

AUC *vs.* some alternate solution biophysical methods for characterizing association and aggregation



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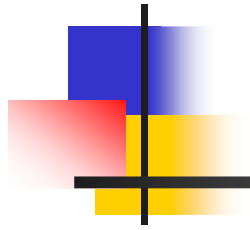
Outline

1. Reversible interactions

- characterizing self-association and receptor-ligand interactions via sedimentation equilibrium
- using on-line light scattering (SEC-MALS) or titration calorimetry to complement sedimentation equilibrium
- using sedimentation velocity to qualitatively assess whether two proteins interact
- some recent light scattering applications

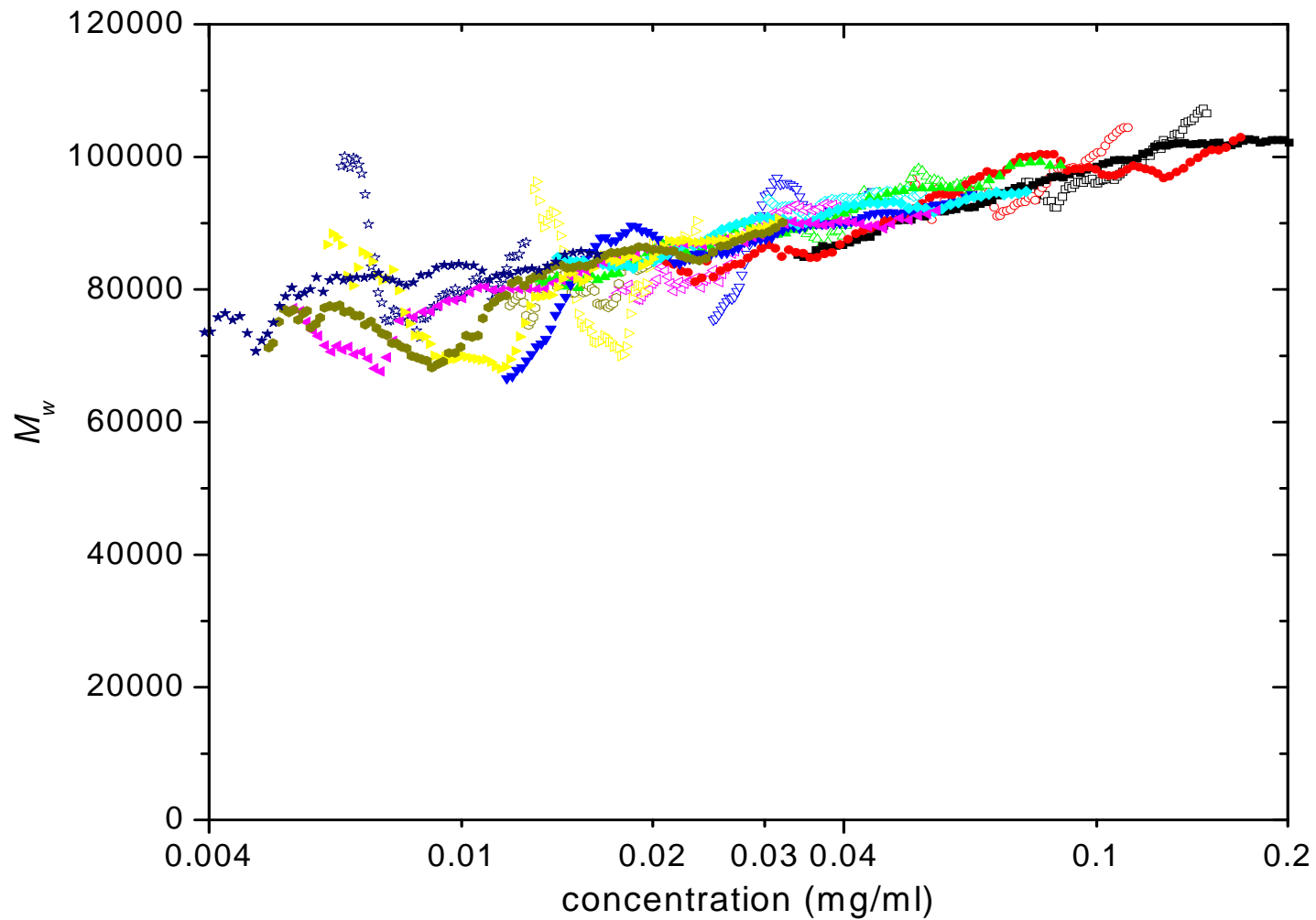
2. “Aggregation” (mostly irreversible) in protein therapeutics

- how we use sedimentation velocity
- some complementary information from SEC-MALS

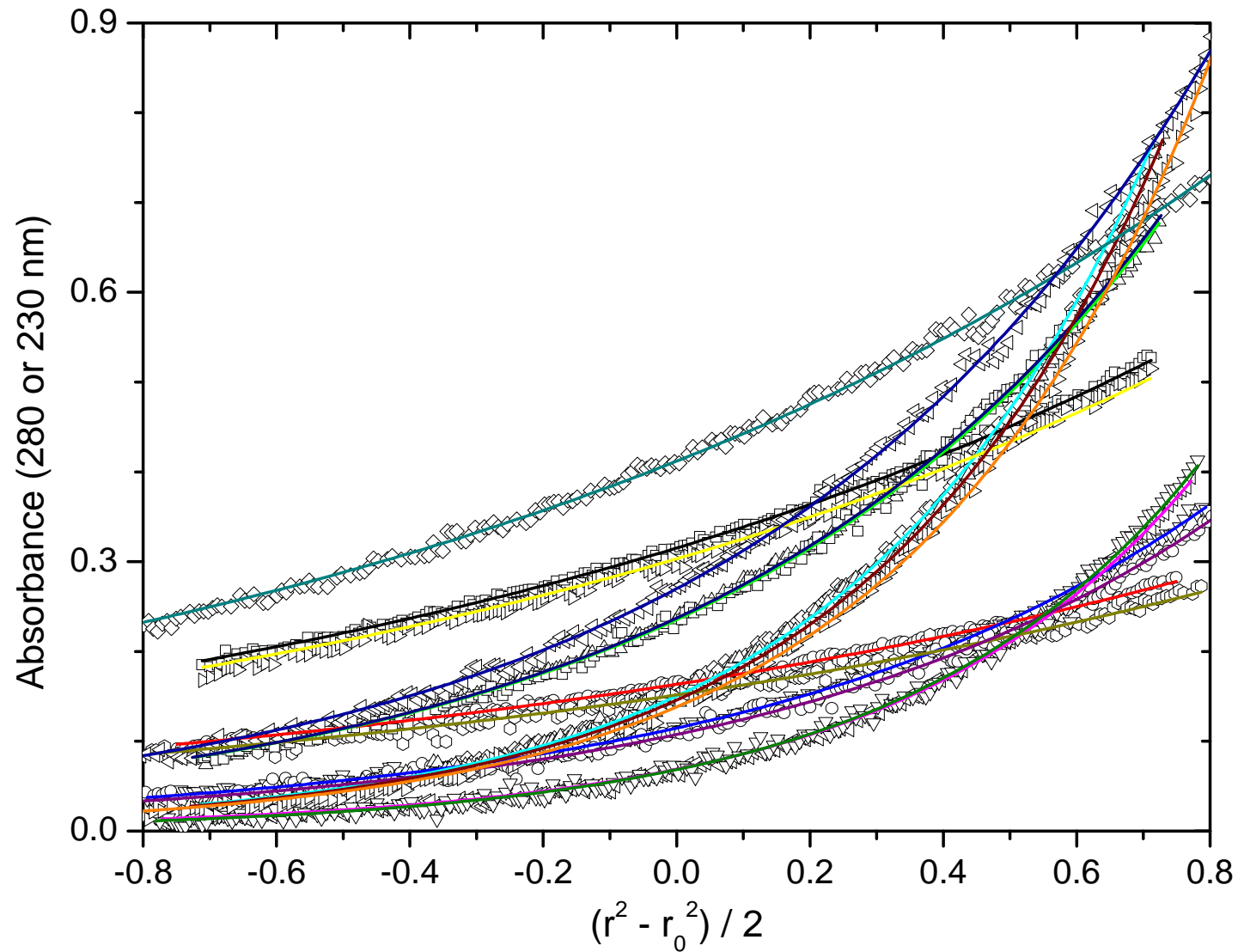


Self-associating systems

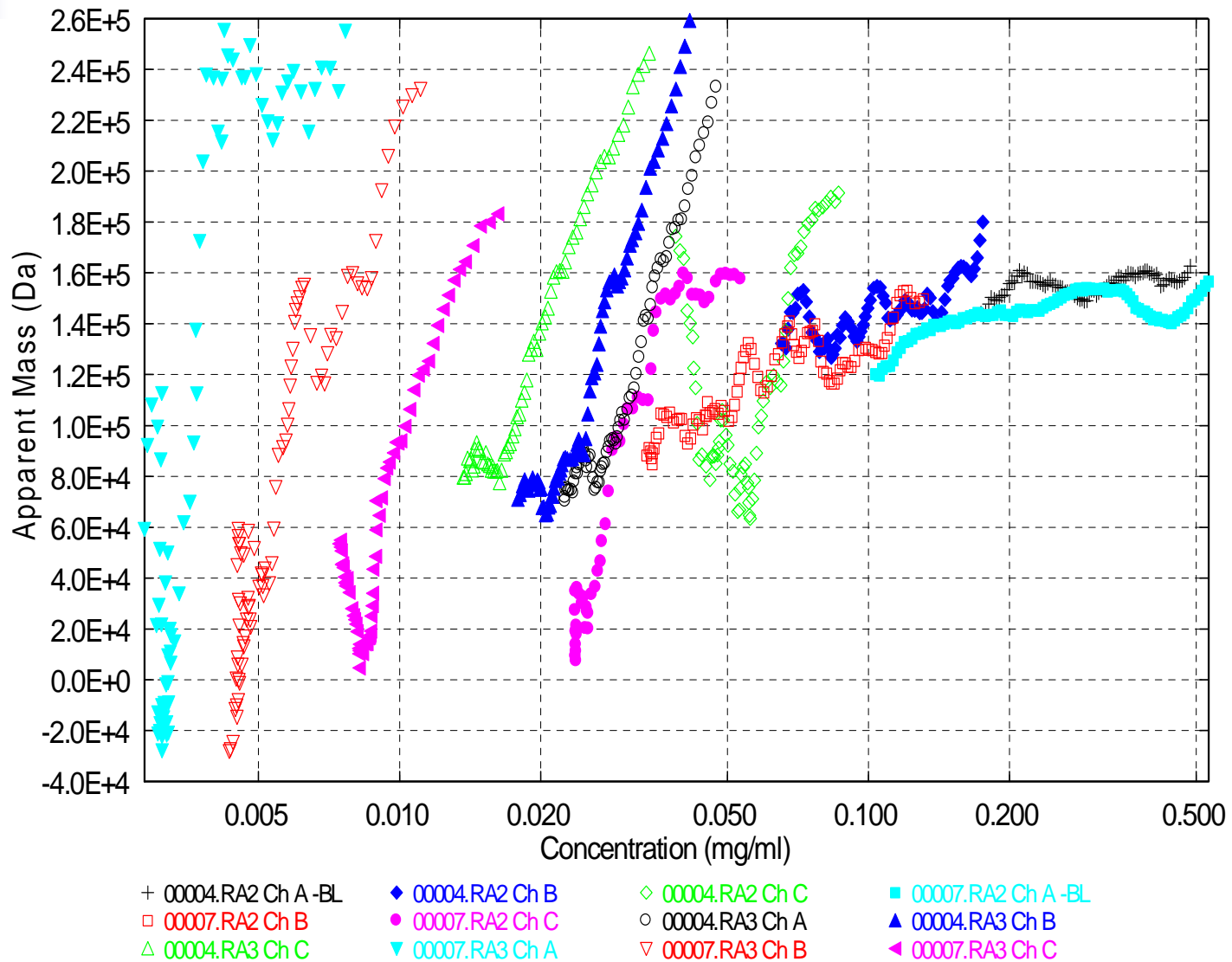
Apparent mass *vs.* concentration for the soluble extracellular domain (ECD) of the atrial natriuretic peptide receptor (monomer mass 58 kDa)



Global analysis shows the ECD dimerizes with $K_d = 520$
+/- 20 nM ($\Delta G = -8570$ +/- 25 cal/mol)



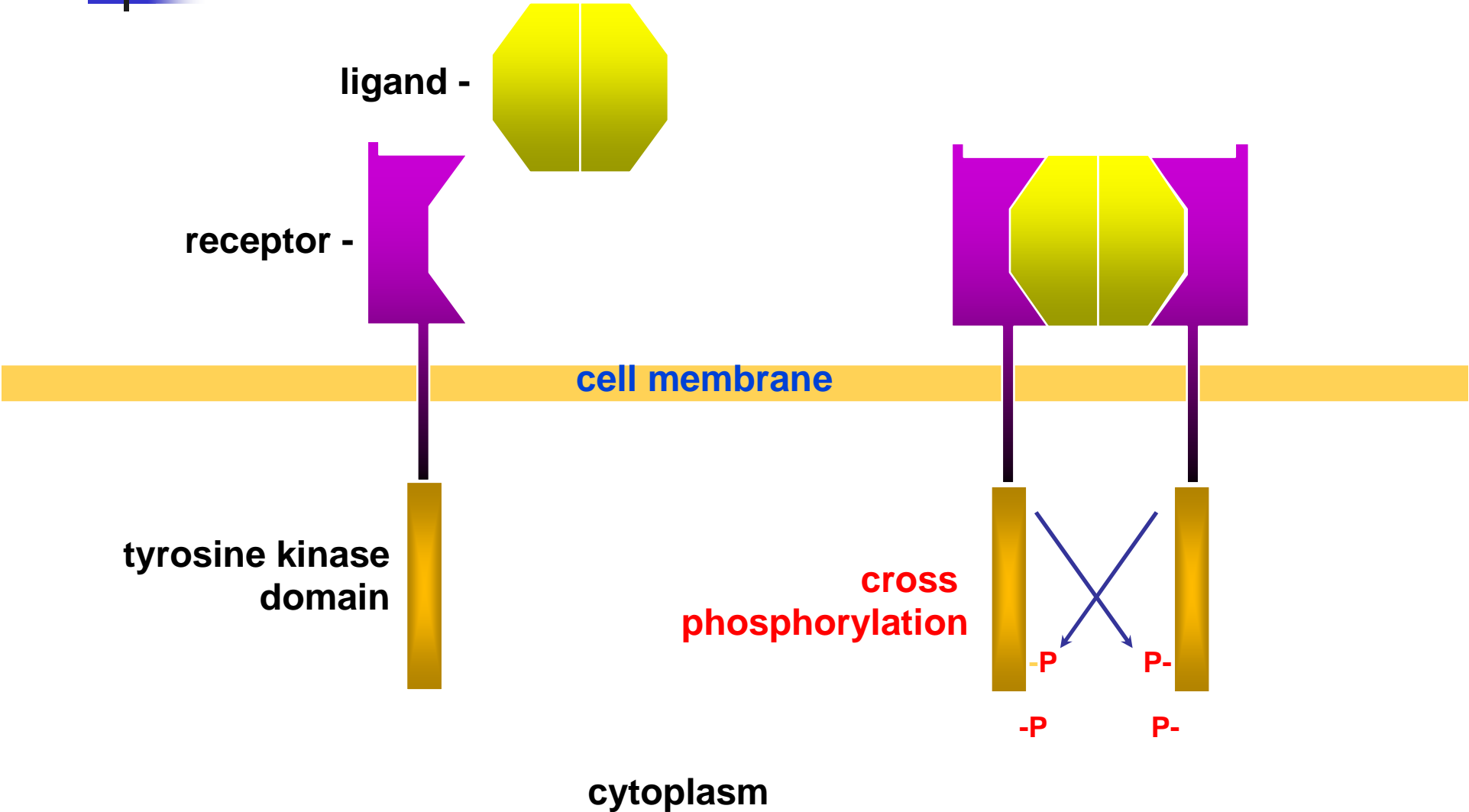
This monomer-dimer-tetramer system acts as a mixture at low concentrations (? due to dilution of its cofactor)



