Some Unusual Aggregation Phenomena in Recombinant Proteins

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ABSTRACT: Biopharmaceuticals exhibit a wide variety of aggregate sizes and types. Here we will describe some interesting and unusual phenomena found in recombinant proteins from 3 different clients.

Protein X is a very sticky glycoprotein that is difficult to measure by SEC and which is subject to freeze/thaw damage. Sedimentation velocity (SV) on freeze/thaw stressed samples showed high levels of non-covalent aggregates that could not be detected by the standard SEC method. After developing an improved SEC method that correlates well with SV data we discovered that freeze/thaw creates metastable aggregates that will slowly dissociate to monomer over hours to days, depending on the temperature.

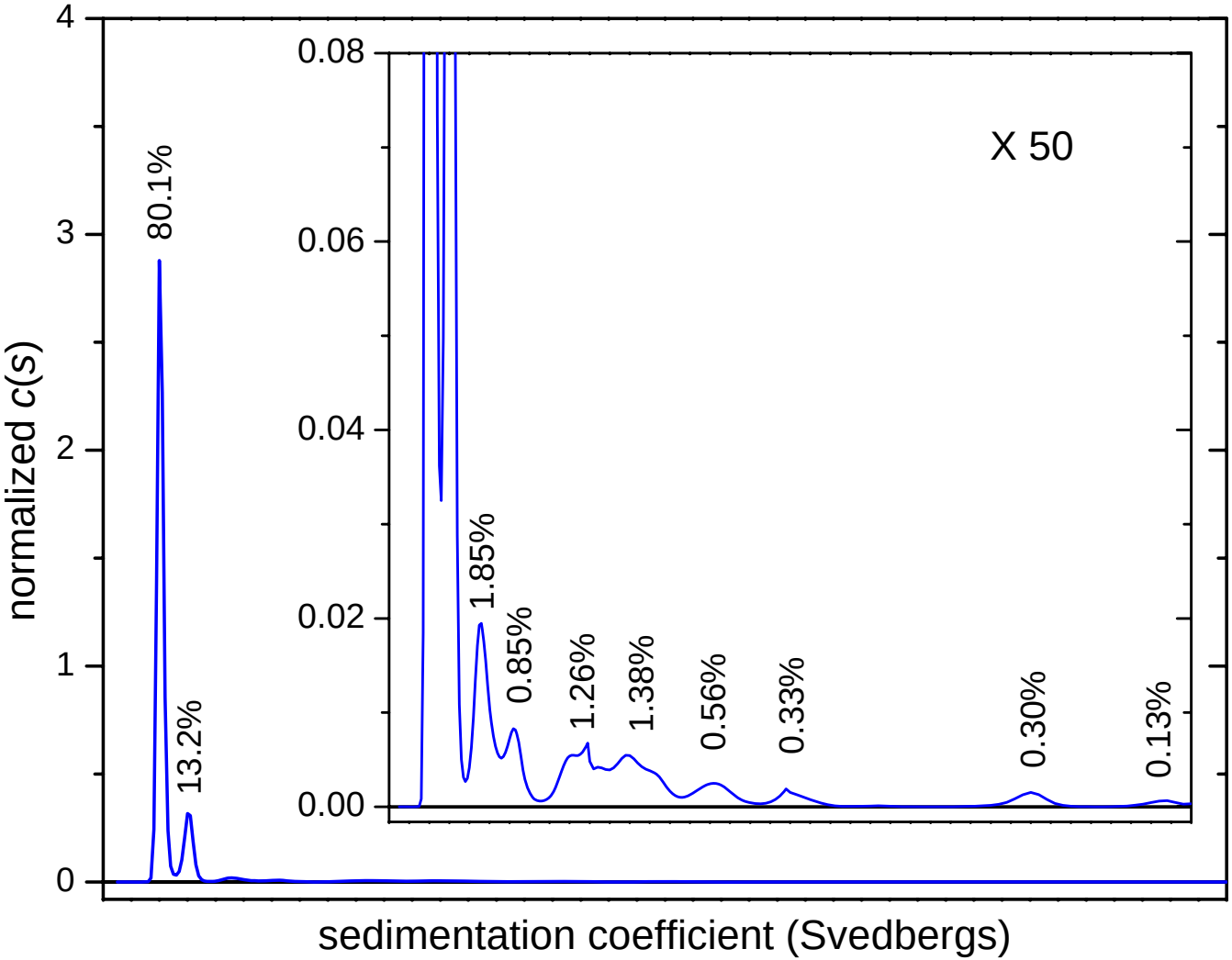
For Protein Y we investigated the appearance of aggregates over time after the lyophilized formulation was reconstituted. SV analysis did show formation of ~0.5% aggregate over 8 hours. More interesting however is that the reconstituted sample initially contained several percent of a partially-unfolded monomer, and this species decreased over time as this species slowly refolded. Aggregation then was arising not from the native state, but from this partially-unfolded state, in a reaction competing with refolding to the native structure.

Protein Z is a test antigen for vaccine development that exhibits high levels of an early-eluting putative dimer peak in stressed samples. However when we used SEC-MALLS to confirm the dimer assignment we discovered this species is actually an expanded, partially-unfolded monomer rather than an aggregate. This interpretation was also confirmed by SV analysis.

