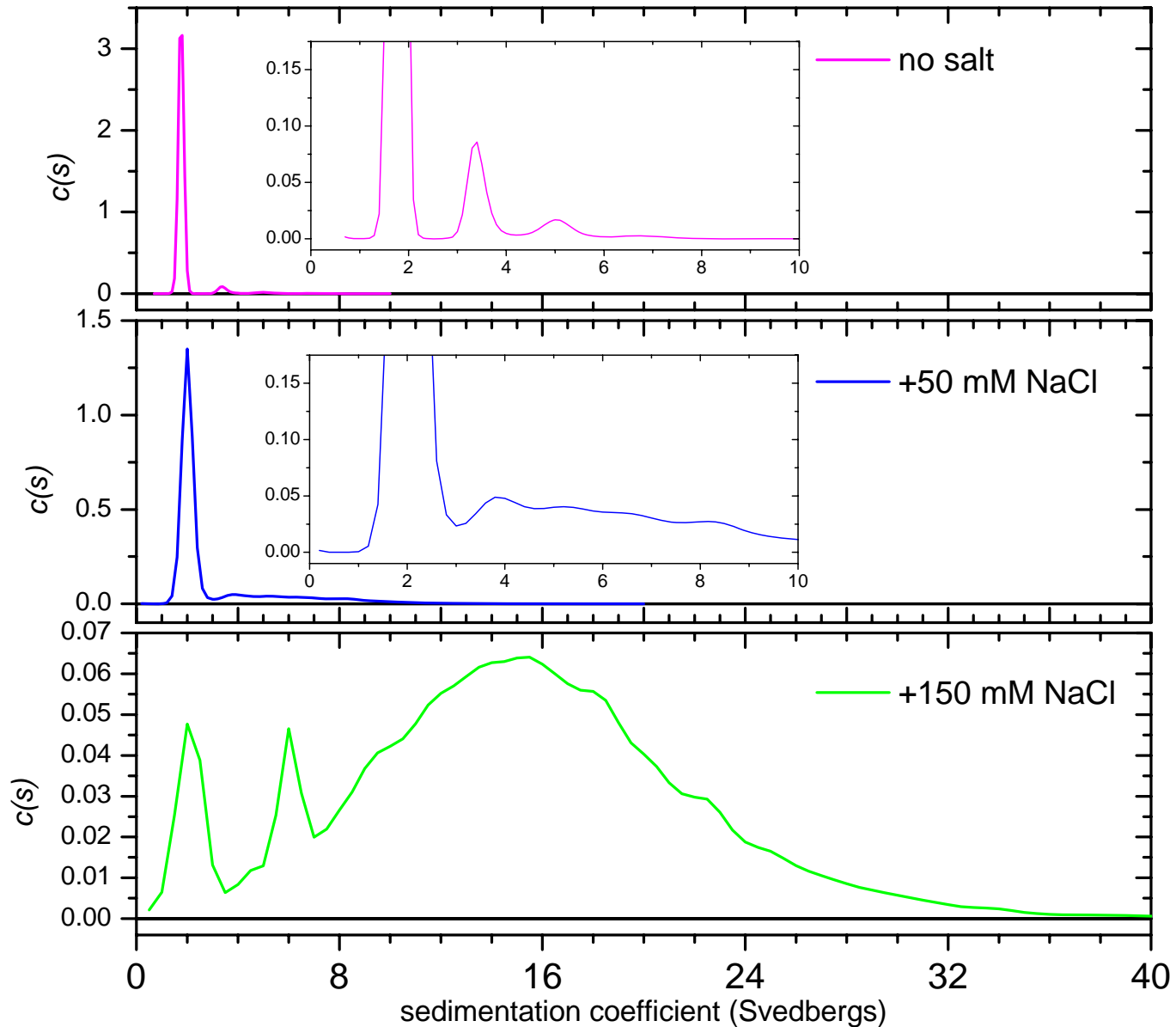
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1. the effective resolution goes down as the fraction of minor peaks goes down
2. the resolution you can achieve for a 150 kDa antibody is much greater than for a 20 kDa cytokine
3. in general it is not possible to uniquely assign a stoichiometry to 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

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



# Adding NaCl to interferon- $\beta$ formulations leads to a broad distribution of non-covalent aggregates out to $\sim 100$ -mers





## Strengths of sedimentation velocity

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1. high resolution (often 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
  - ★ Tween and high levels of sugars do cause some interference
5. little dilution of sample (~25%)
6. absolute method; requires no molecular standards
7. strong theoretical background; “first principles” method




## Weaknesses of sedimentation velocity

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1. low throughput (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
5. never been validated for lot release