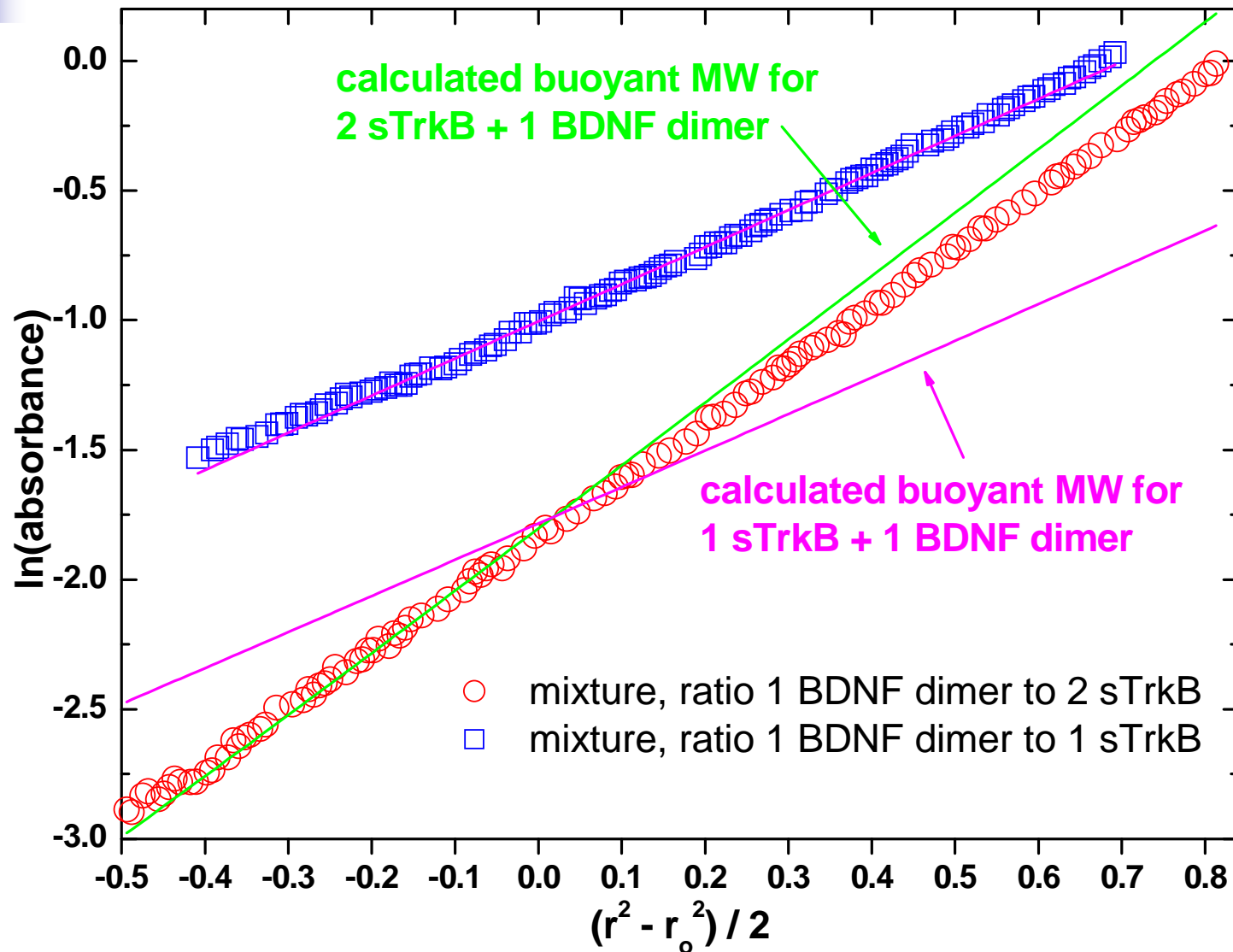


Mixed (hetero) associations

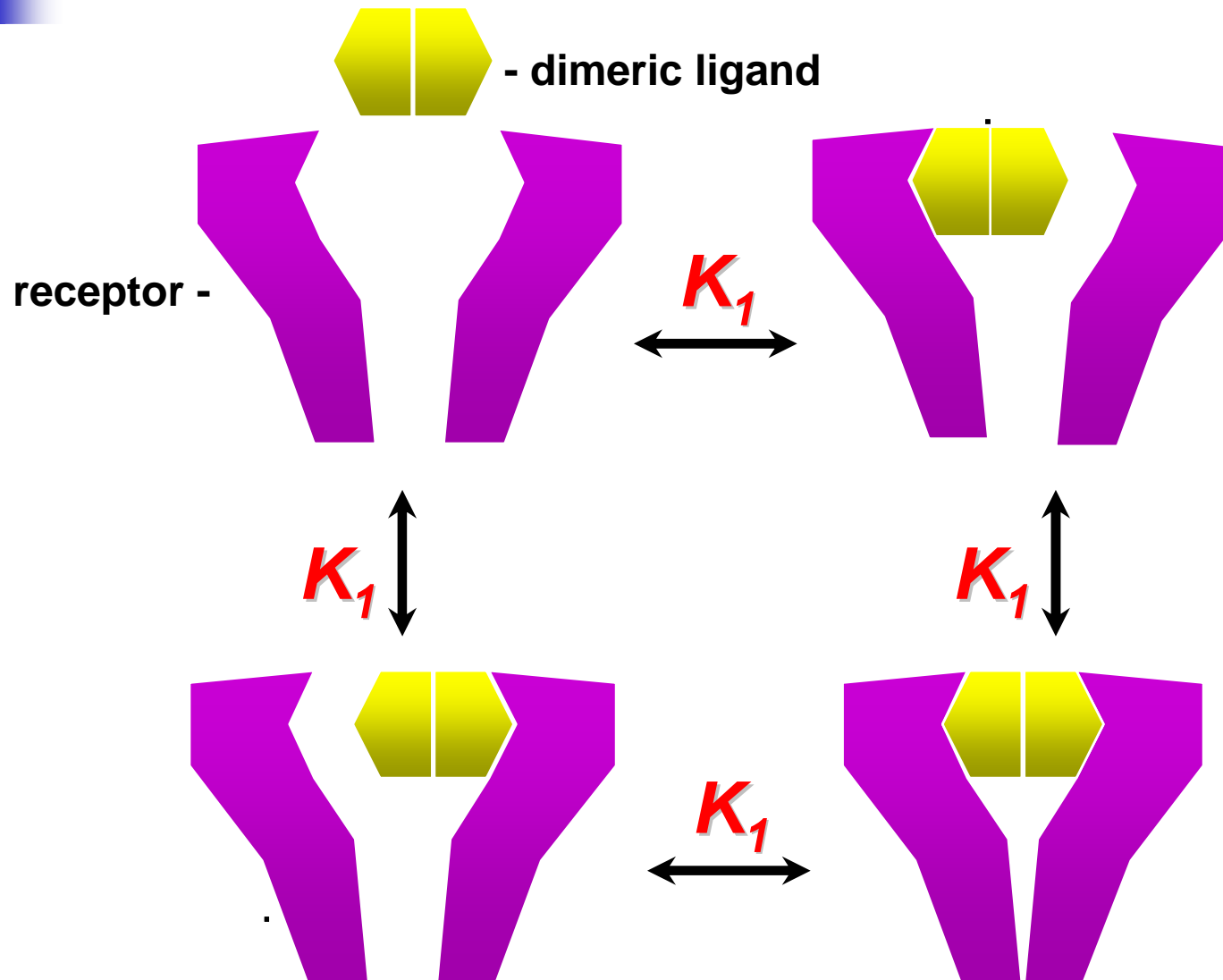
Initiation of signaling by receptor dimerization



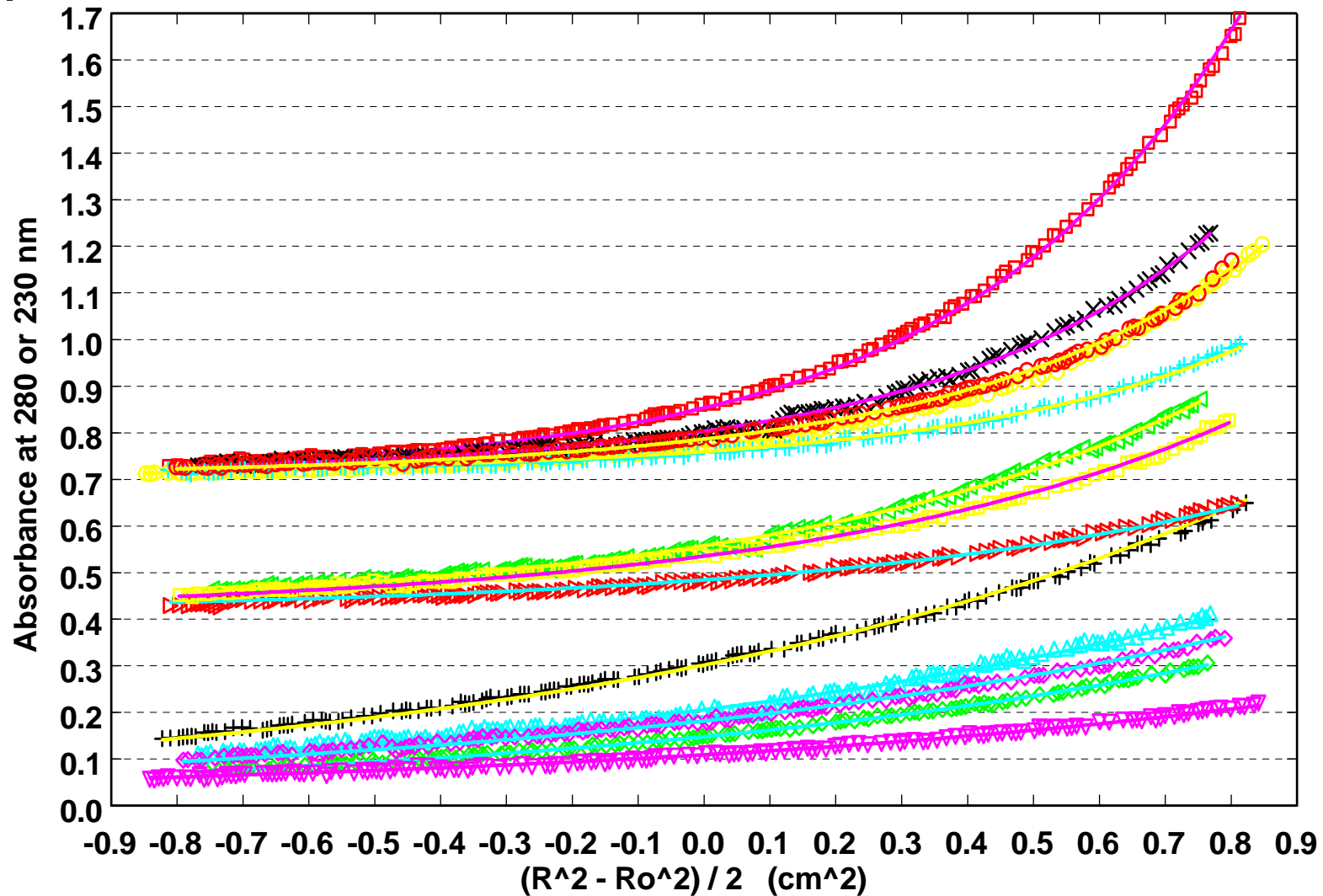
Linearized plots of sedimentation equilibrium data at 11,000 rpm for sTrkB + BDNF mixtures



2 equivalent, independent binding sites model

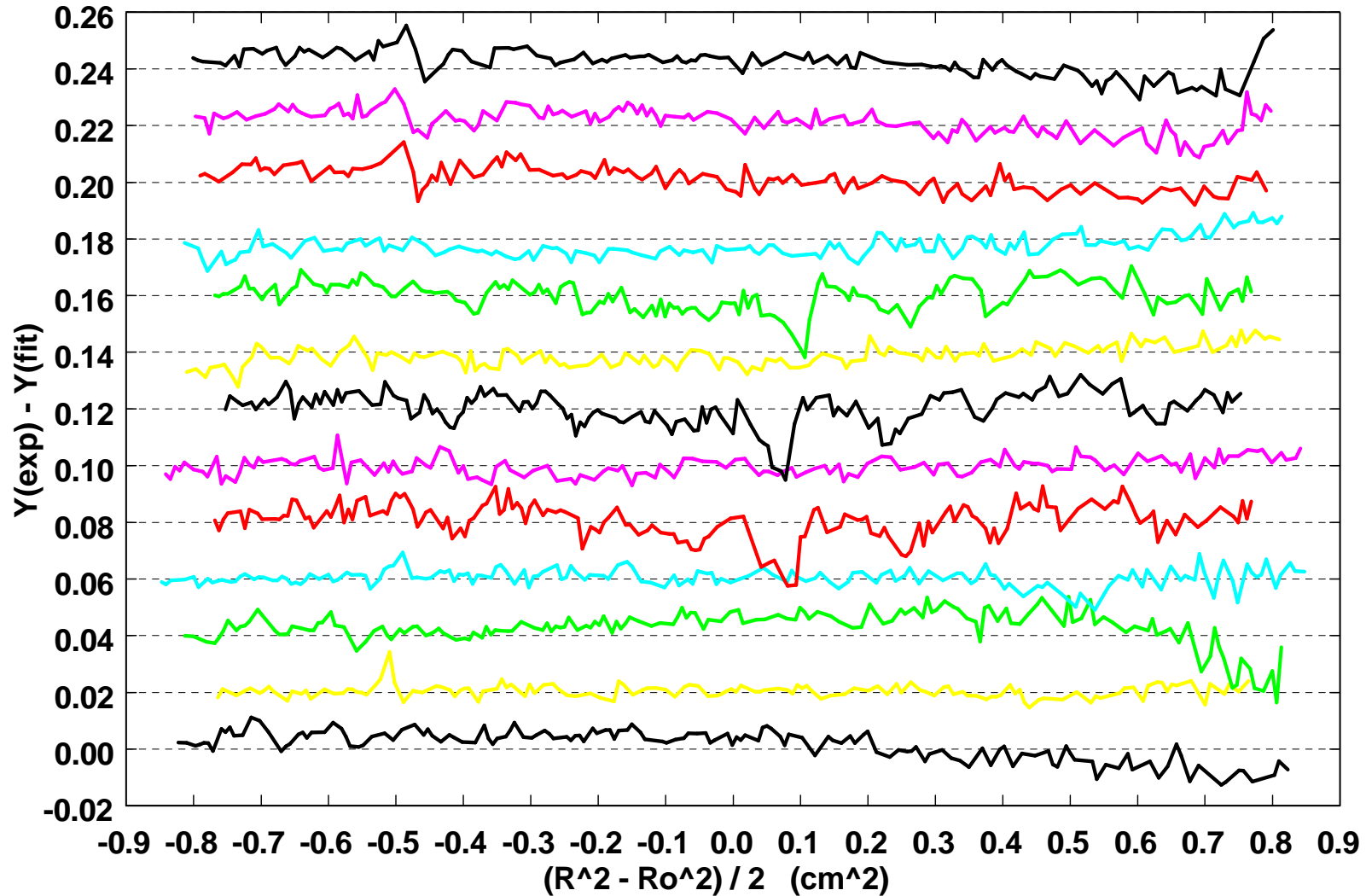


Global fit of 13 data sets for sTrkB + BDNF mixtures to an equivalent, non-interacting binding sites model; the fit returns $K_d = 8.3$ nM [95% confidence 7.0 to 9.4 nM]



Residual plot for global fit of sTrkB + BDNF data

r.m.s residual = 0.0053 AU

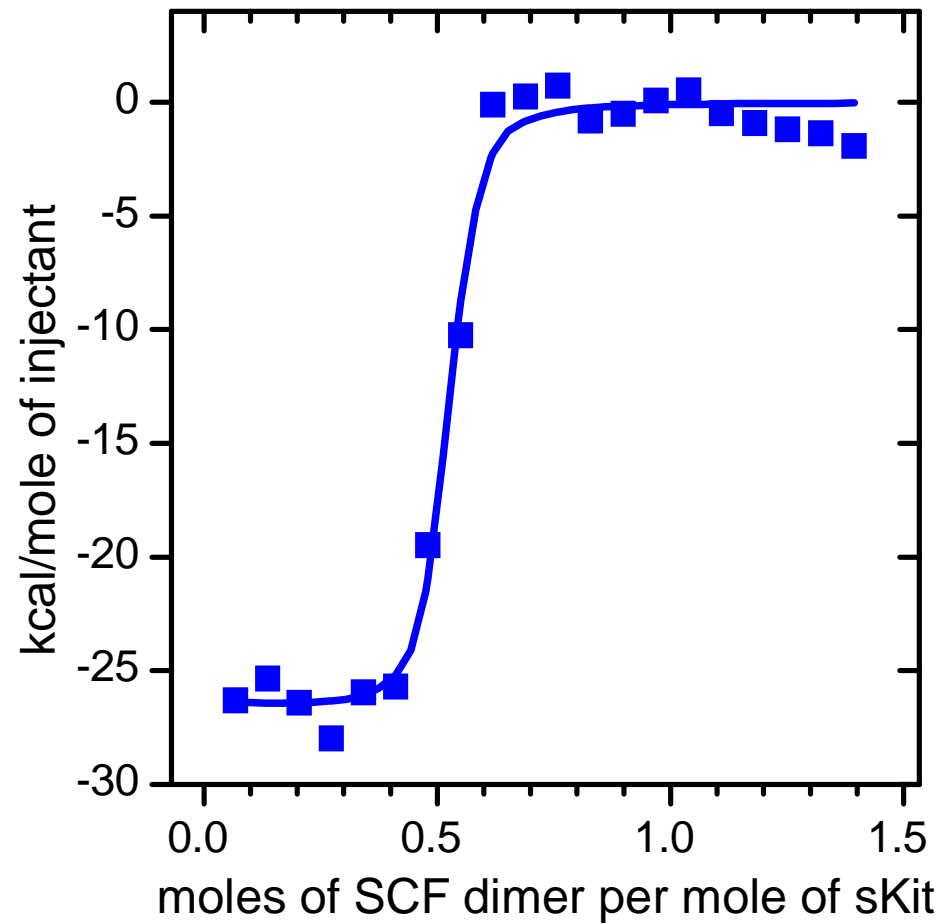
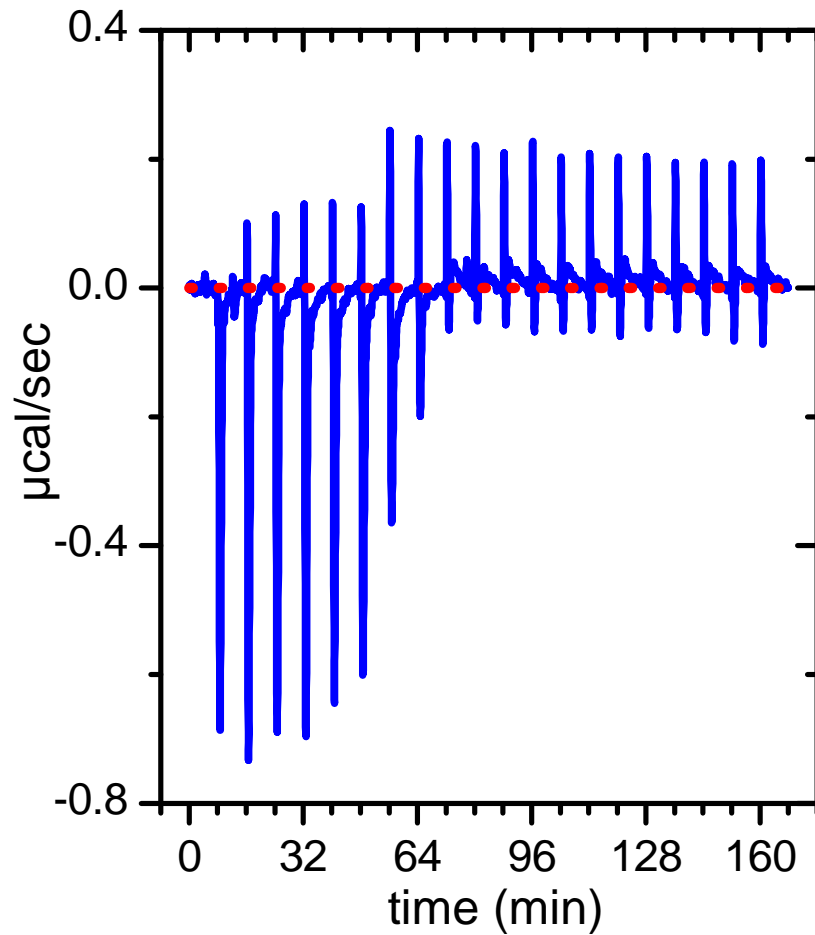


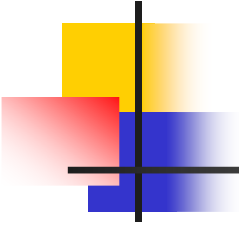


But this isn't the whole story...