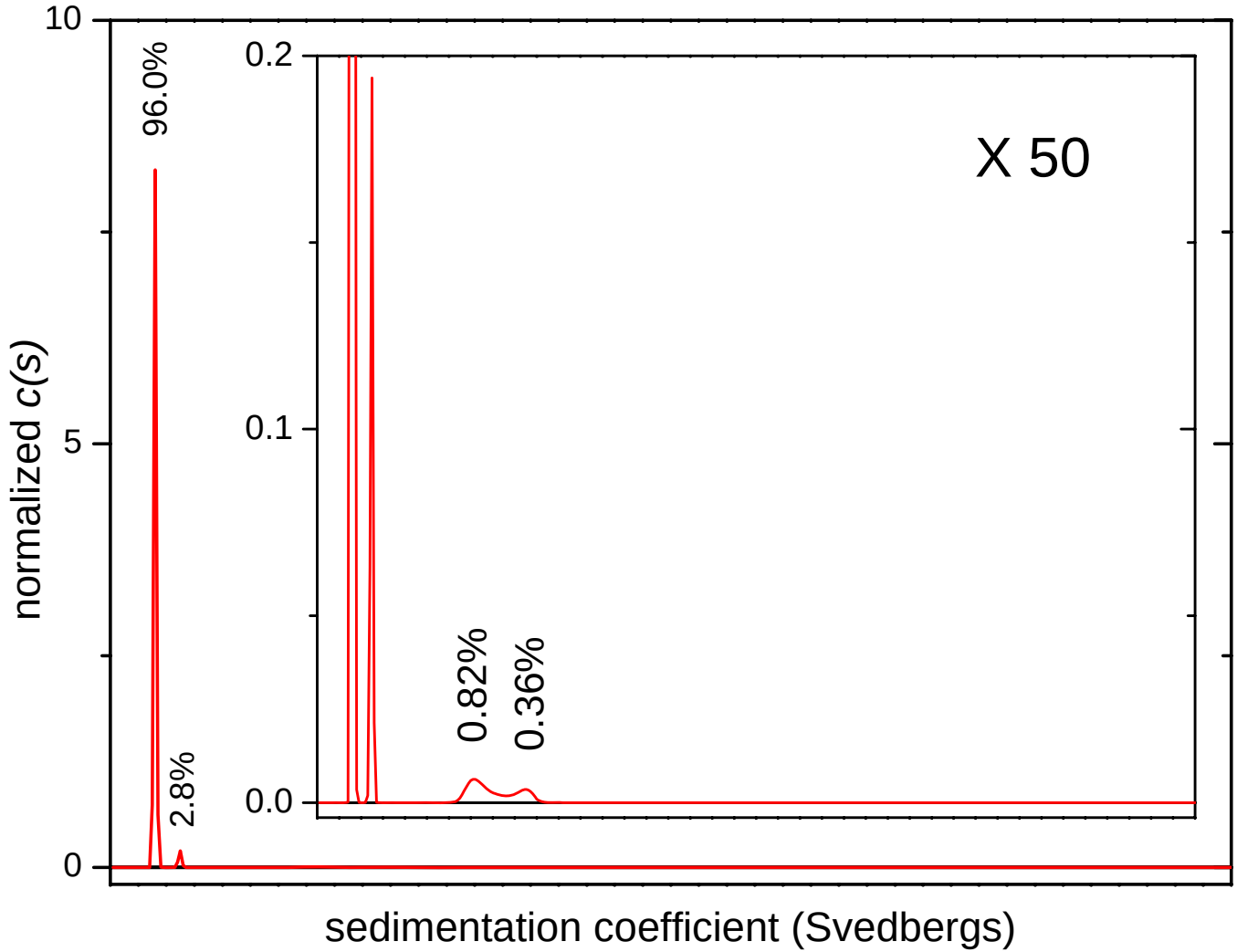
Protein X:
metastable non-covalent aggregates

Sedimentation velocity reveals high levels of aggregates in a Protein X drug substance sample stressed by 4 freeze/thaw cycles