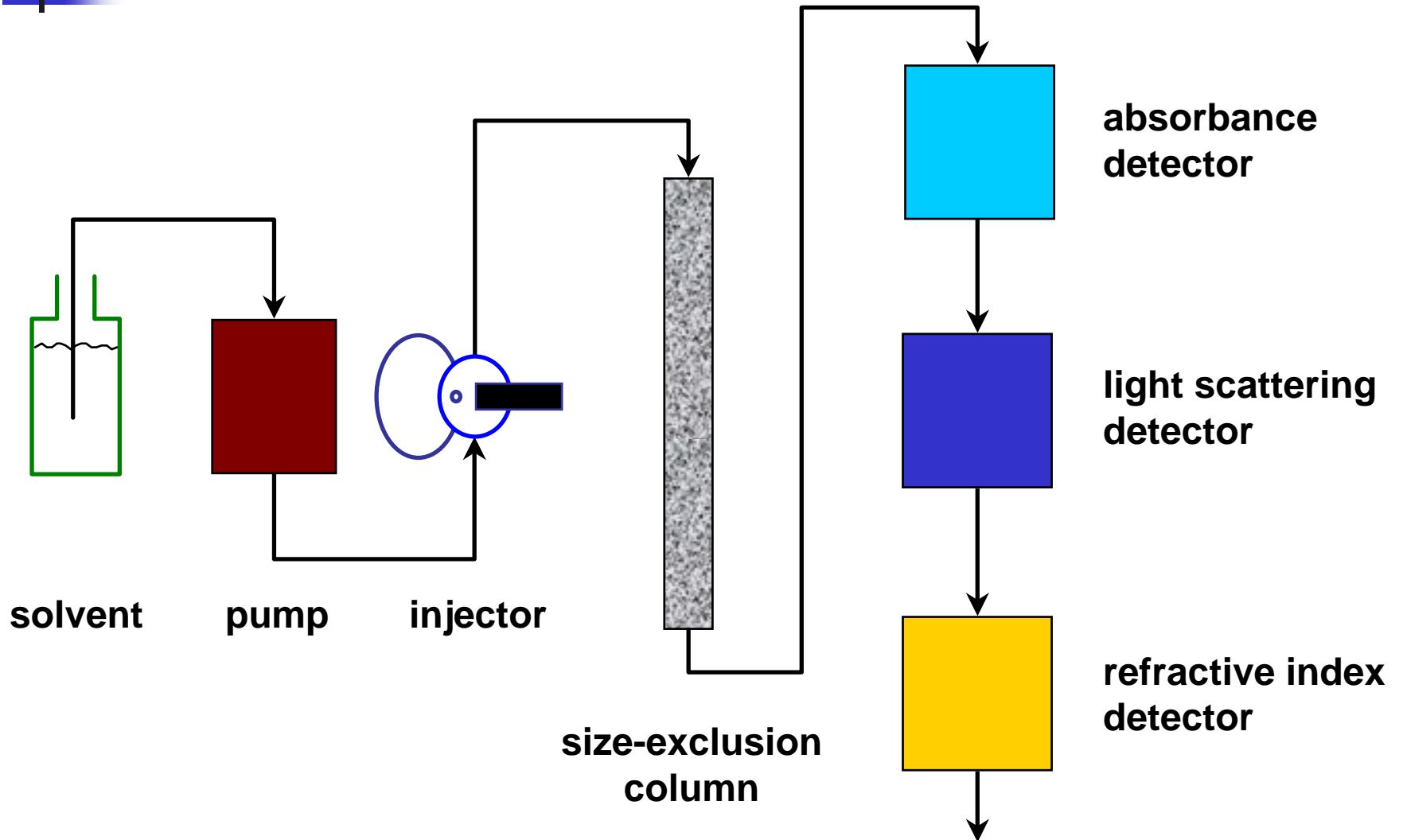
**Sedimentation velocity can not replace SEC, but it is an excellent tool to test whether SEC is missing important features. It can also serve as a “gold standard” to help improve SEC methods.**