- For analyzing mixed associations having the correct binding model is critically important
- One key to having the correct model is to nail down the stoichiometry
- Both light scattering and isothermal titration calorimetry (ITC) can be quite helpful for defining stoichiometry (or at least molar ratios for ITC)

ITC confirms a molar ratio of 2 c-KIT receptors binding to one SCF dimer

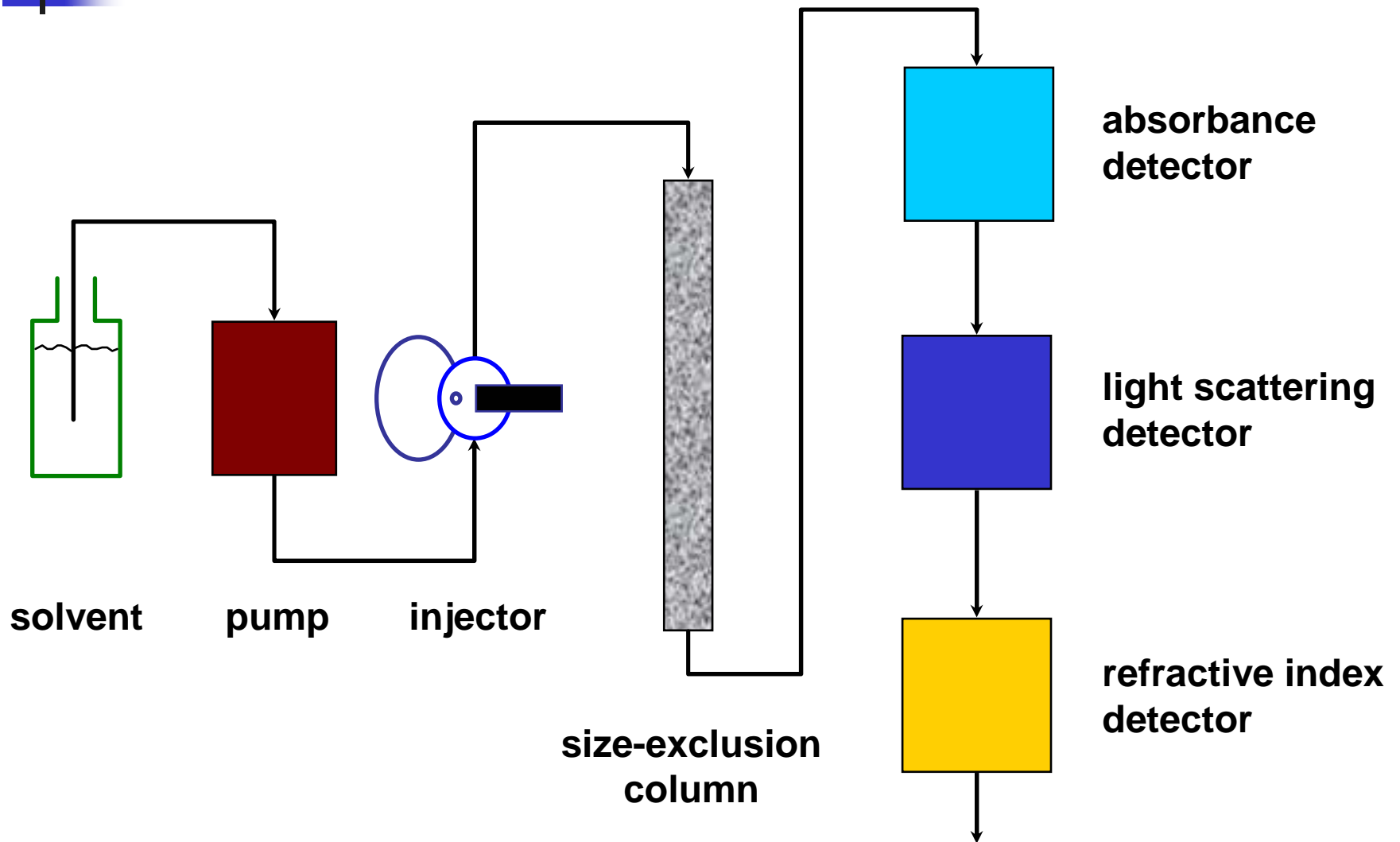




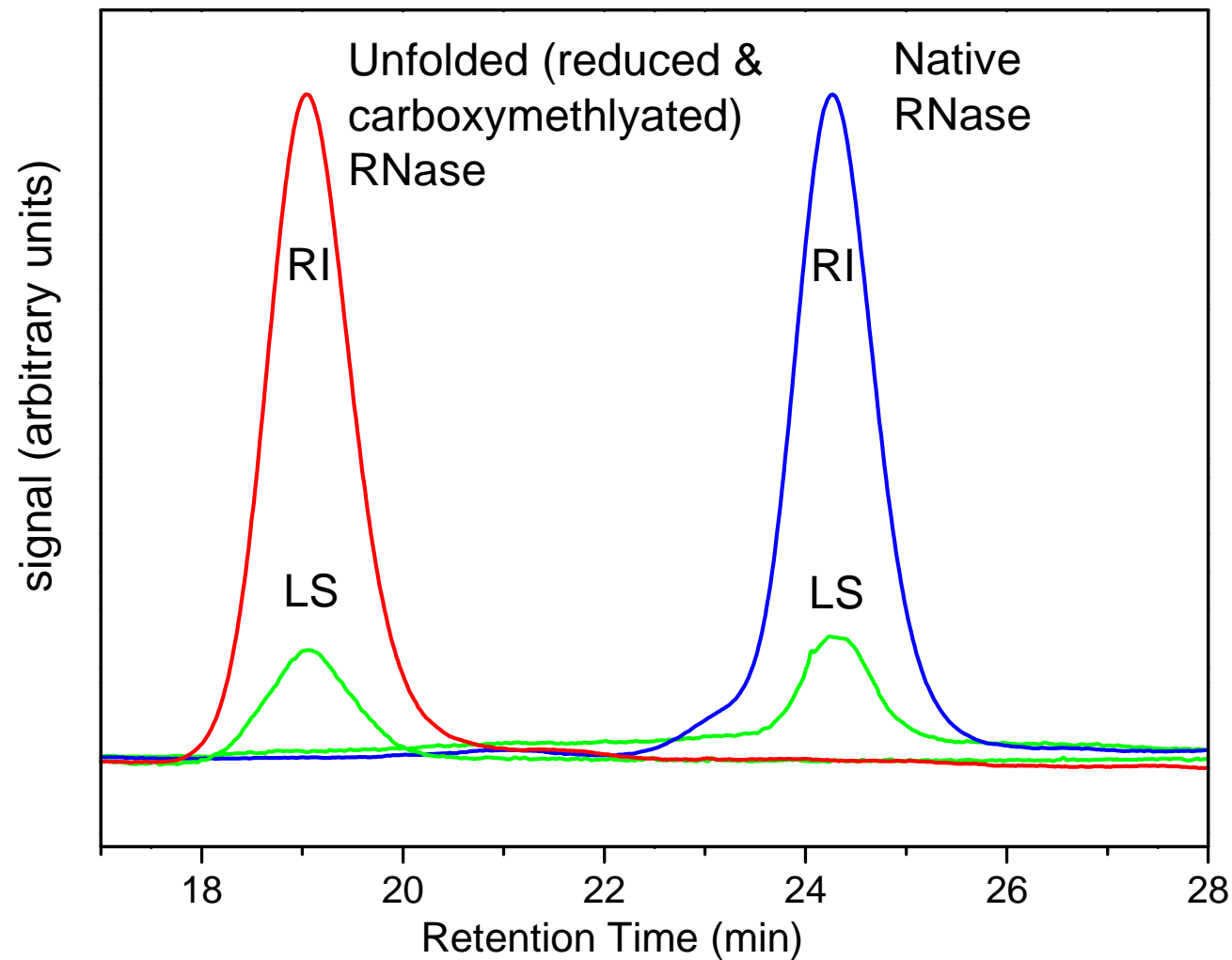
Size exclusion chromatography with on-line classical light scattering detection (SEC-MALS) can also be very useful for interaction studies

- SEC-MALS can easily determine the stoichiometry of the complex (protein-protein, protein-DNA, *etc.*) in many cases
 - typically if the K_d is $\sim 1 \mu\text{M}$ or less the complex will not dissociate on the SEC column

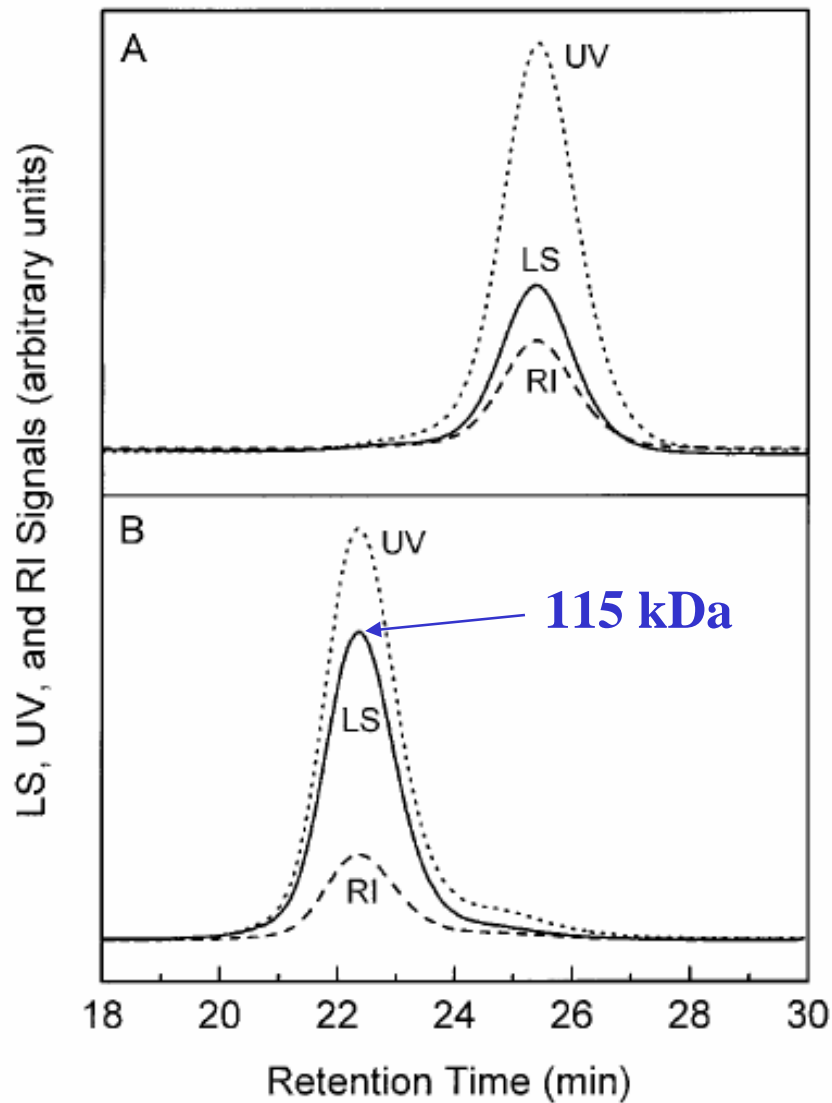
Typical setup for size-exclusion chromatography with on-line light scattering detection (SEC-MALS)



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



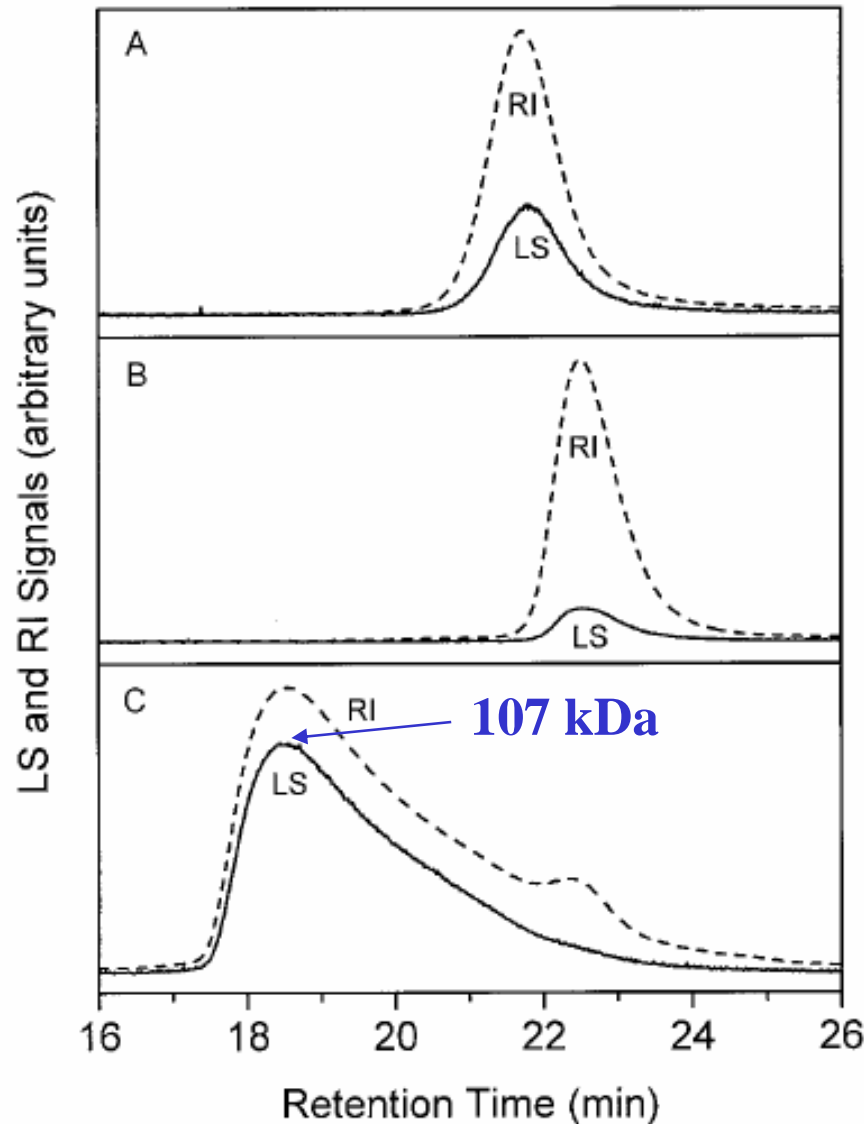
Complexes of sTrkB with BDNF dimer (27 kDa)



sTrkB alone
(44 kDa)

sTrkB + BDNF mixture, ratio
2 sTrkB per BDNF dimer

SEC-MALS determines stoichiometry for complexes of sTNFR with TNF



TNF trimer alone
(52 kDa)

sTNFR alone
(17 kDa)