19.9% total aggregate is measured by SV, after 4X freeze/thaw

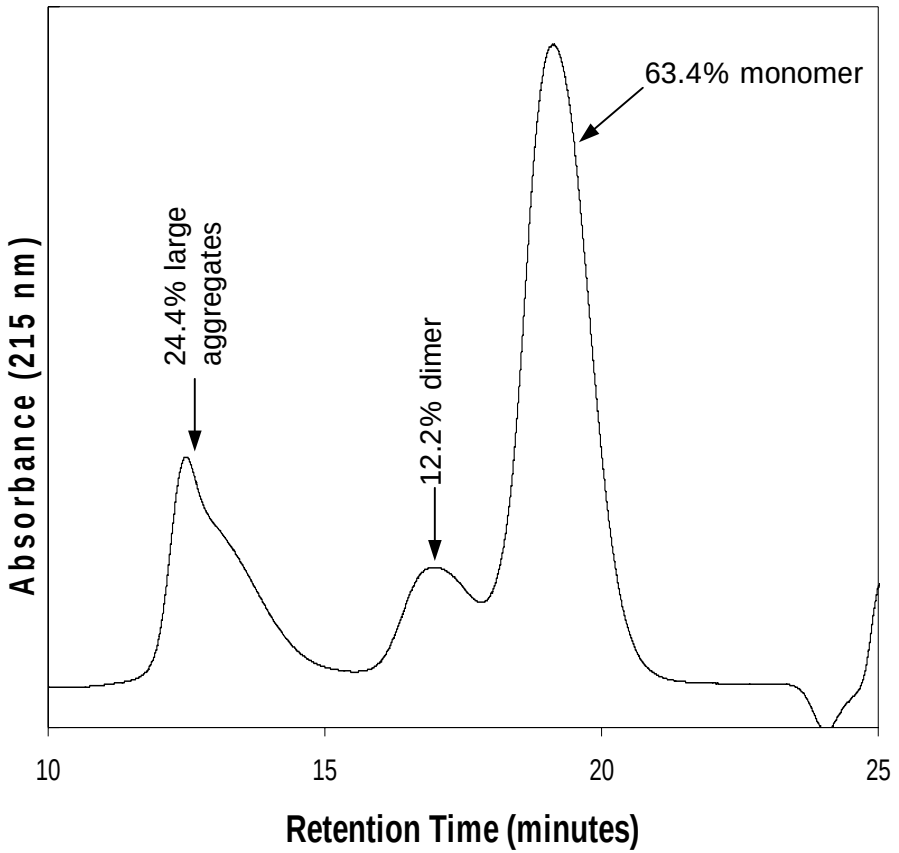


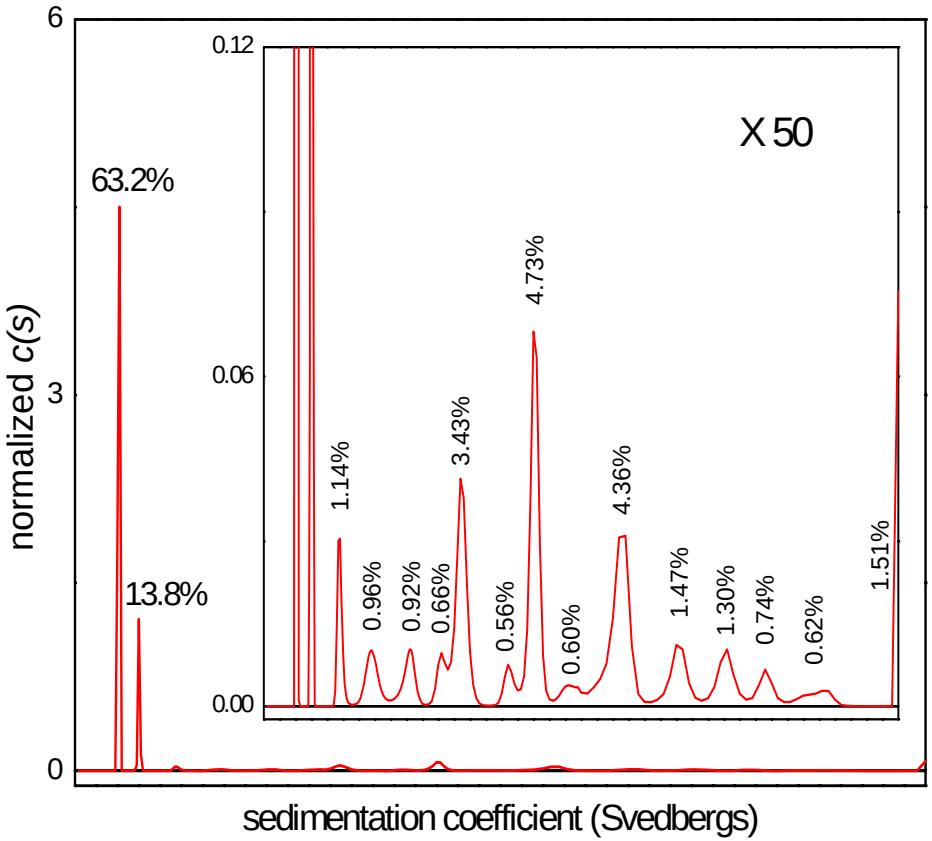
The same sample measured by SV in the standard SEC elution buffer for Protein X shows only 4% aggregate (because the buffer dissociates non-covalent aggregates)