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



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



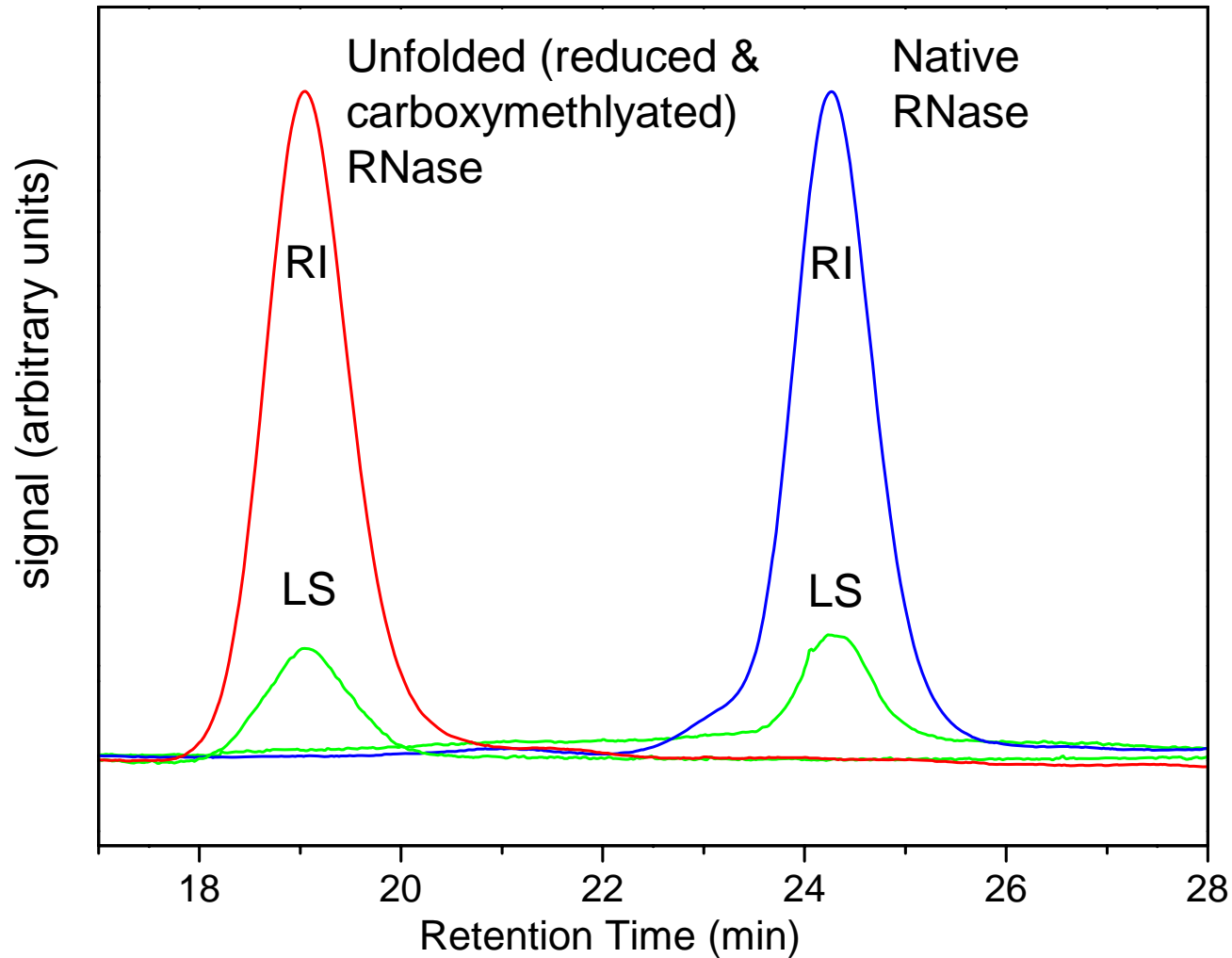


# Getting molecular mass from static light scattering: the basic idea

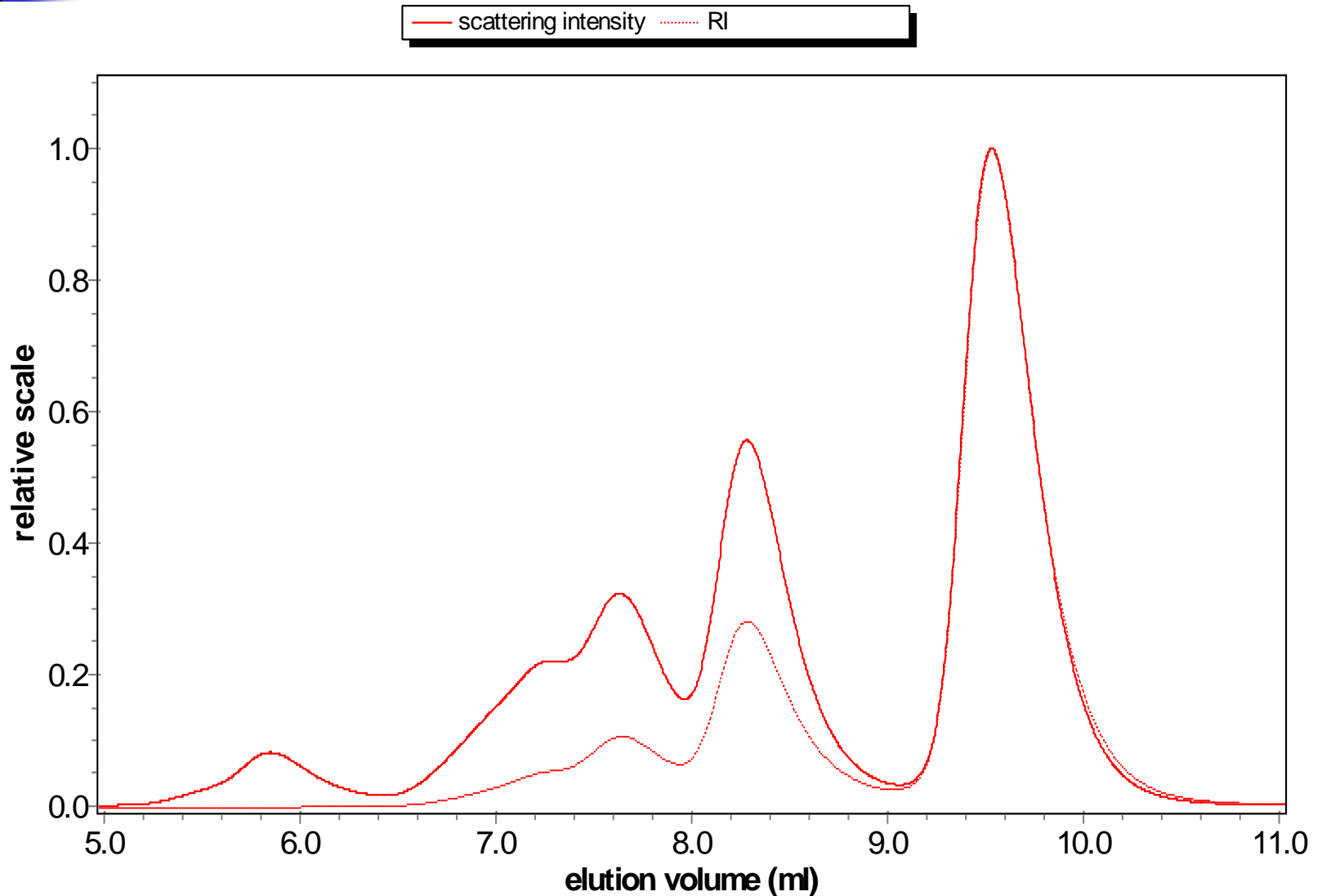
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- the light scattering signal is proportional to the product  $c \times M$
- we measure  $c$  simultaneously with a UV or RI detector
- then the ratio of the scattering to concentration signals will be proportional to  $M$
- masses obtained this way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