mixture, ratio
3 sTNFR per TNF trimer

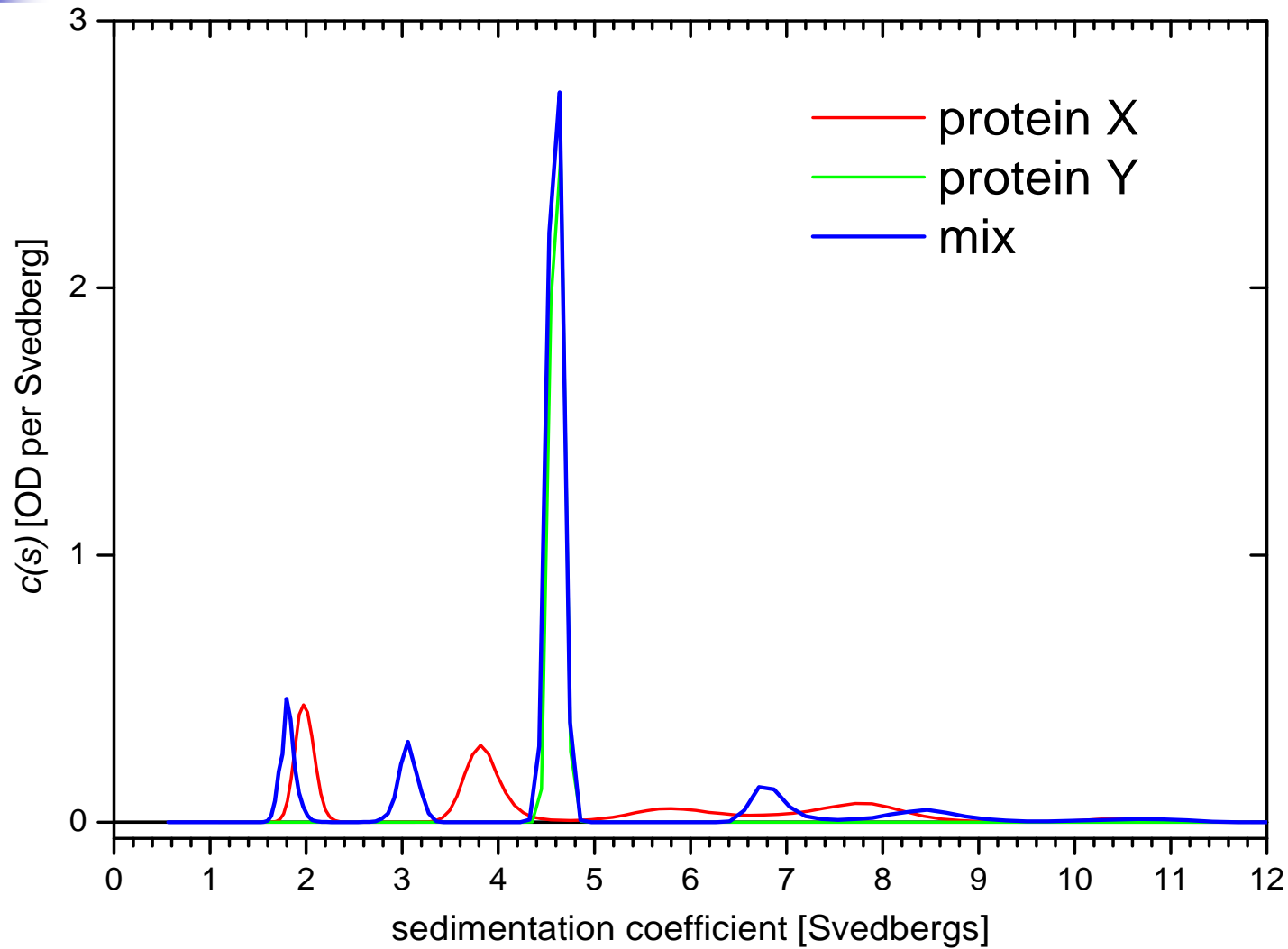


The biotech industry is starting to produce “combo” products which contain 2 or more different proteins

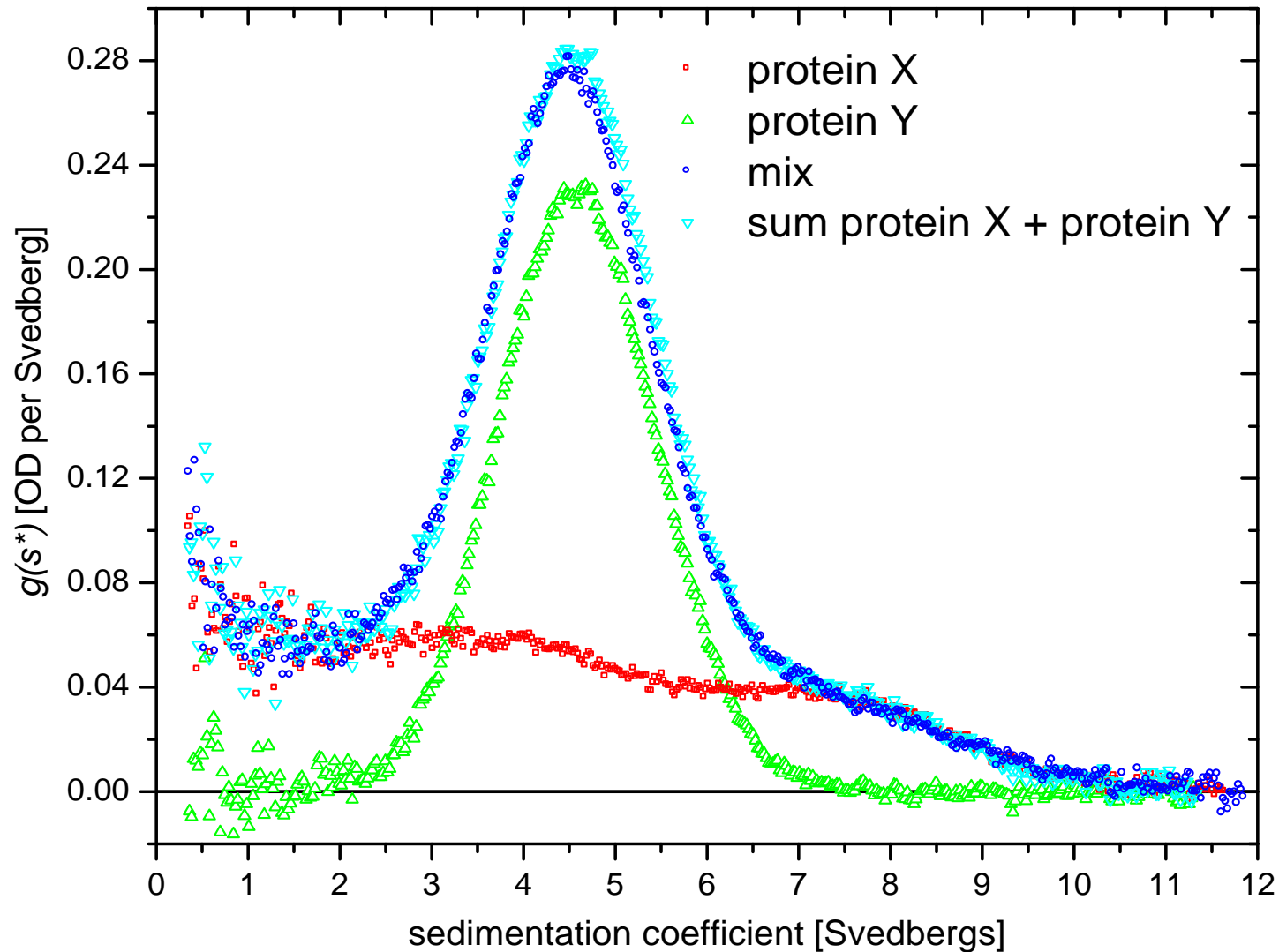
- combining products into one syringe would minimize the anxiety associated with injections
- one protein may enhance the delivery or effectiveness of another
- however if these proteins interact to form complexes, that could potentially have negative consequences:
 - changed serum lifetime or distribution in the body
 - increased immunogenicity due to new epitopes

Can you tell whether these proteins bind?

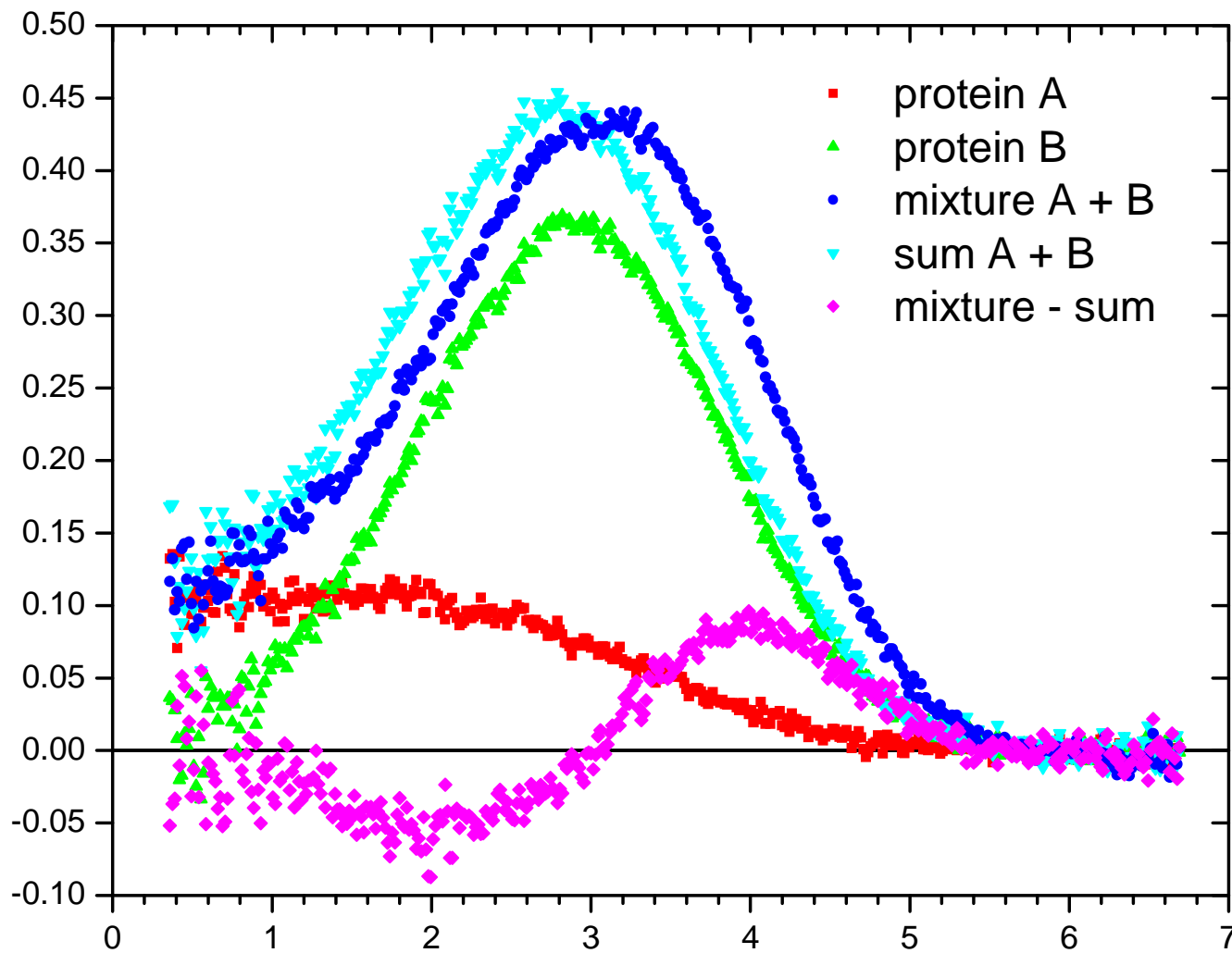
$c(s)$ analysis of pure protein X, pure protein Y, and their mixture



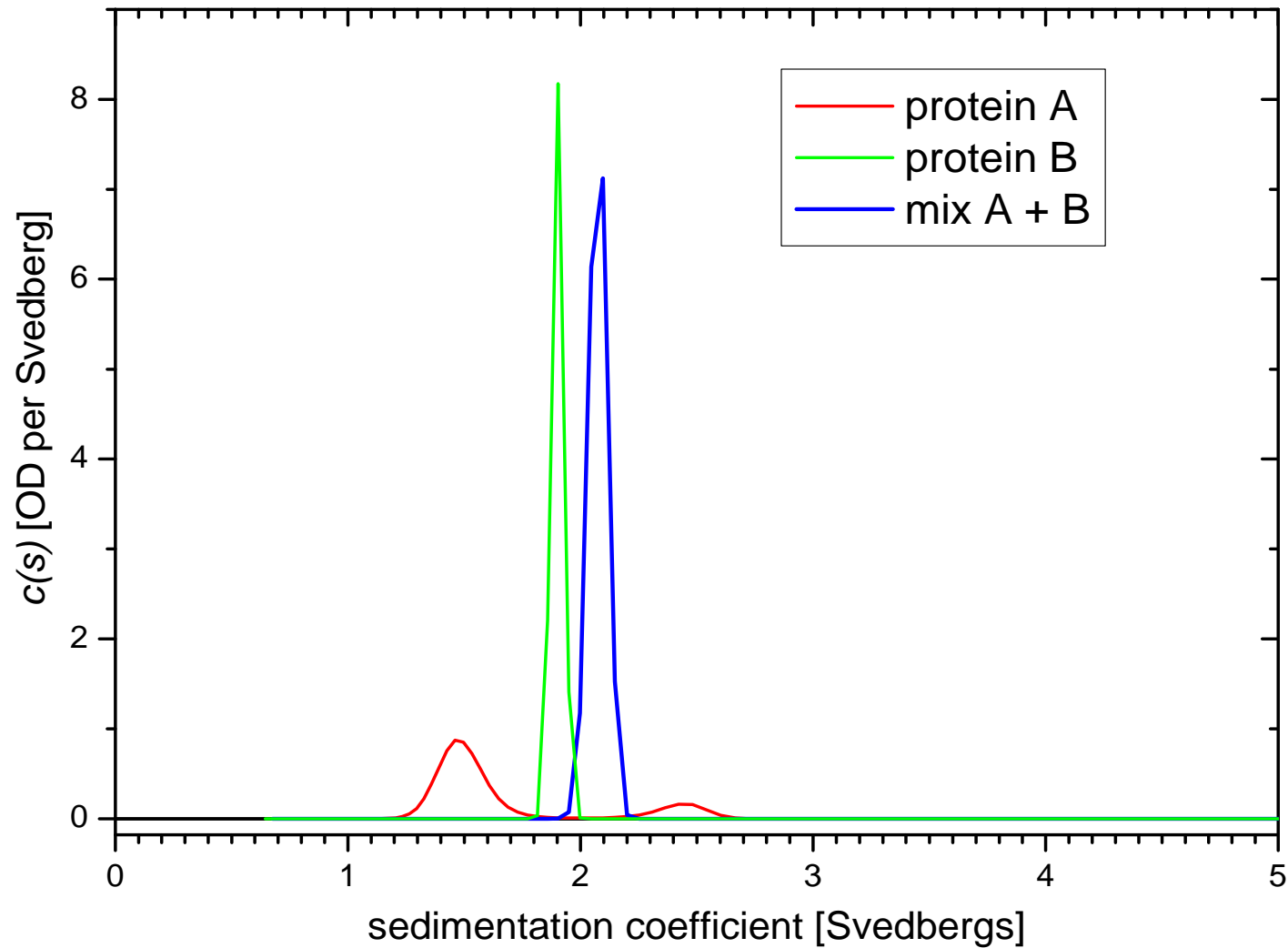
The same data analyzed via $g(s^*)$ are unambiguous:
there is no detectable interaction



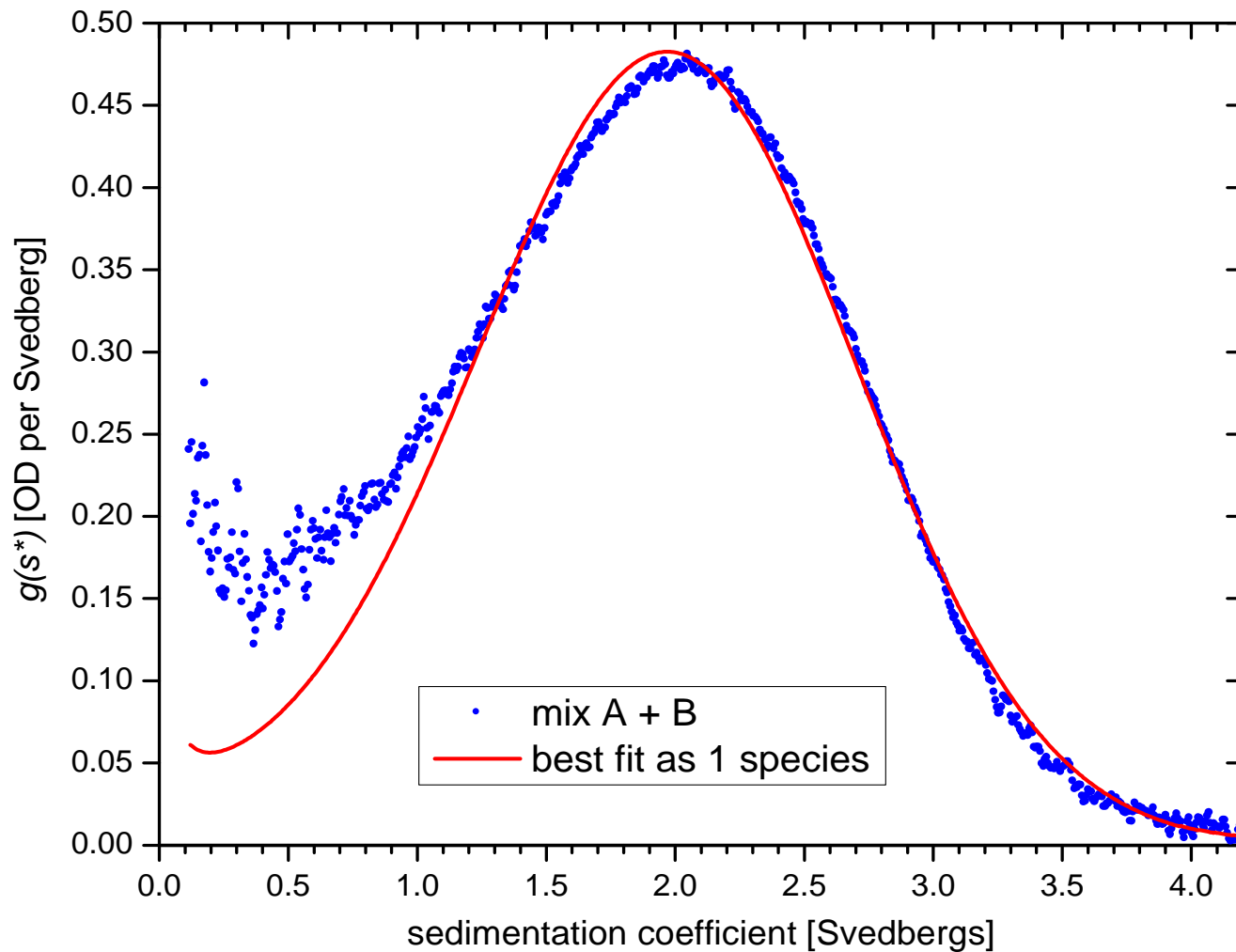
In contrast $g(s^*)$ analysis clearly shows
proteins A and B interact