A new SEC method gives good correlation with SV results

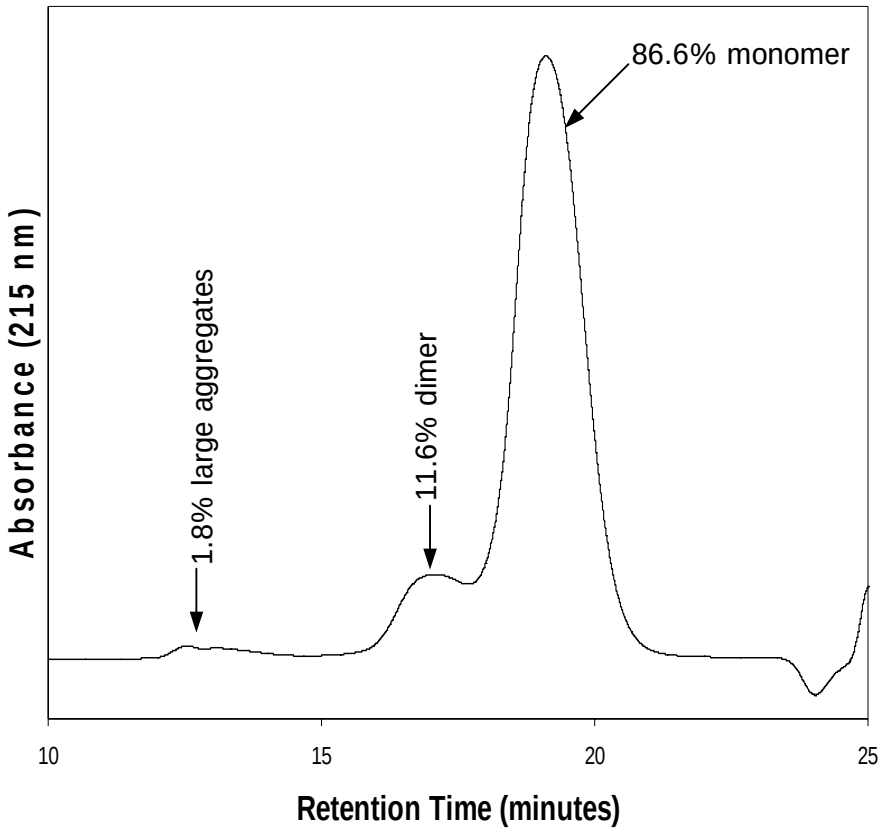
A sample highly stressed by freeze/thaw cycles gives 63.4% monomer by SEC, 63.2% by SV; 12.2% dimer by SEC, 11.4% by SV (note that dimer is much better resolved by SV, so the ‘dimer’ peak in SEC is probably contaminated by trimer)

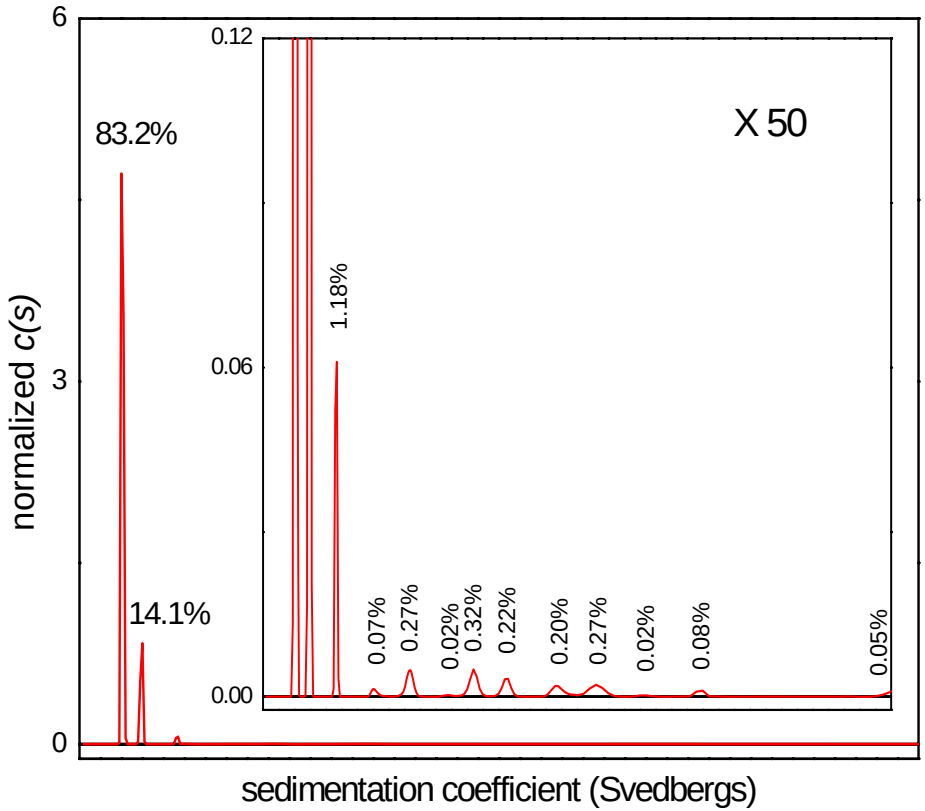




A freshly-thawed* sample of bulk drug substance shows similar levels of dimer but much lower levels of larger aggregates

This sample is 86.6% monomer by SEC, 83.2% by SV;
11.6% dimer by SEC, 14.1% by SV