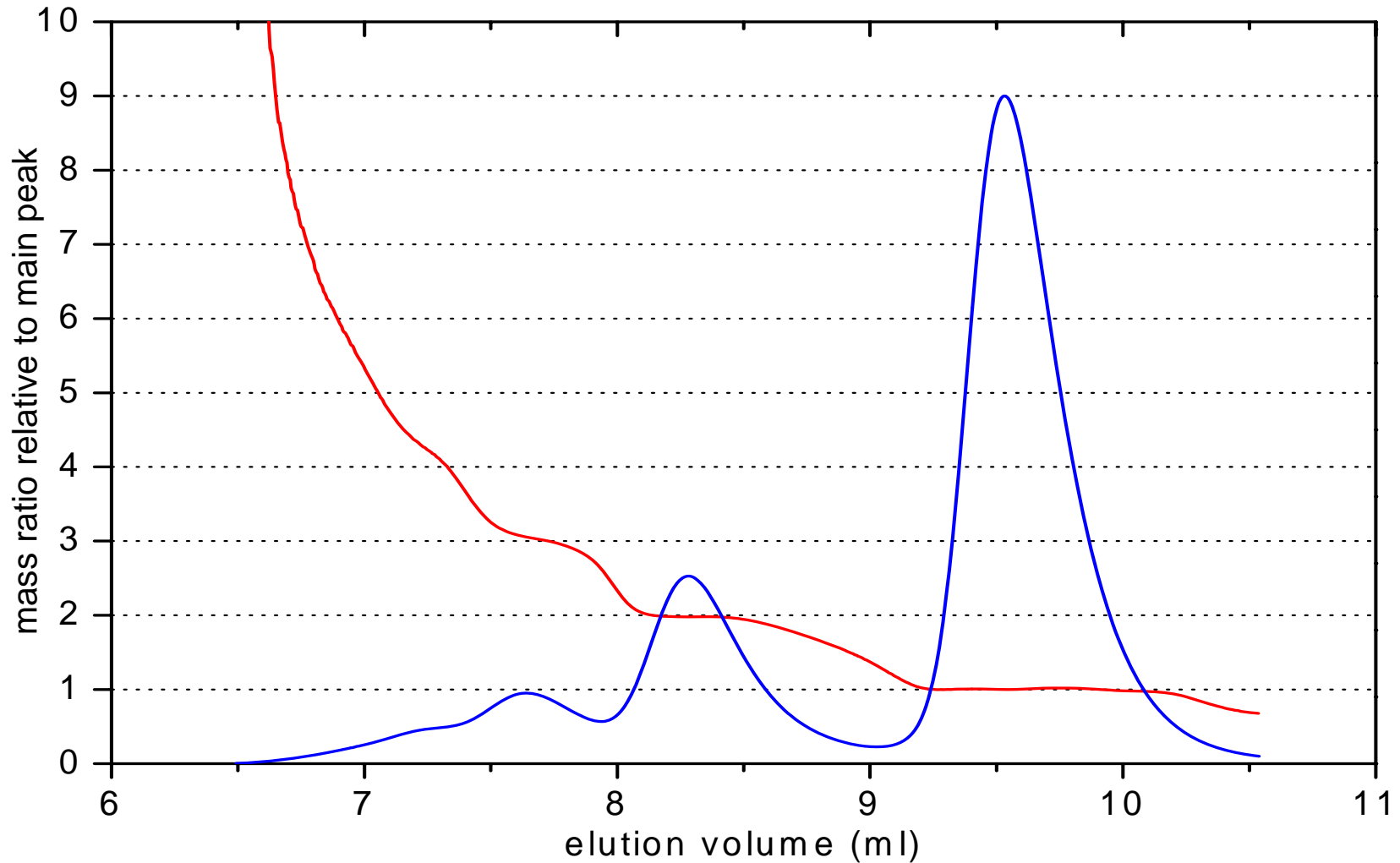
Demonstrating that scattering is independent of elution position and molecular conformation: the ratio of LS to RI signals is the same whether the protein is folded or unfolded



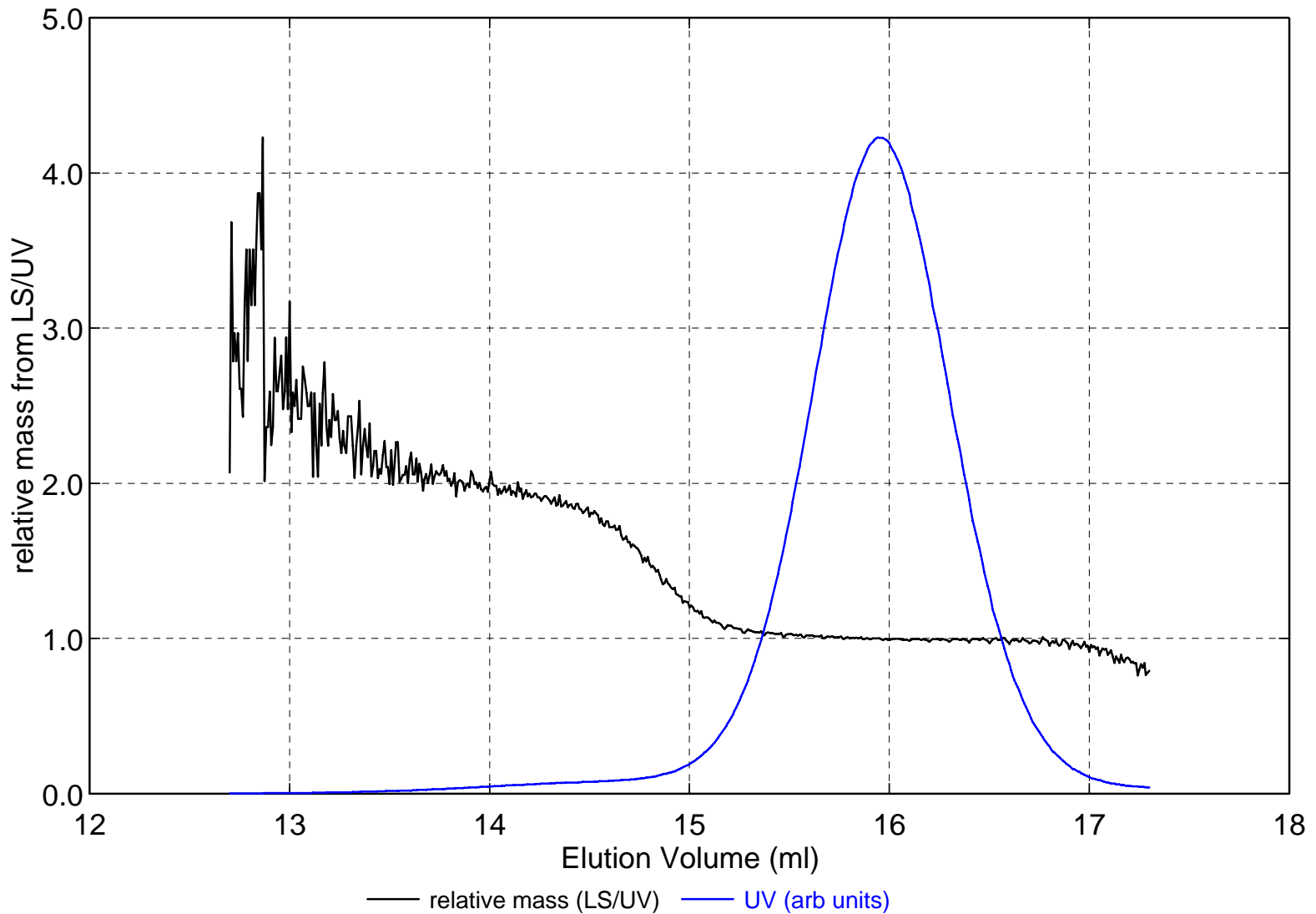
An example for an Fc-fusion protein: the aggregate signals are much stronger in 90° scattering than in the UV chromatogram