However $c(s)$ falsely implies that 100% of the mixture is in the form of a single type of complex



Attempting to fit the $g(s^*)$ distribution for the mixture as a single species clearly fails (and implies a mass below that of B monomer)



Allen Minton at NIH has pioneered a new application of these LS detectors he calls "composition-gradient static light scattering" (CG-MALS)

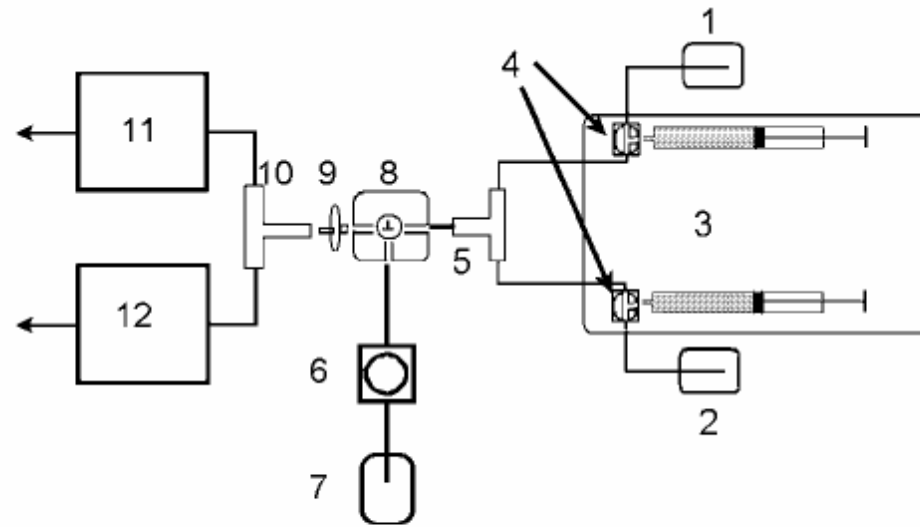
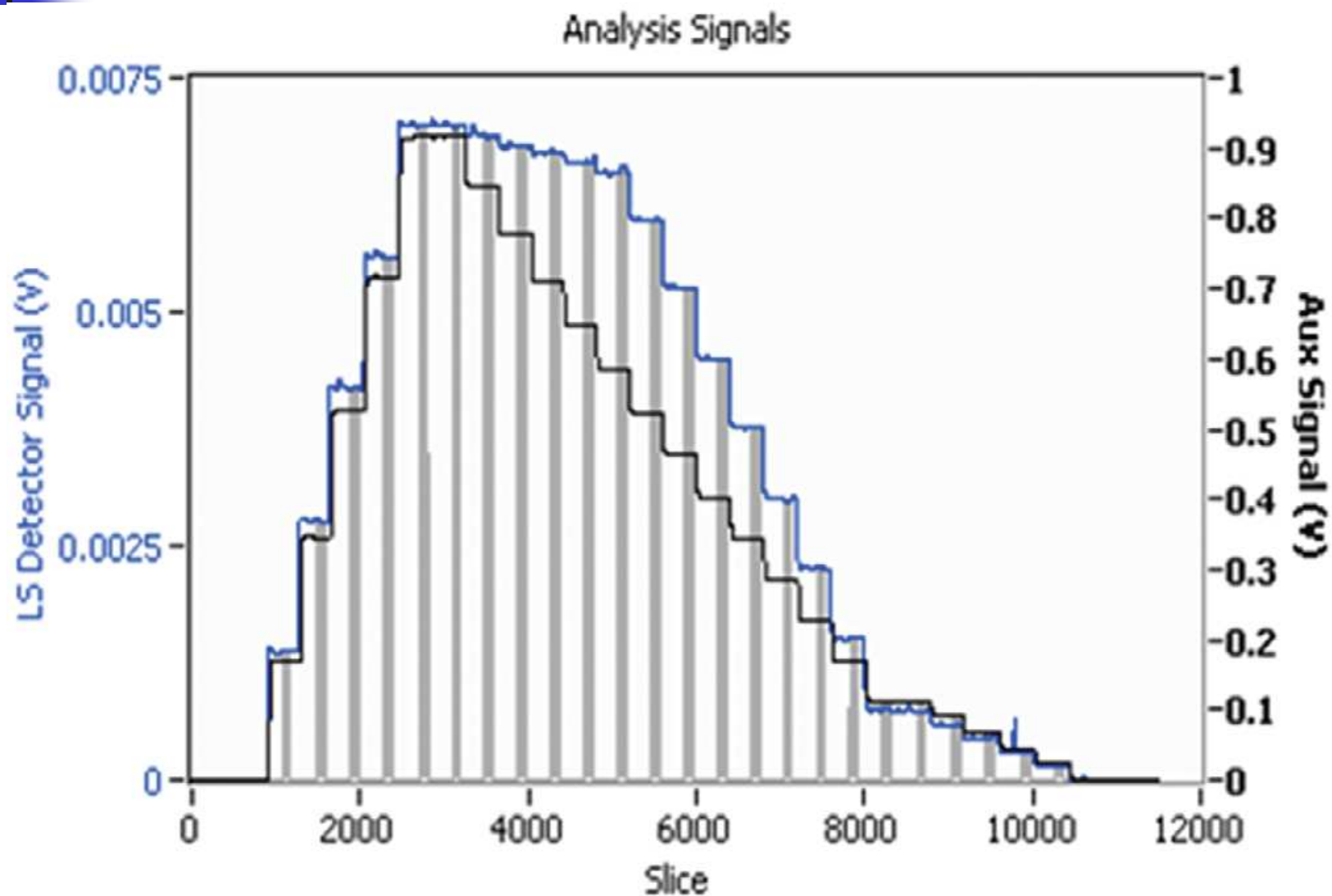


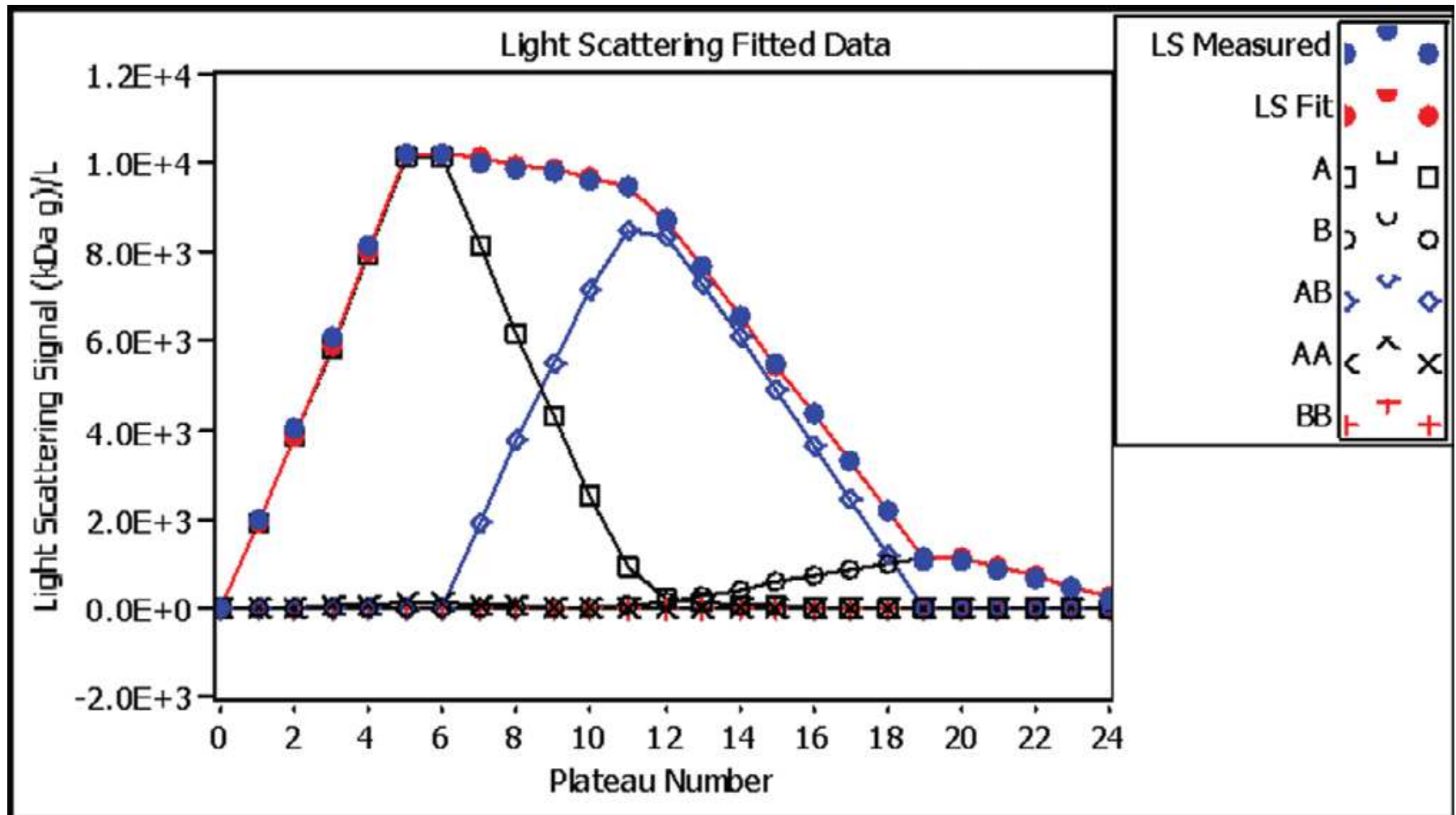
FIGURE 1 Schematic illustration of instrumentation used to perform composition gradient light scattering measurements: (1–2) Reservoirs for solutions A and B. (3) Programmable dual syringe pump. (4) Programmable valves for switching syringes between filling and delivery modes. (5) T-junction for mixing of input streams from the two syringes. (6) Peristaltic pump for delivery of buffer in reservoir (7). (8) Valve for switching apparatus input between peristaltic pump (6) and syringe pump (3). (9) Inline filter. (10) T-junction for splitting solution mixture into parallel streams for concurrent measurement in absorbance detector flow cell (11) and multi-angle light scattering detector flow cell (12). Figure reproduced from Attri and Minton (4).

K. Kameyama and A. P. Minton (2006) *Biophys. J.* 90, 2164-2169

Cross-titration of chymotrypsin with bovine pancreatic trypsin inhibitor (BPTI)



Fit of the chymotrypsin-BPTI binding to a 1:1 binding model



Self-association of FtsZ

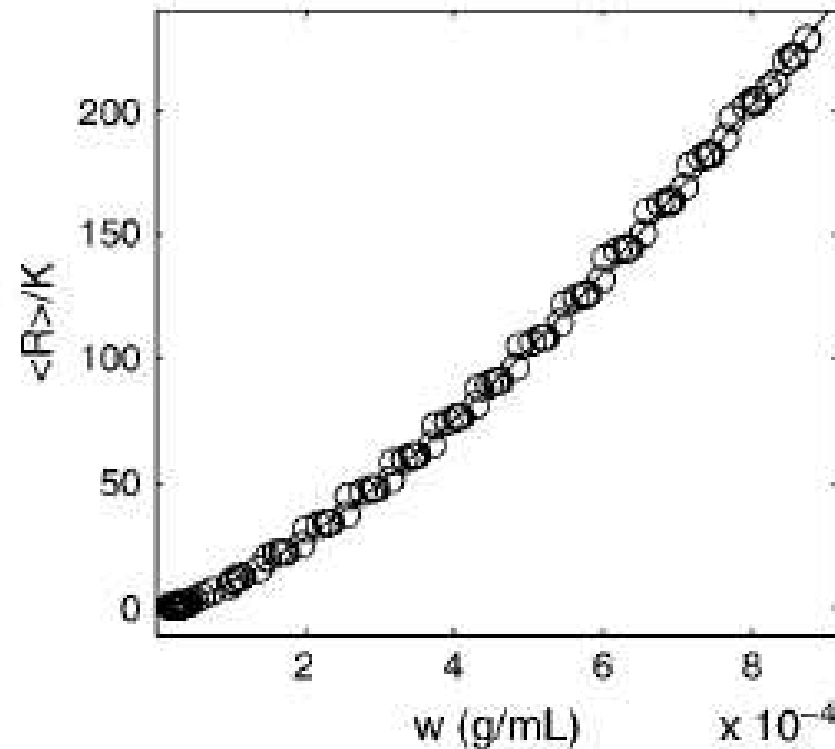


FIGURE 5 Concentration-dependent scattering of FtsZ as a function of total protein concentration. (*Points*, experimental data; *curve*, dependence calculated from inverse-decay model, using any of a several sets of correlated parameter values leading to identical fits of the data.)

For self-association studies Minton has also created a much simpler cuvette-based approach for automated dilutions