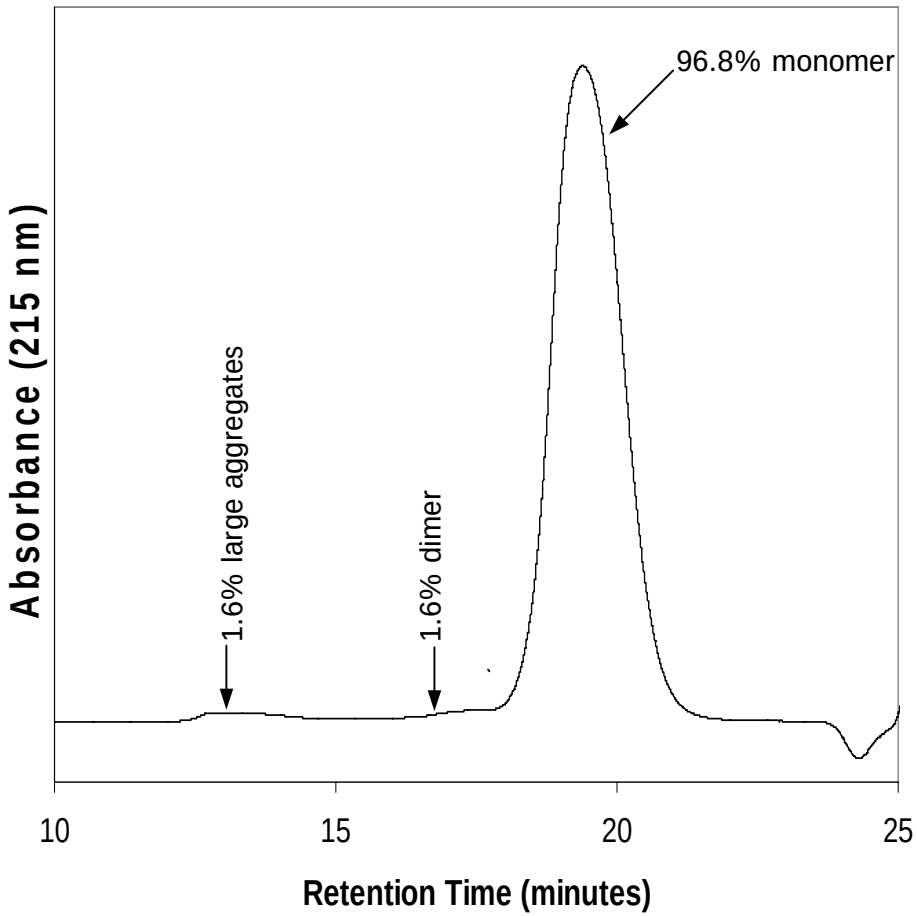


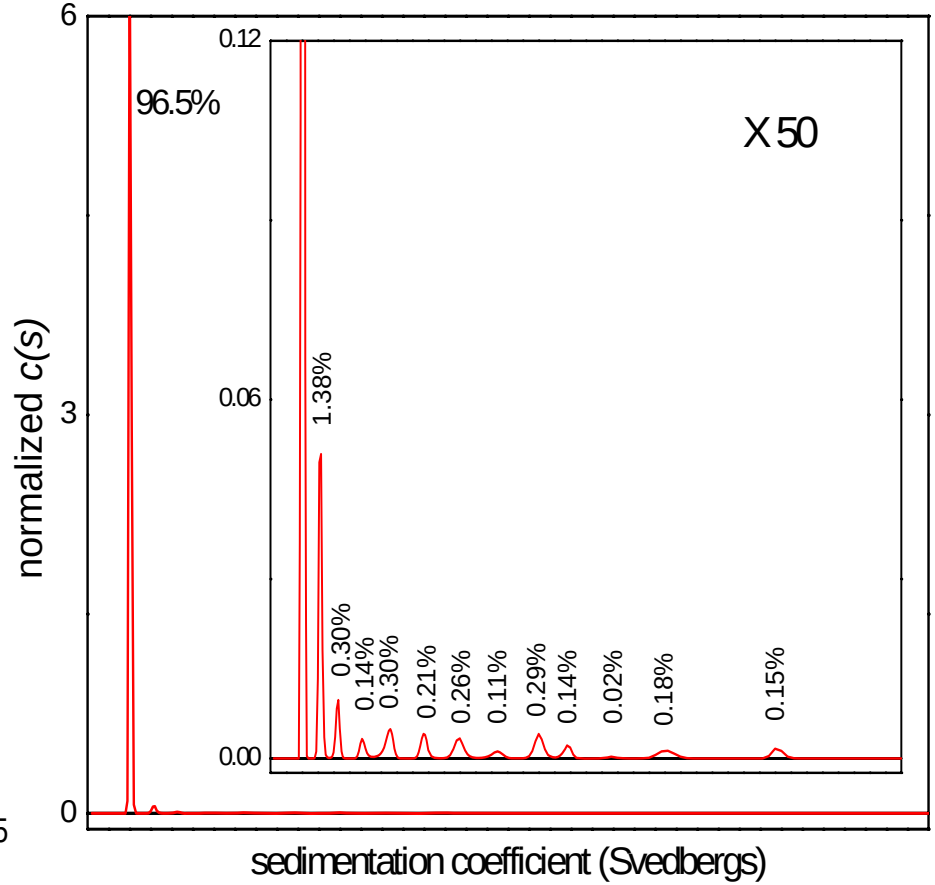


*Note however that the SV measurement requires about 6 hr.