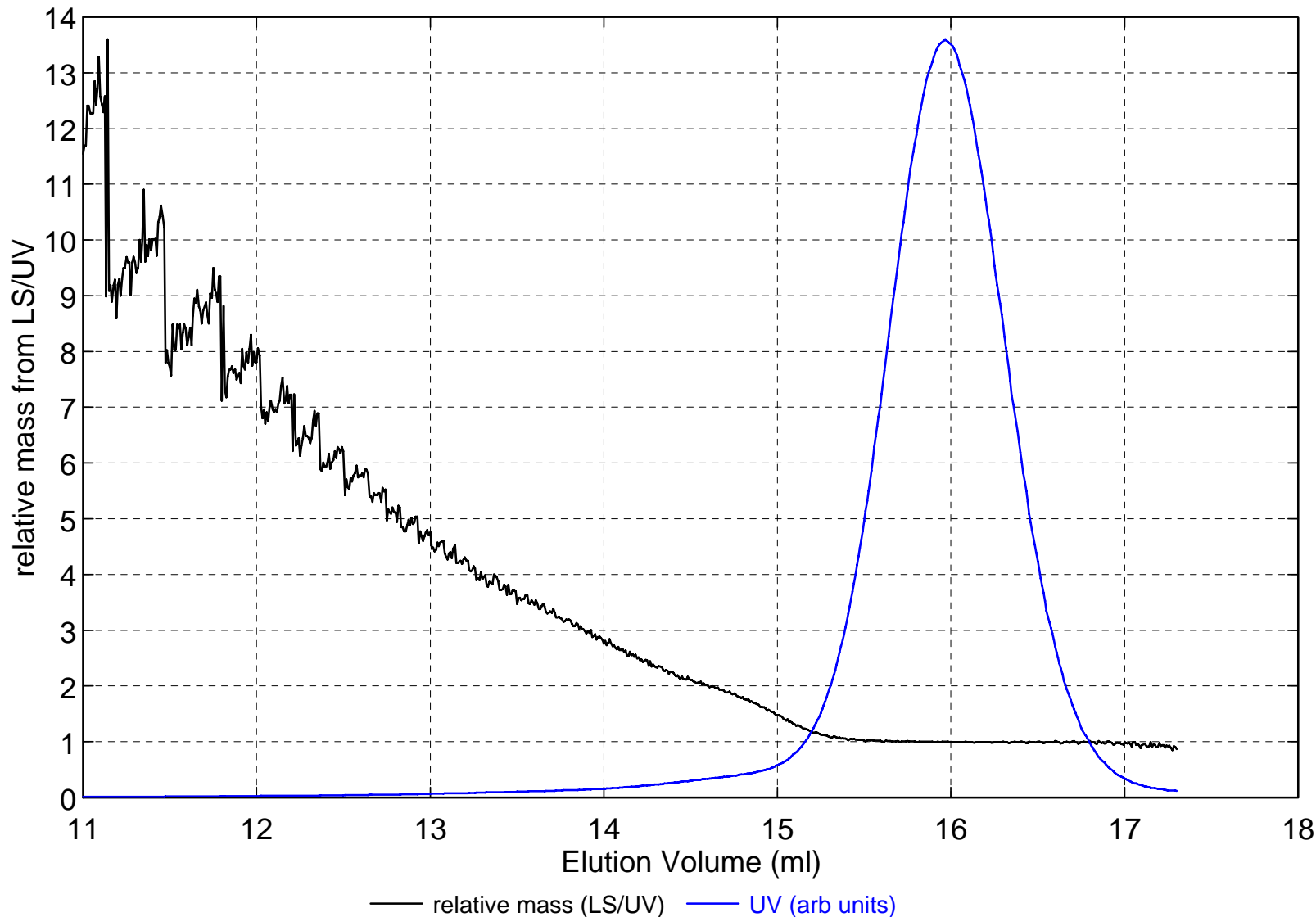
# "Oligomer hunting": display the absolute molecular weight from LS in units of monomers