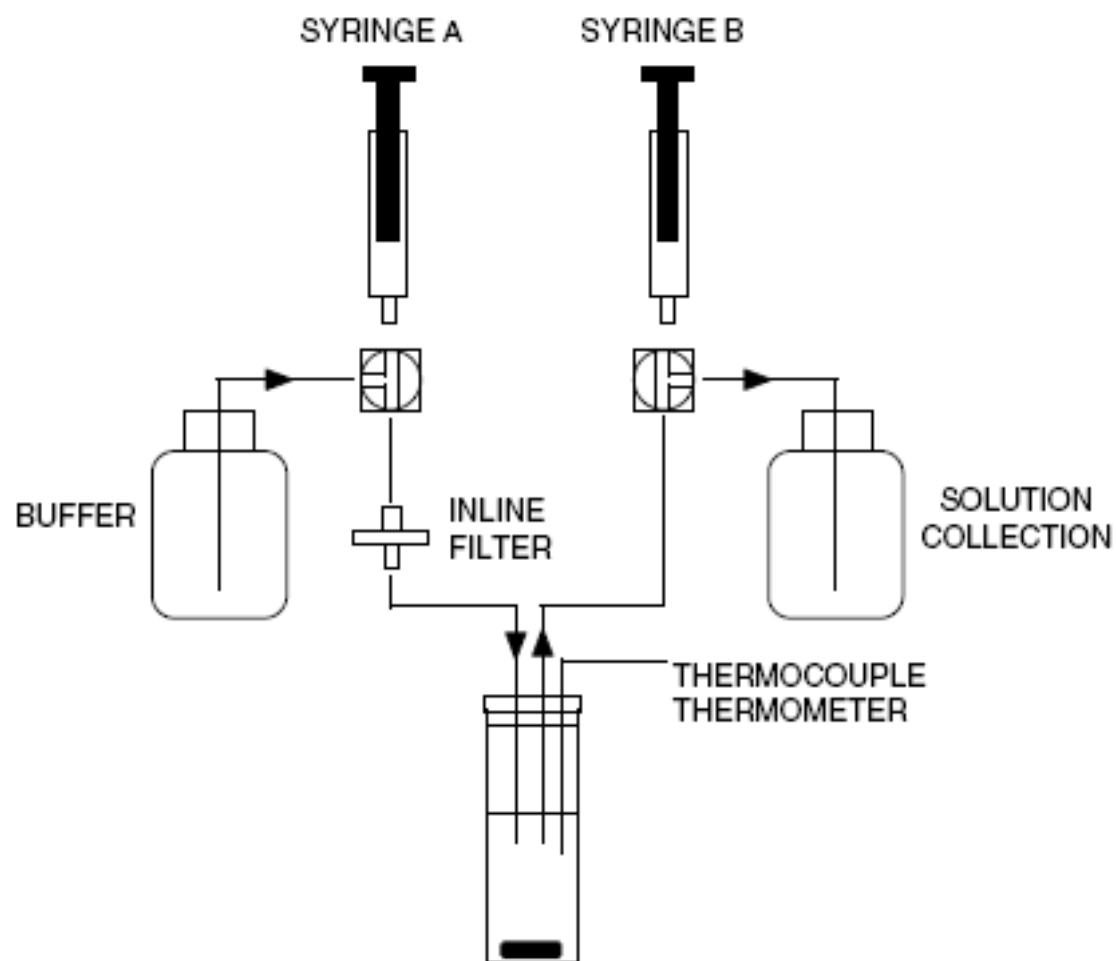
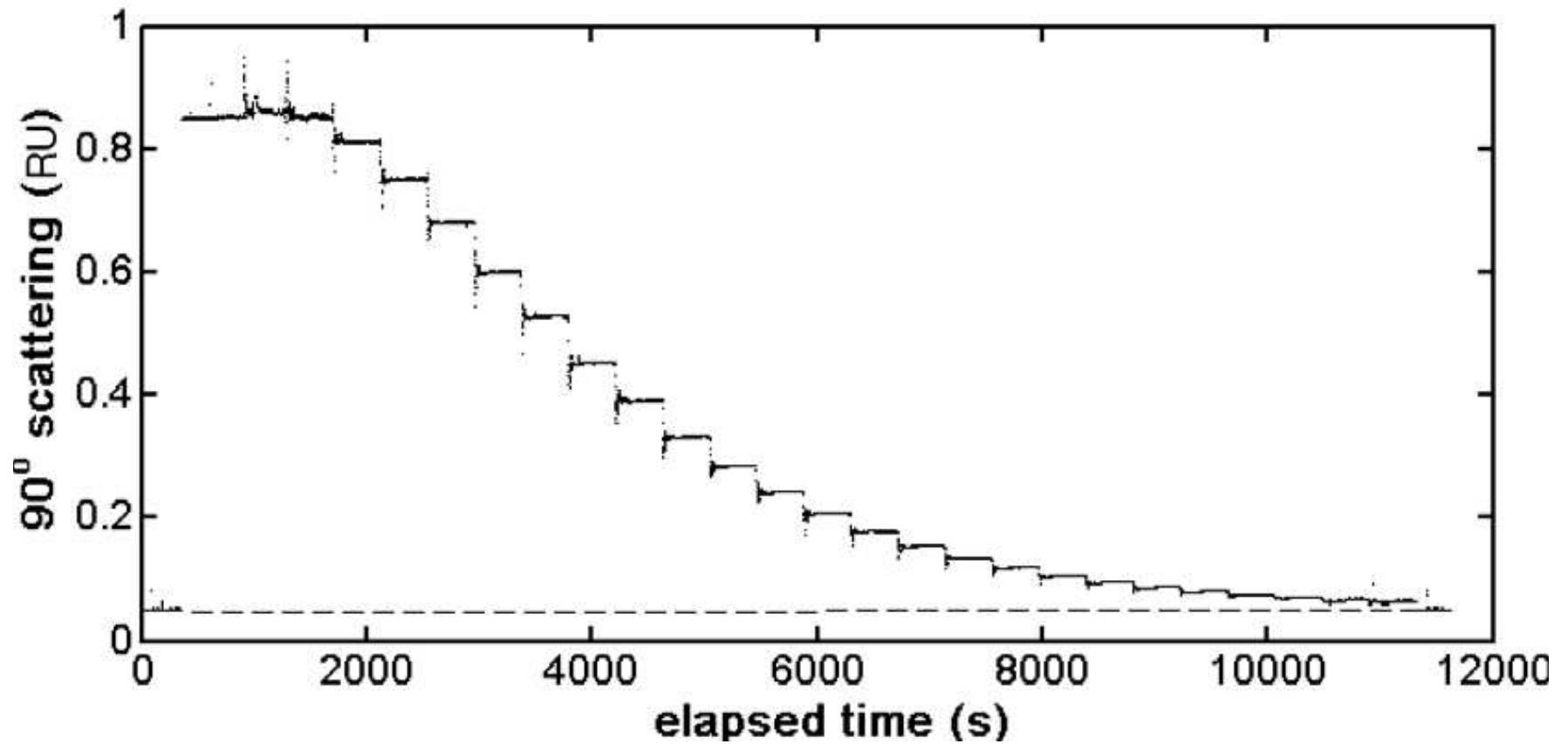


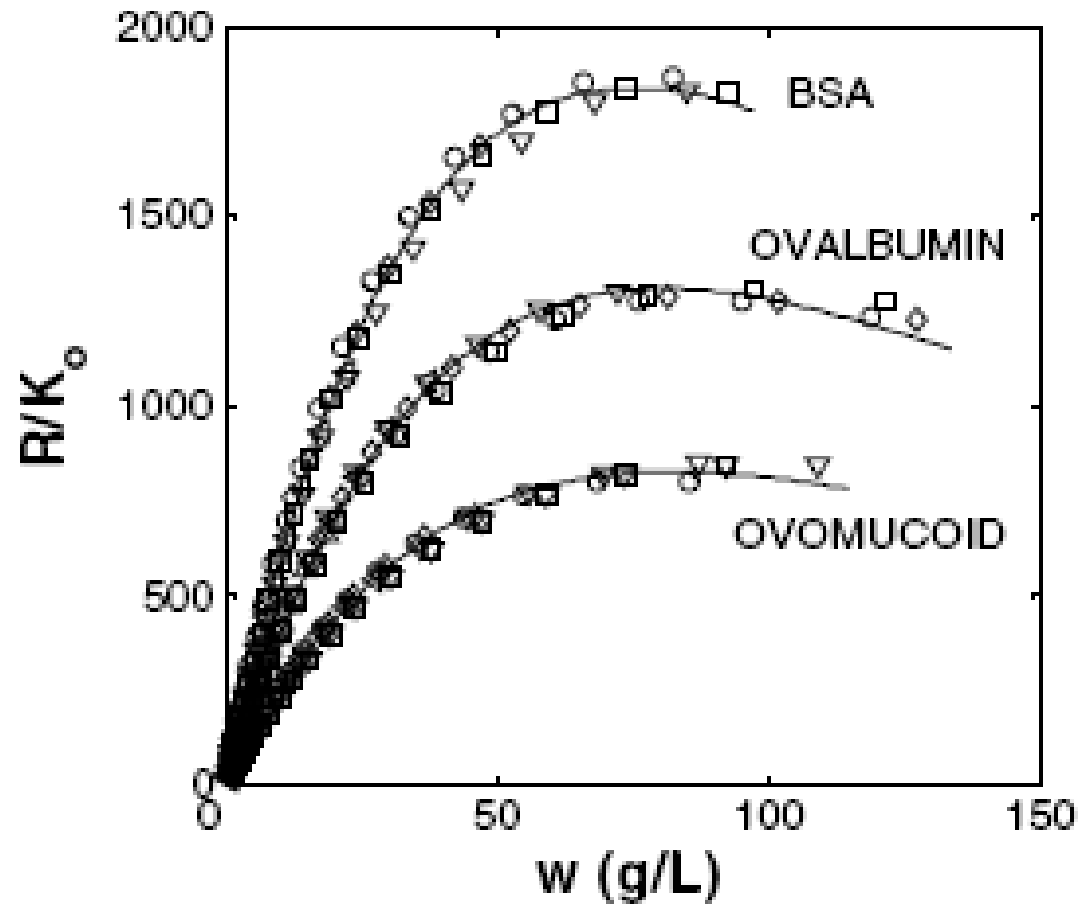
Fig. 1. Schematic diagram of the automated dilution system.

Fernandez, C. and Minton, A. P. (2008) *Anal. Biochem.* 381, 254-257.

Here is an example of a dilution series



With this approach it is possible to study proteins at concentrations up to ~ 200 mg/mL





Aggregation in protein therapeutics



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**



The analytical challenge

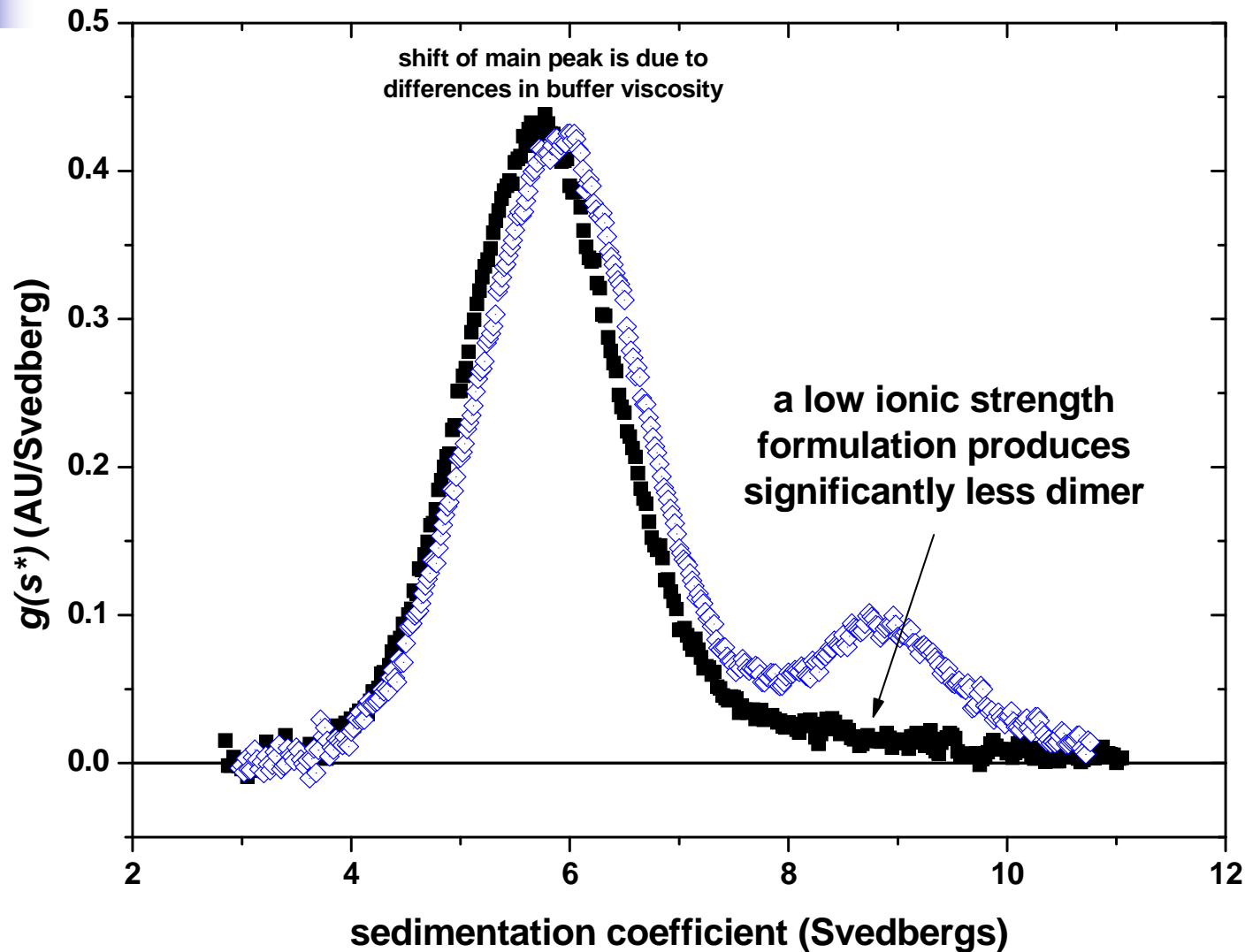
1. Any protein sample may contain aggregates with a wide range of sizes, types, and lifetimes
2. Any one analysis method may not detect all the aggregate sizes or types that are present
3. The measurement itself may perturb the aggregate distribution that was initially present



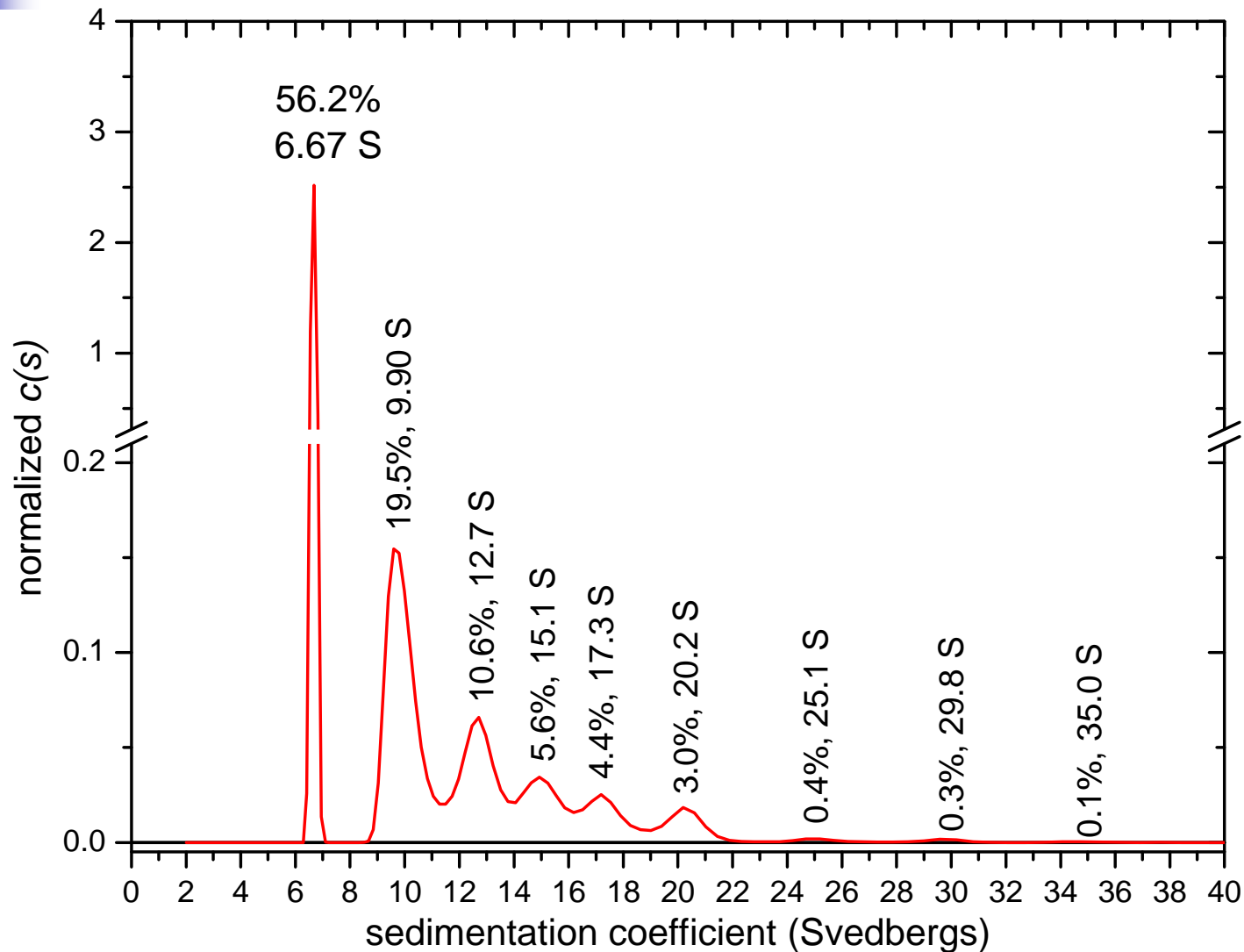
The measurement itself may create or destroy aggregates

dissociation or loss of aggregates can be caused by:	SEC	SV	FFF
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