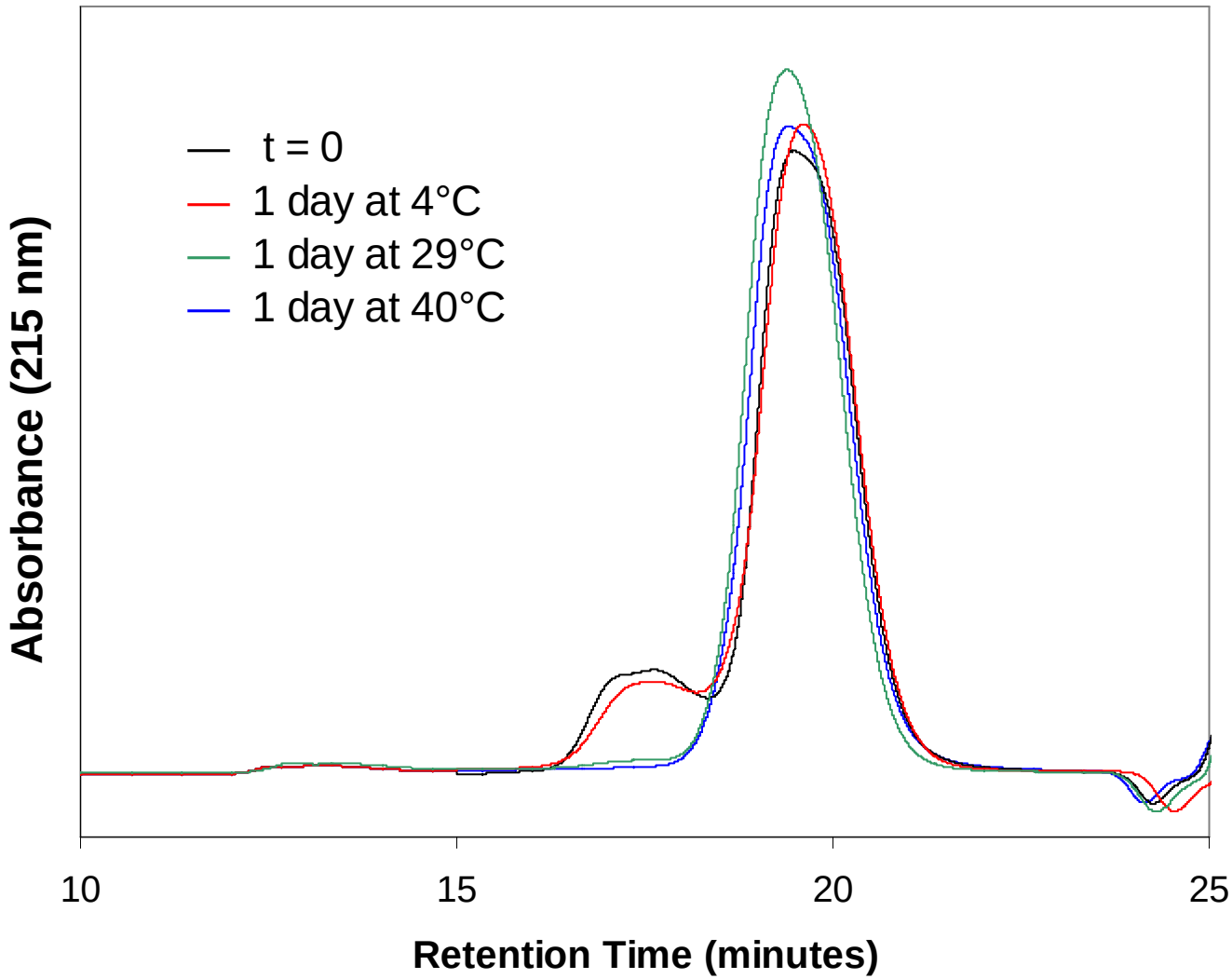
After 15 hr at 29 °C, the dimer content of a freshly-thawed sample drops from 13% down to 1.5%

There is a corresponding increase in monomer, and also a small decrease in the larger oligomers





The rate of dissociation of dimer to monomer is strongly dependent on temperature