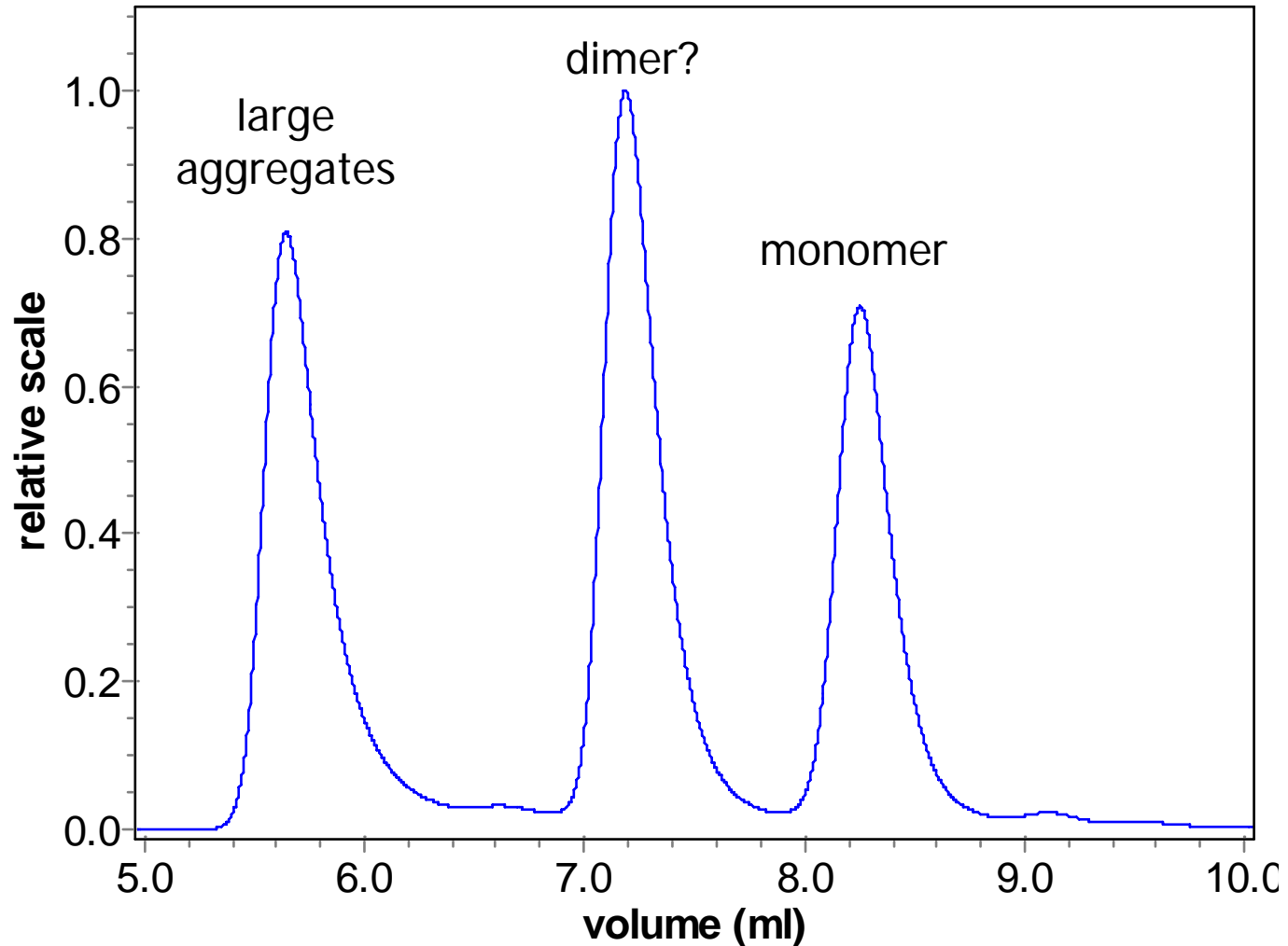
This antibody sample has traces of dimer and trimer



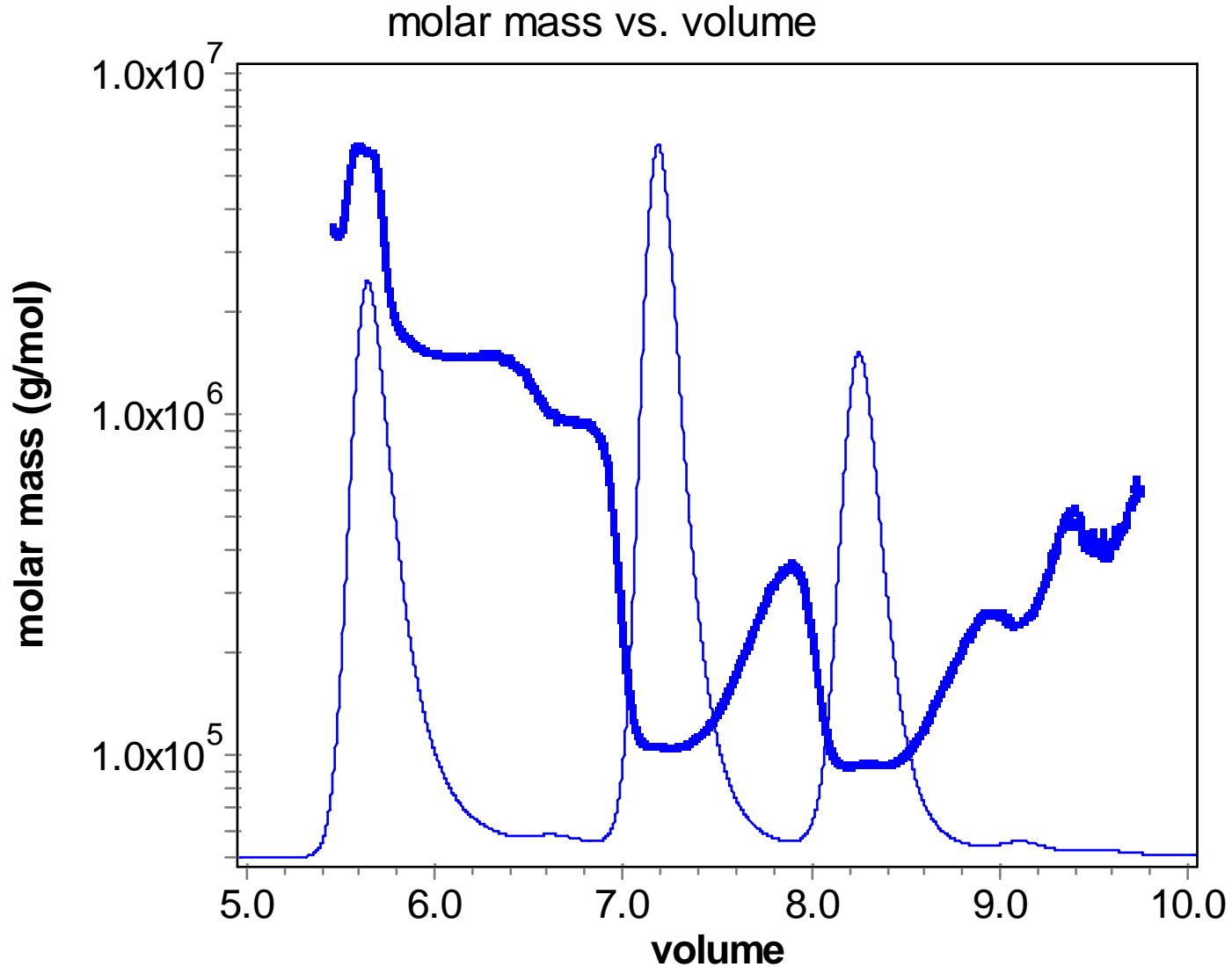
A different lot contains more higher oligomers, and they are so sticky that even dimer is no longer resolved



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!