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

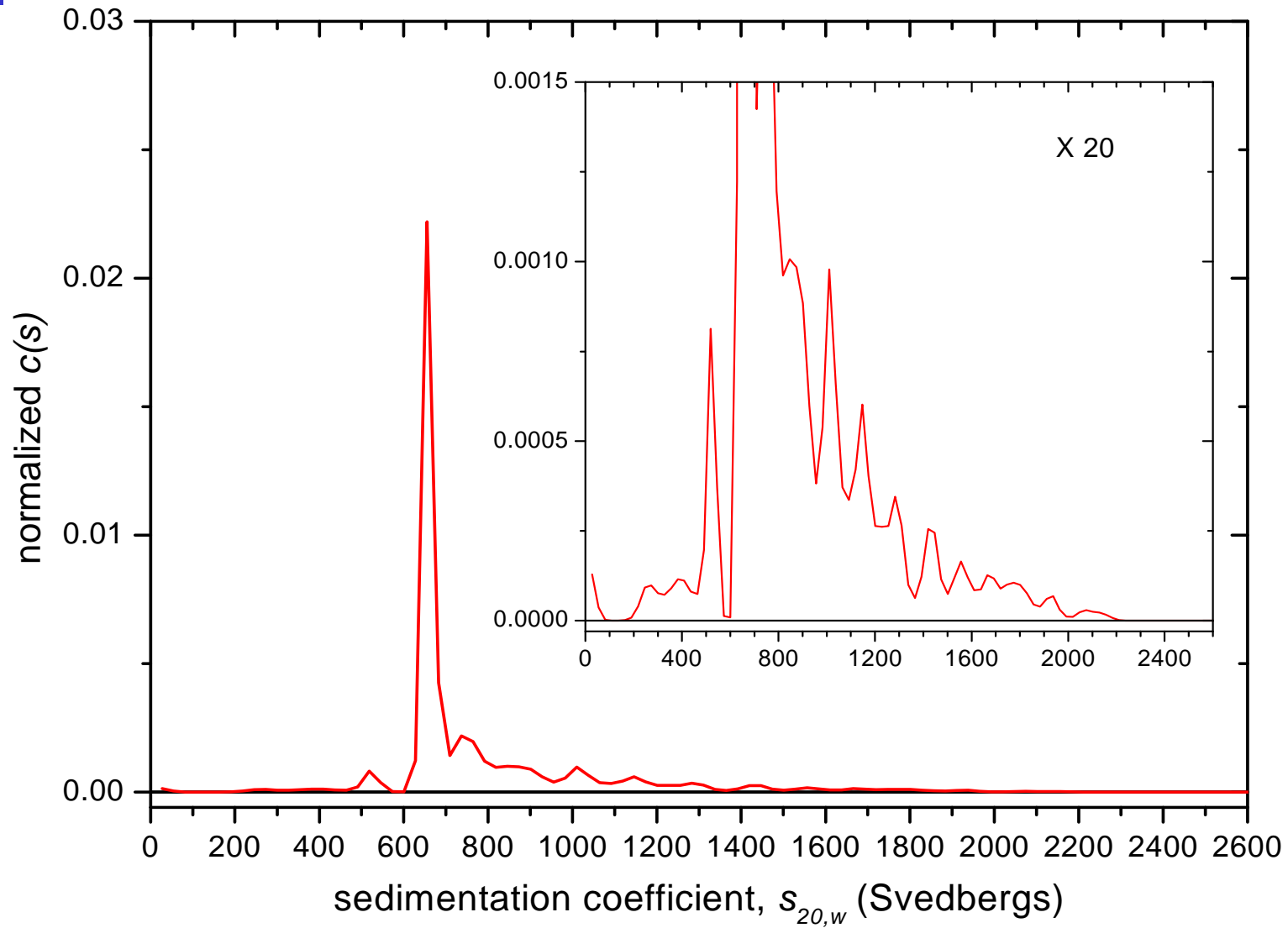




Often a significant fraction of the aggregates never elute from the SEC column

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%

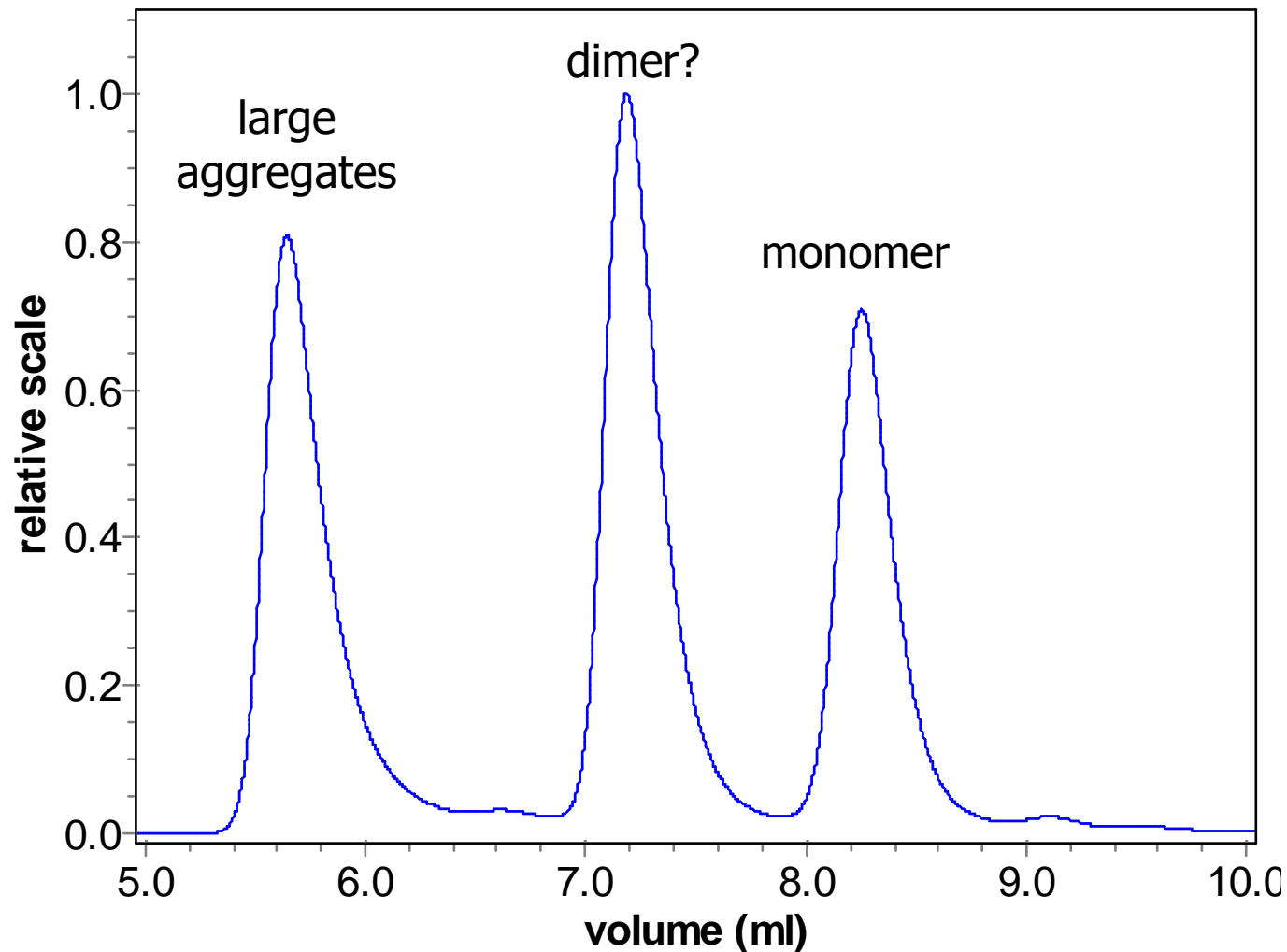
Heterogeneity and aggregation in an adenovirus gene therapy vector



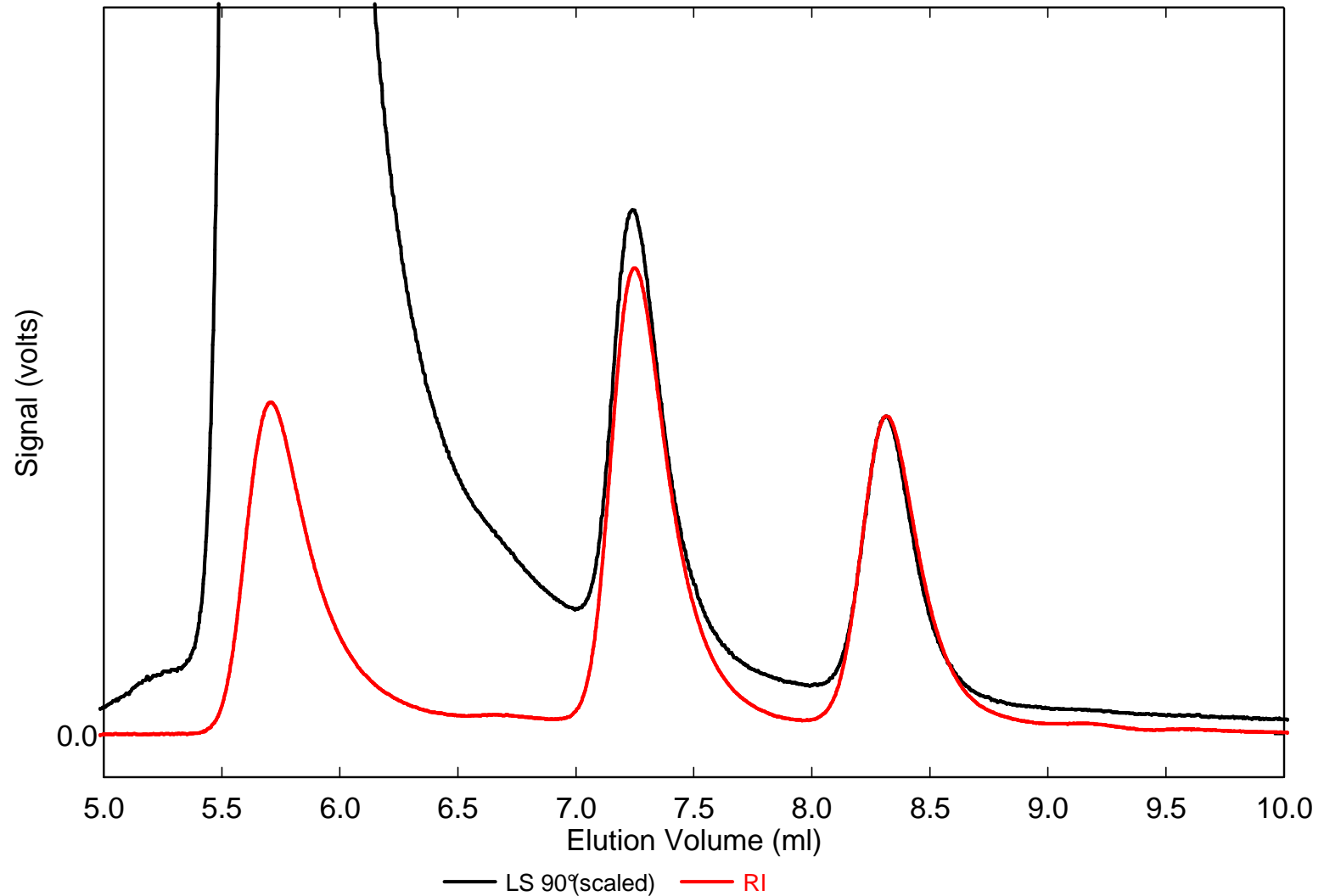
Methods other than AUC can be helpful and complementary: SEC-MALS shows an “aggregate” isn’t an aggregate



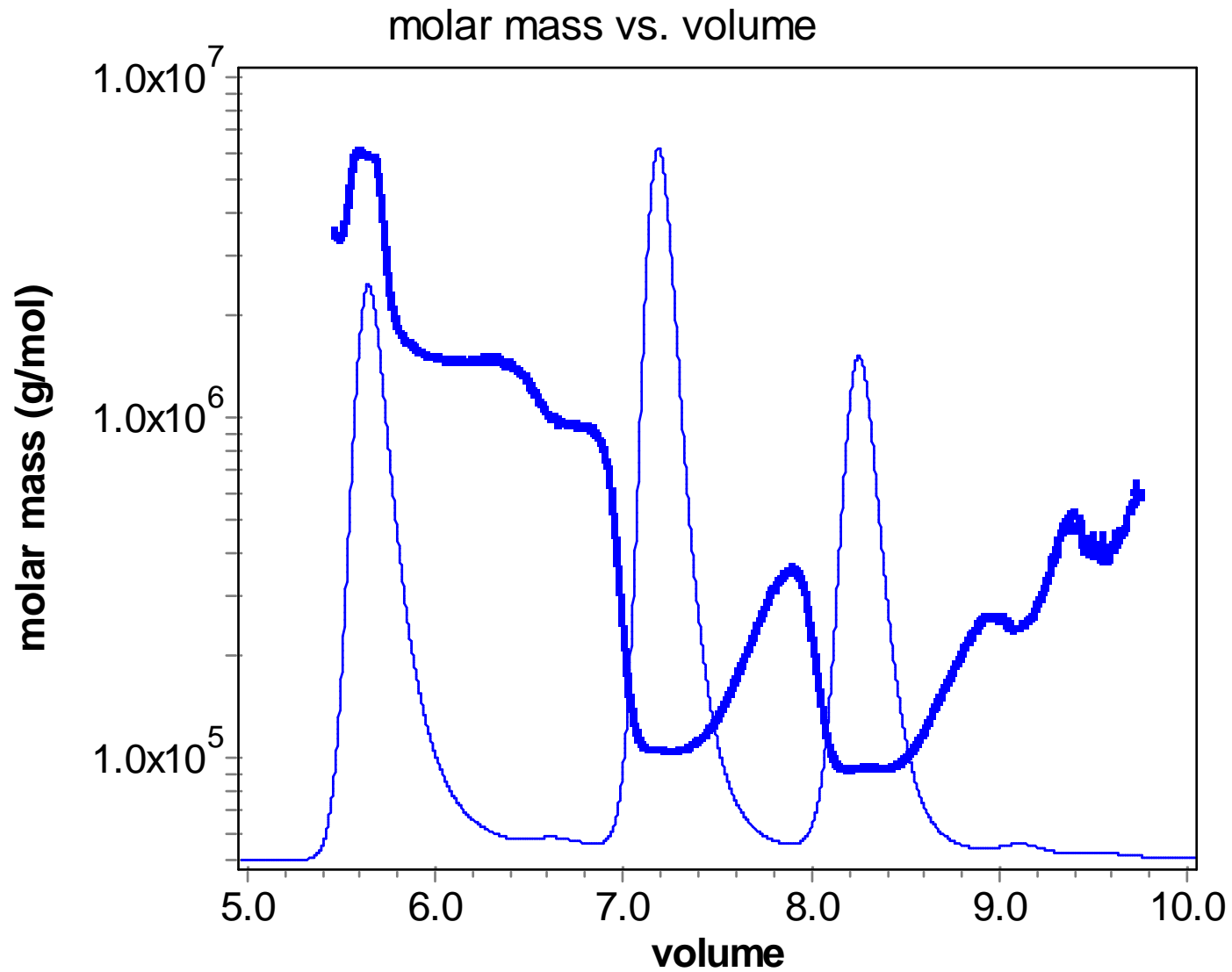
This highly stressed sample of a VaxGen test antigen showed high levels of an SEC peak eluting near the position expected for a dimer



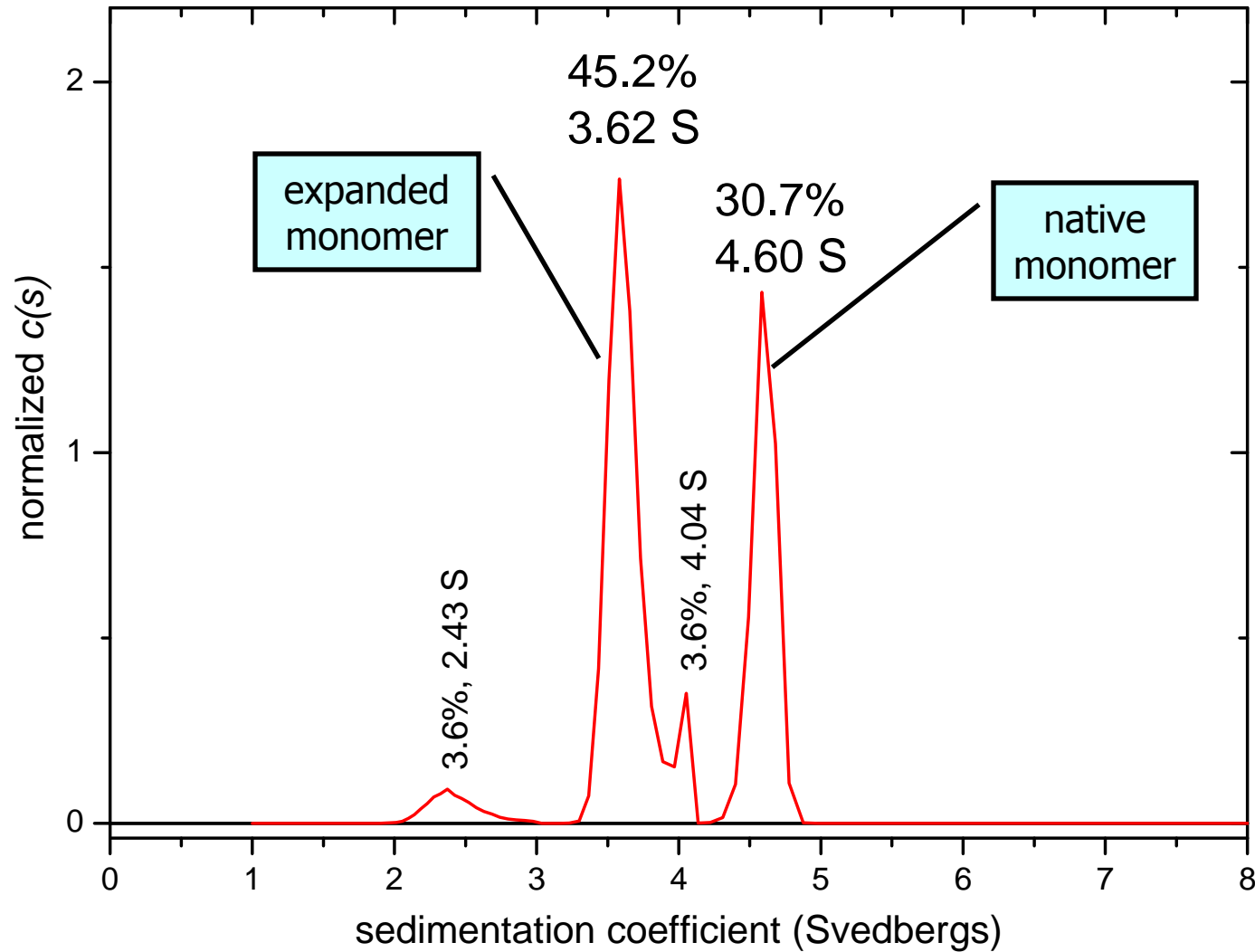
However SEC-MALLS immediately shows that alleged aggregate is actually an altered form of monomer!