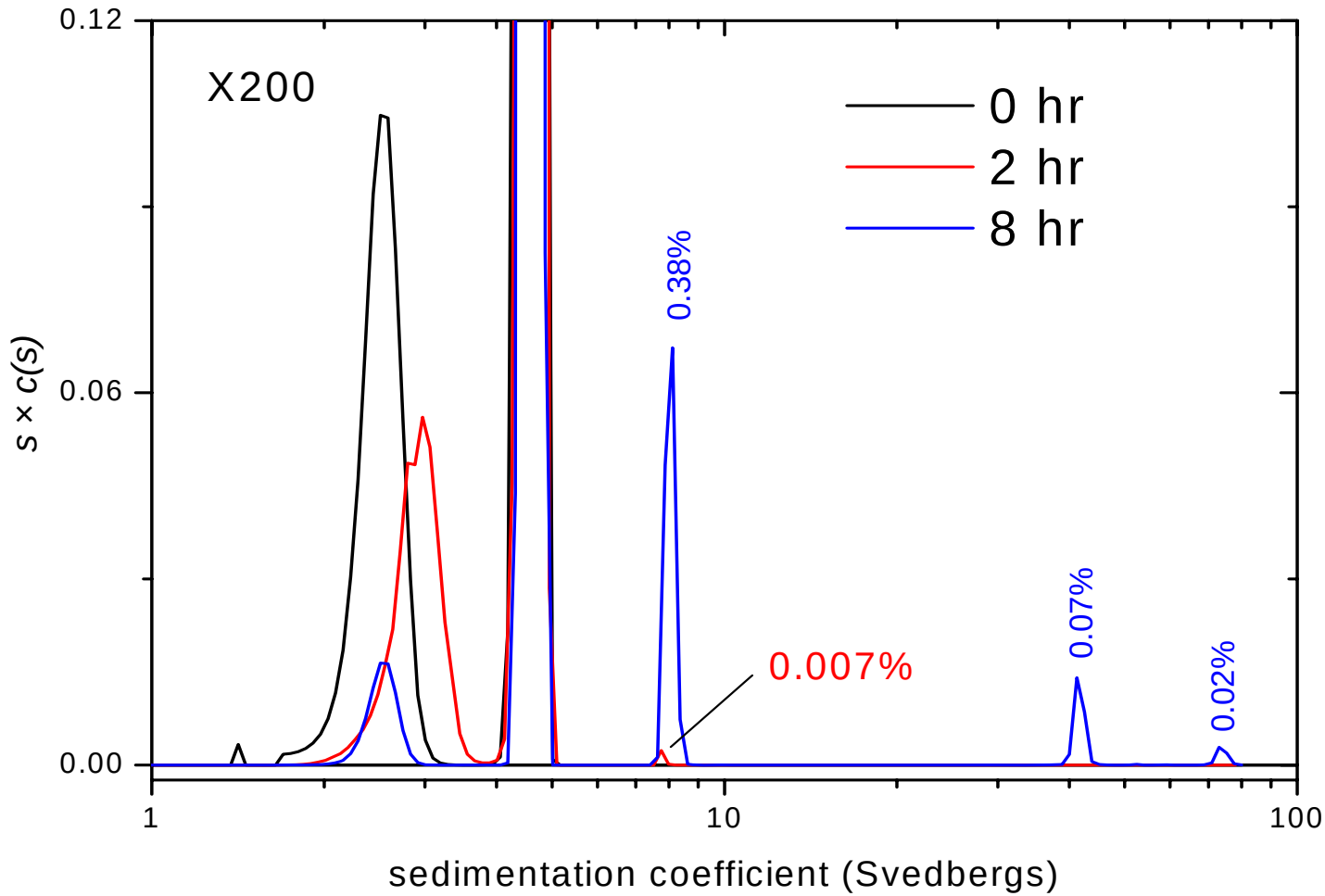
Take-home lessons from Protein X

1. Sedimentation velocity can serve as a “gold standard” to help develop better SEC methods
   - the goal is high correlation between SEC and SV, not perfect quantitative agreement
2. Metastable aggregates are not uncommon!
   - it may take hours to days for re-equilibration after any change in concentration, solvent, or temperature

Protein Y:
after reconstitution the lyophilized product contains transient partially-unfolded monomers

Sedimentation velocity analysis of lyophilized Protein Y over time after re-hydration

1. new large aggregates appear and increase
2. slowly-sedimenting partially-unfolded monomer decreases
3. native monomer increases