## Strengths of SEC + classical LS

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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. high throughput, low cost (comparable to the HPLC it is used with), fairly easy
5. absolute method; requires no molecular standards
6. strong theoretical background; "first principles" method



## Weaknesses of SEC + classical LS

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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 elution region near the column's exclusion limit
4. good signal/noise may require larger injection amounts than are normally used in standard SEC

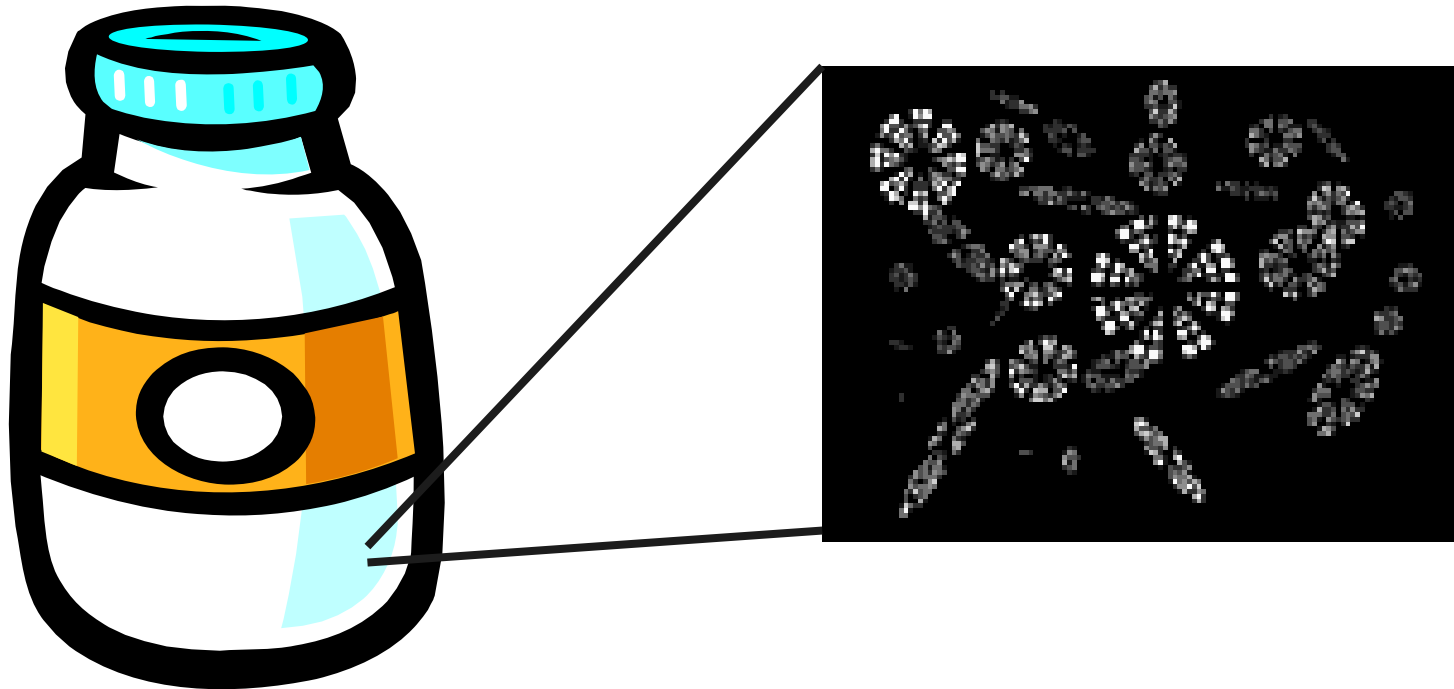


# Batch-mode dynamic light scattering (DLS)

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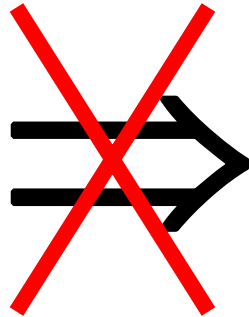
also known as quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS)

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



- 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'