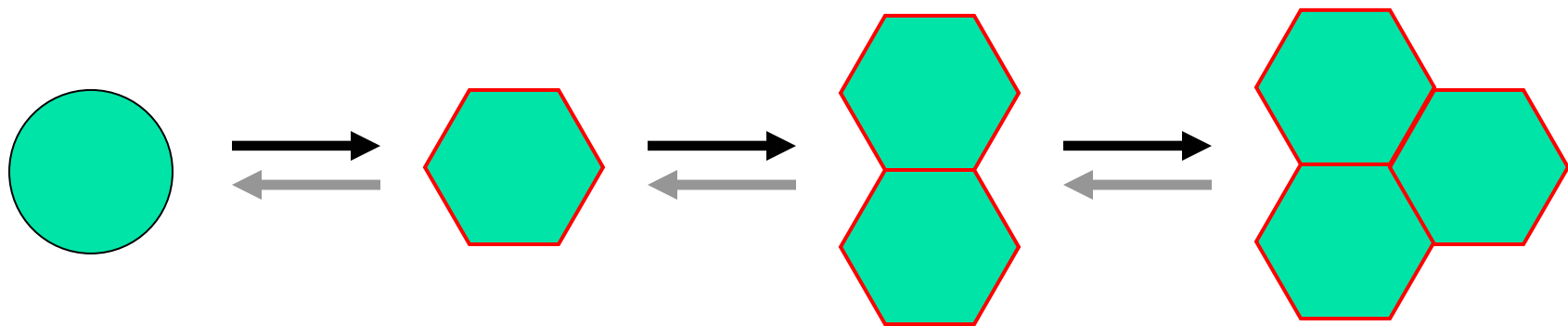
Here is the molecular mass chromatogram calculated from the light scattering data



Sedimentation velocity confirms formation of an expanded monomer that sediments slowly



Aggregation mechanisms (2): oligomerization following conformational change

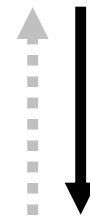


**Native
protein**

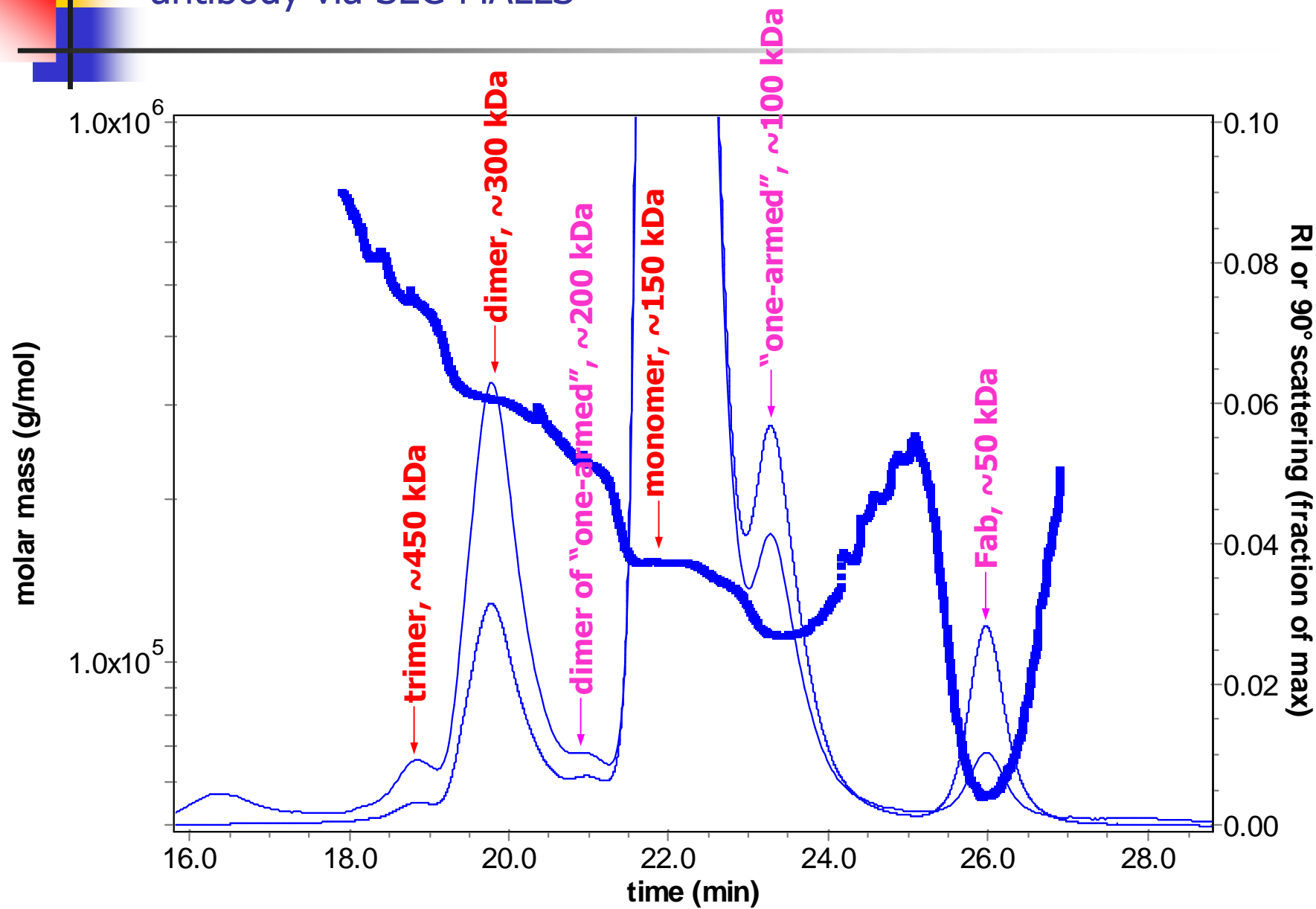
**Conformational
change or partial
unfolding**

**Oligomerization
of non-native
protein**

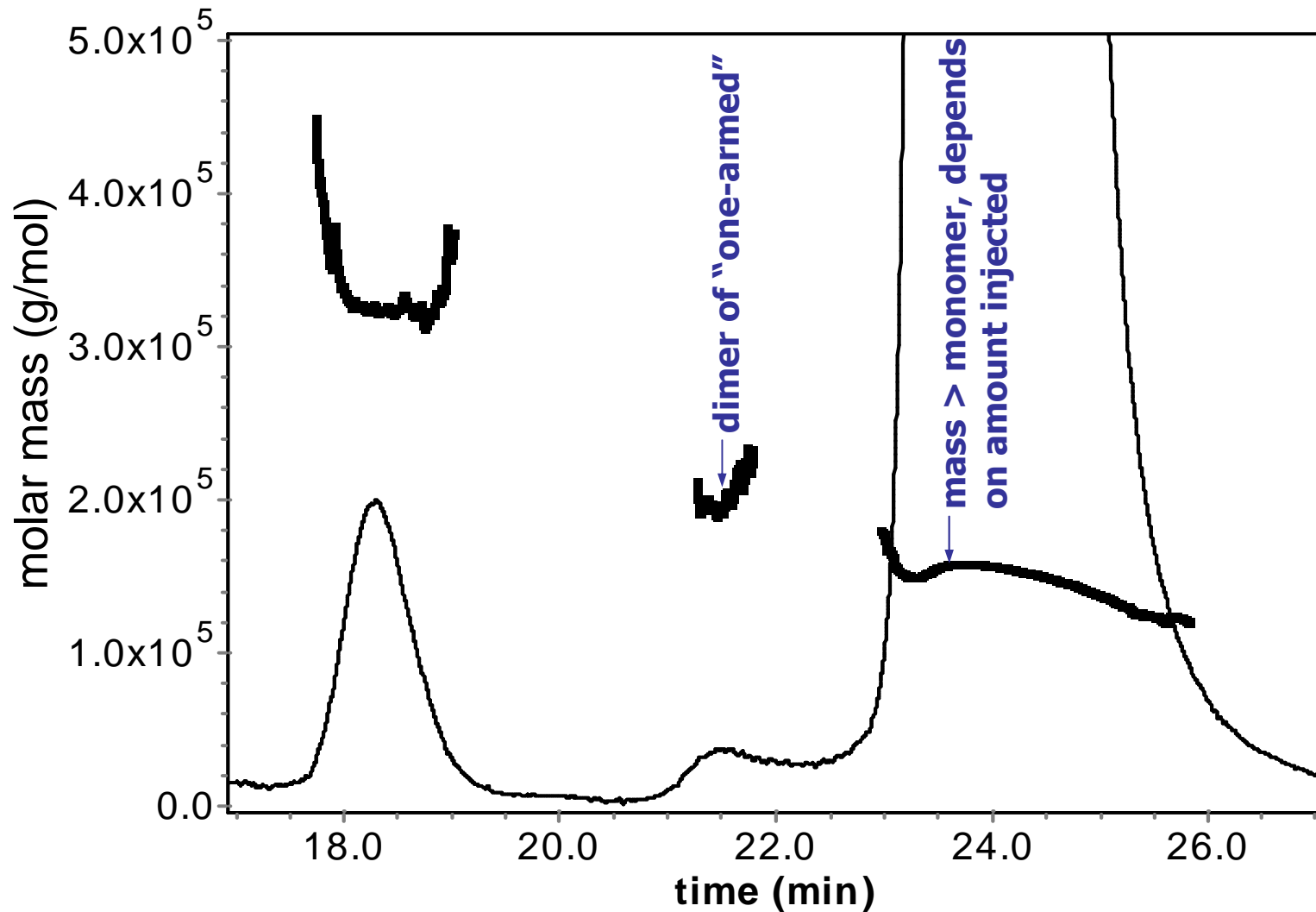
**Higher oligomers
(probably
irreversible)**



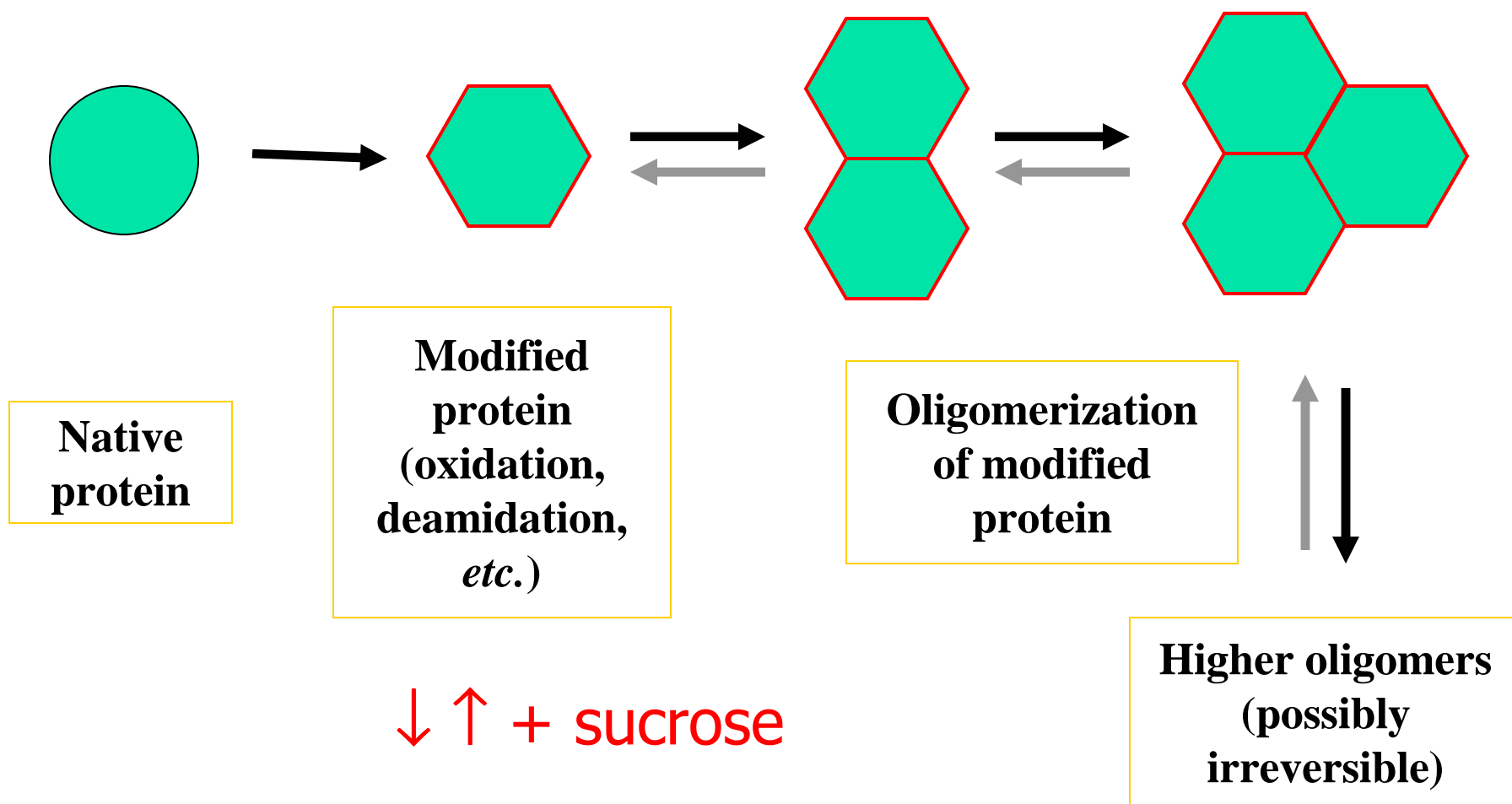
Identifying minor components in a heat-stressed monoclonal antibody via SEC-MALLS



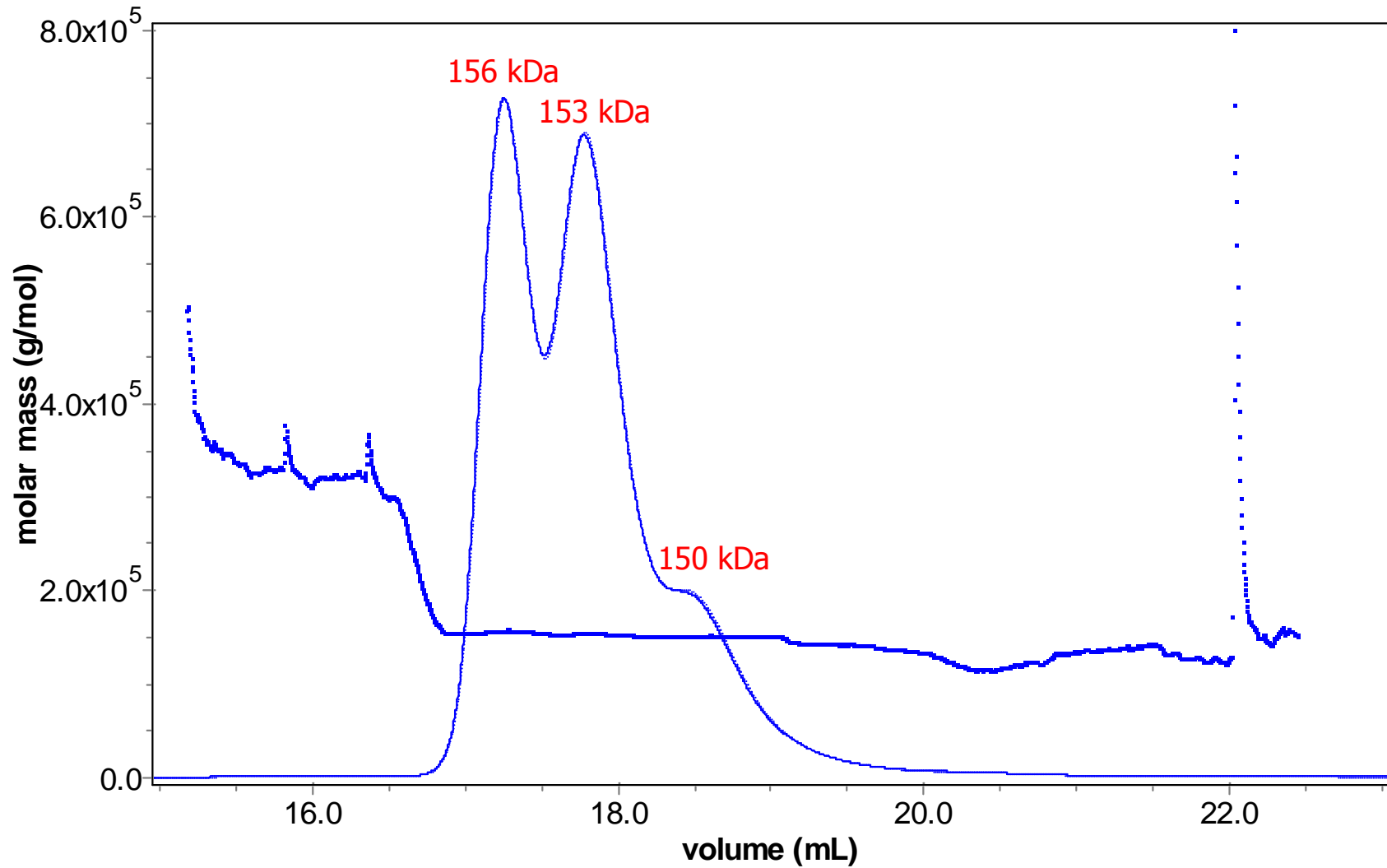
This antibody not only shows dimers of 1-armed fragments, the monomer is weakly reversibly associating



Aggregation mechanisms (3): oligomerization driven by covalent modification



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





Summary

1. Some simple graphical transformations of sedimentation equilibrium data can be quite useful as diagnostics (reversible or not?) and to illustrate what types of complexes or oligomers are formed
2. For mixed associations (heteroassociation) it is very helpful to determine stoichiometry by SEC-MALS or ITC
3. Sedimentation velocity is very useful for measuring long-lived aggregates in biotherapeutics, and for proving more routine assays are working correctly
4. SEC-MALS can be quite useful for identifying degradation products and when samples have unusual chromatographic behavior