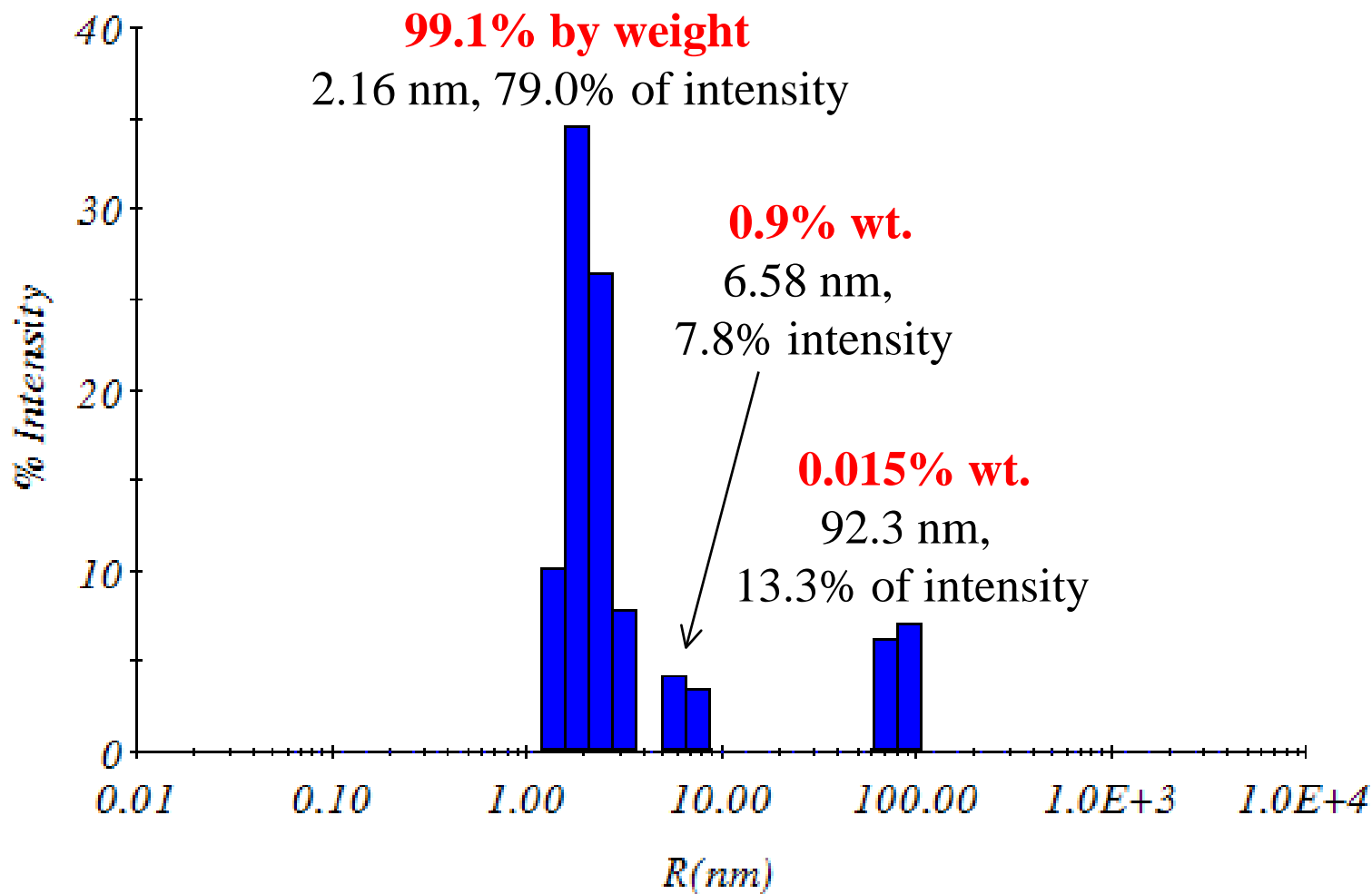


# Dynamic light scattering: the basic idea

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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



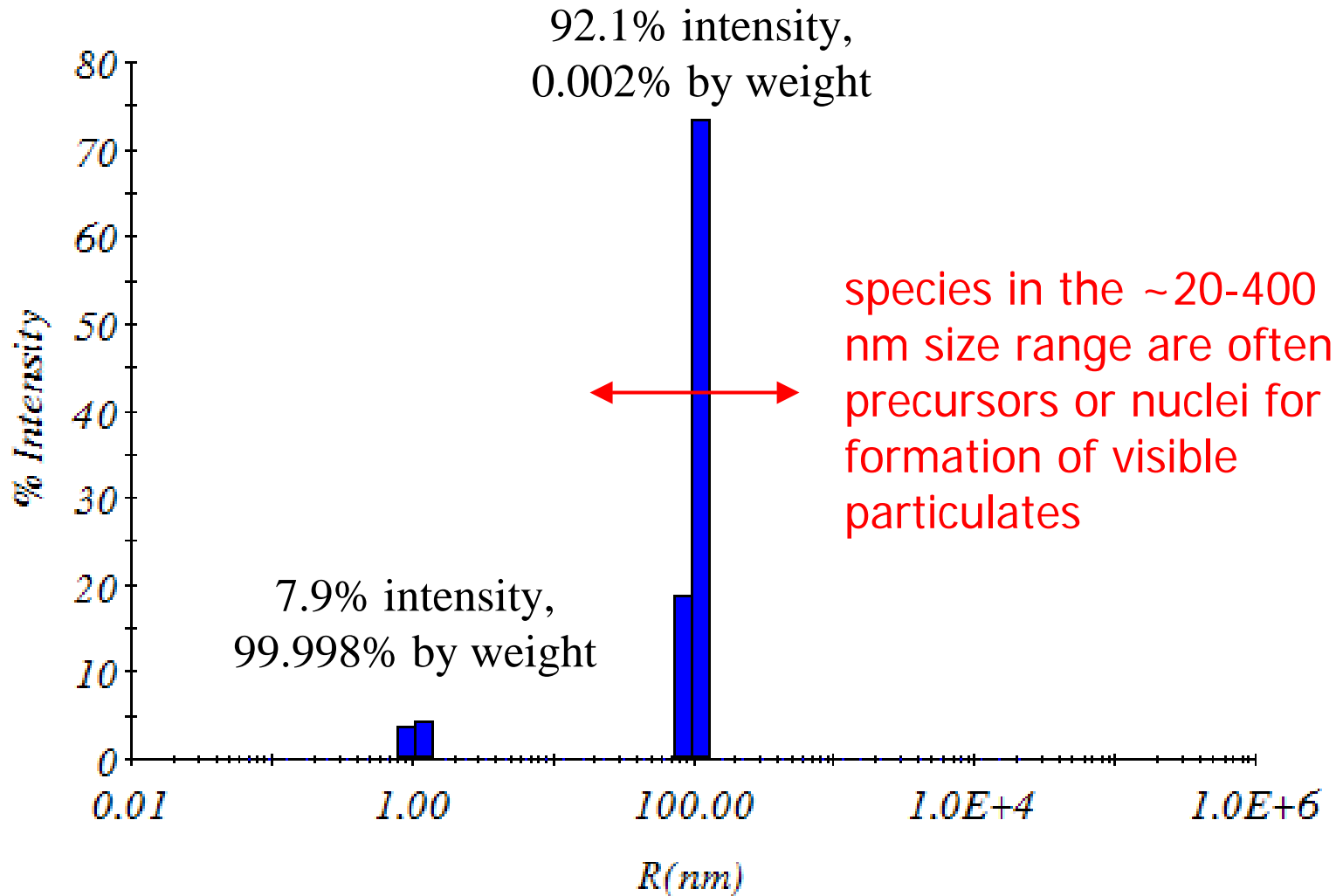


## Two key weaknesses of DLS

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1. Low resolution
  - two species are not resolved as separate peaks unless their radii differ  $\sim 2$ -fold ( $\sim 8$ -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 our hands DLS has been the most effective tool for detecting precursors of visible particulates

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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 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



# Strengths of DLS

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1. high sensitivity to large aggregates that may be immunogenic and/or precursors to visible particulates
2. covers an enormous range of sizes 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