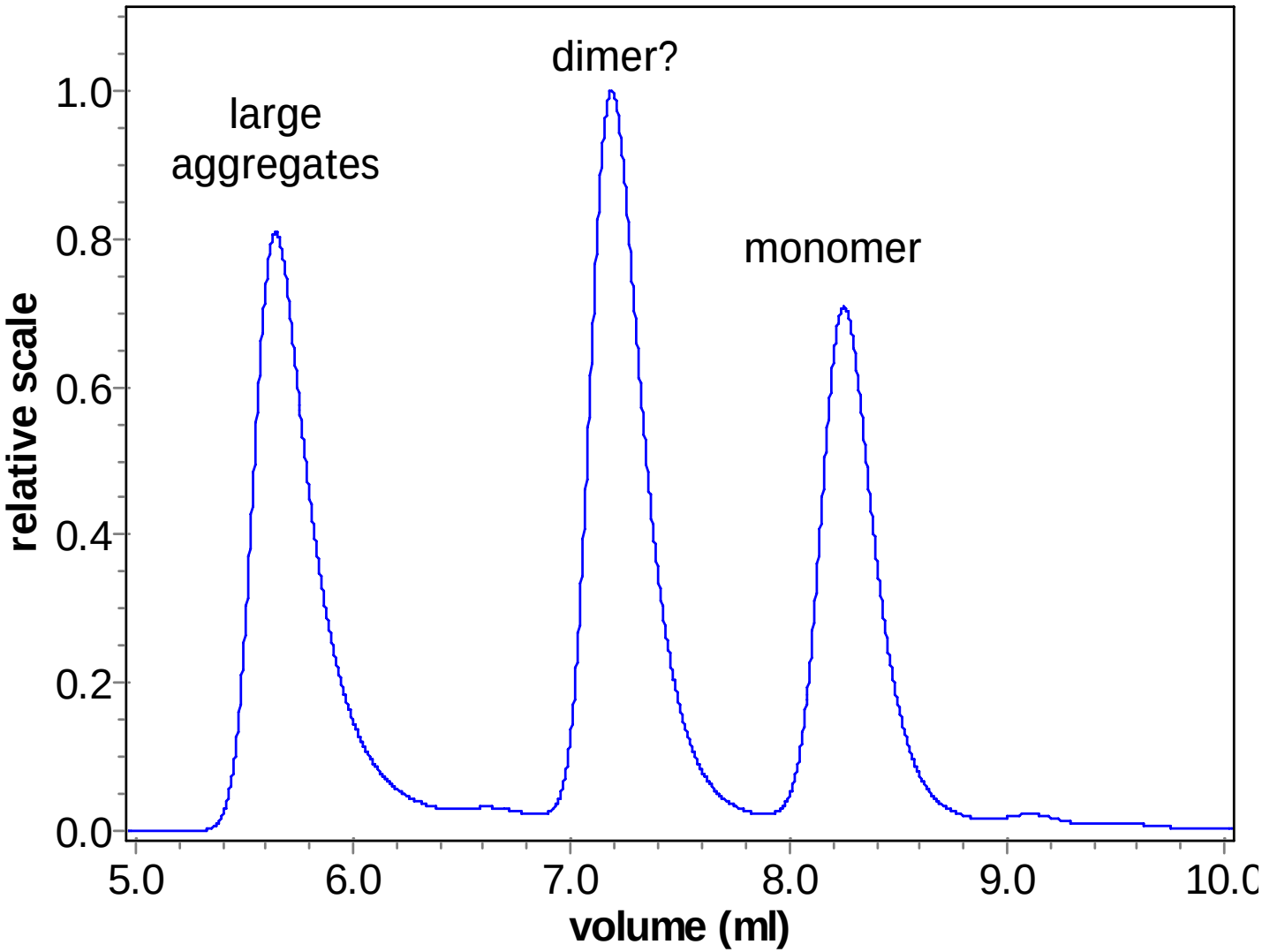


Conclusions about Protein Y

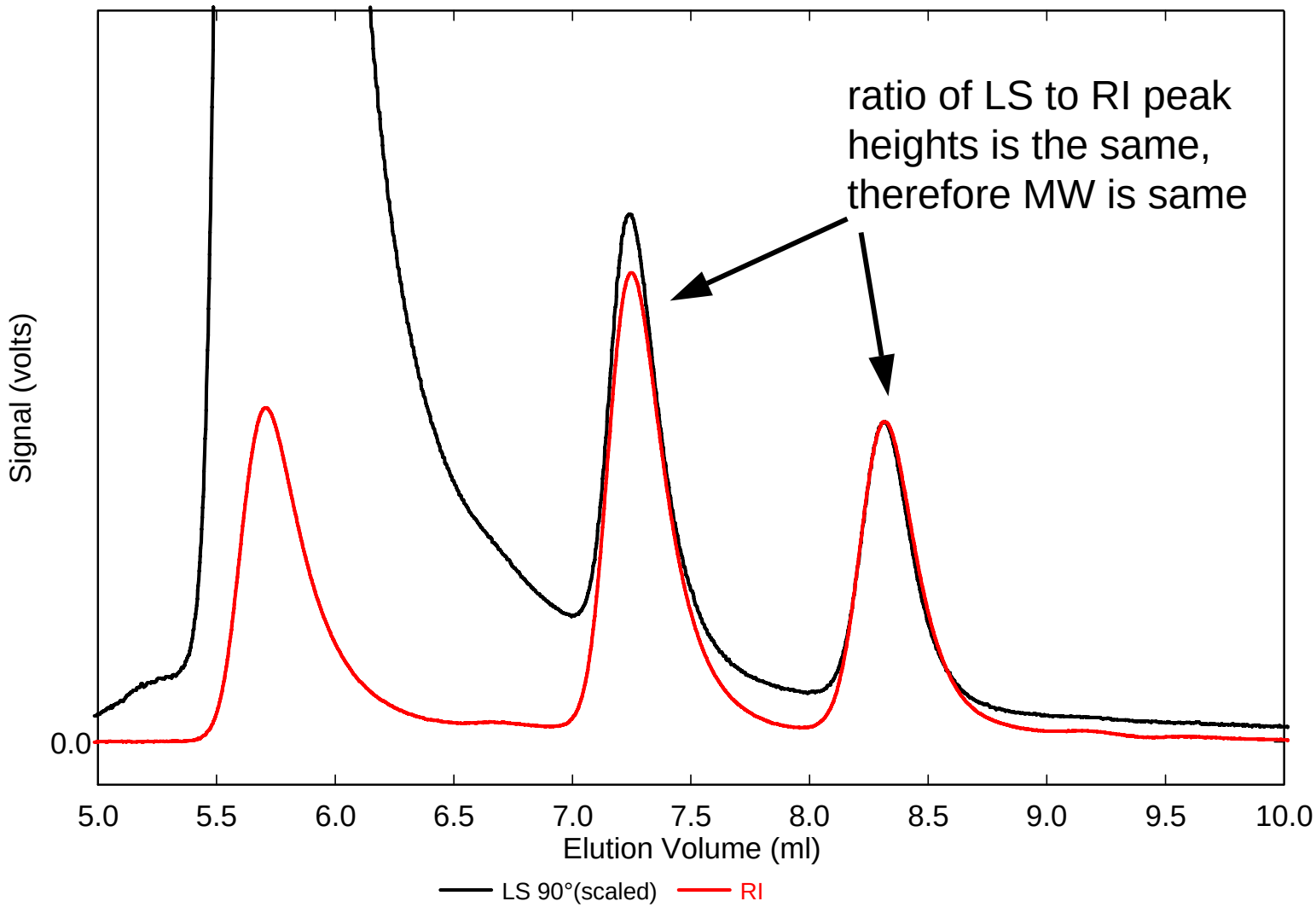
1. The reconstituted protein initially contained several percent of a partially unfolded monomer, which could slowly refold to the native state on a time scale of a few hours
2. The ~0.5% total aggregate that formed over 8 hours was arising from association of the partially unfolded monomer
   - the aggregation reaction competes with the re-folding reaction

Protein Z:
An “aggregate” that 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

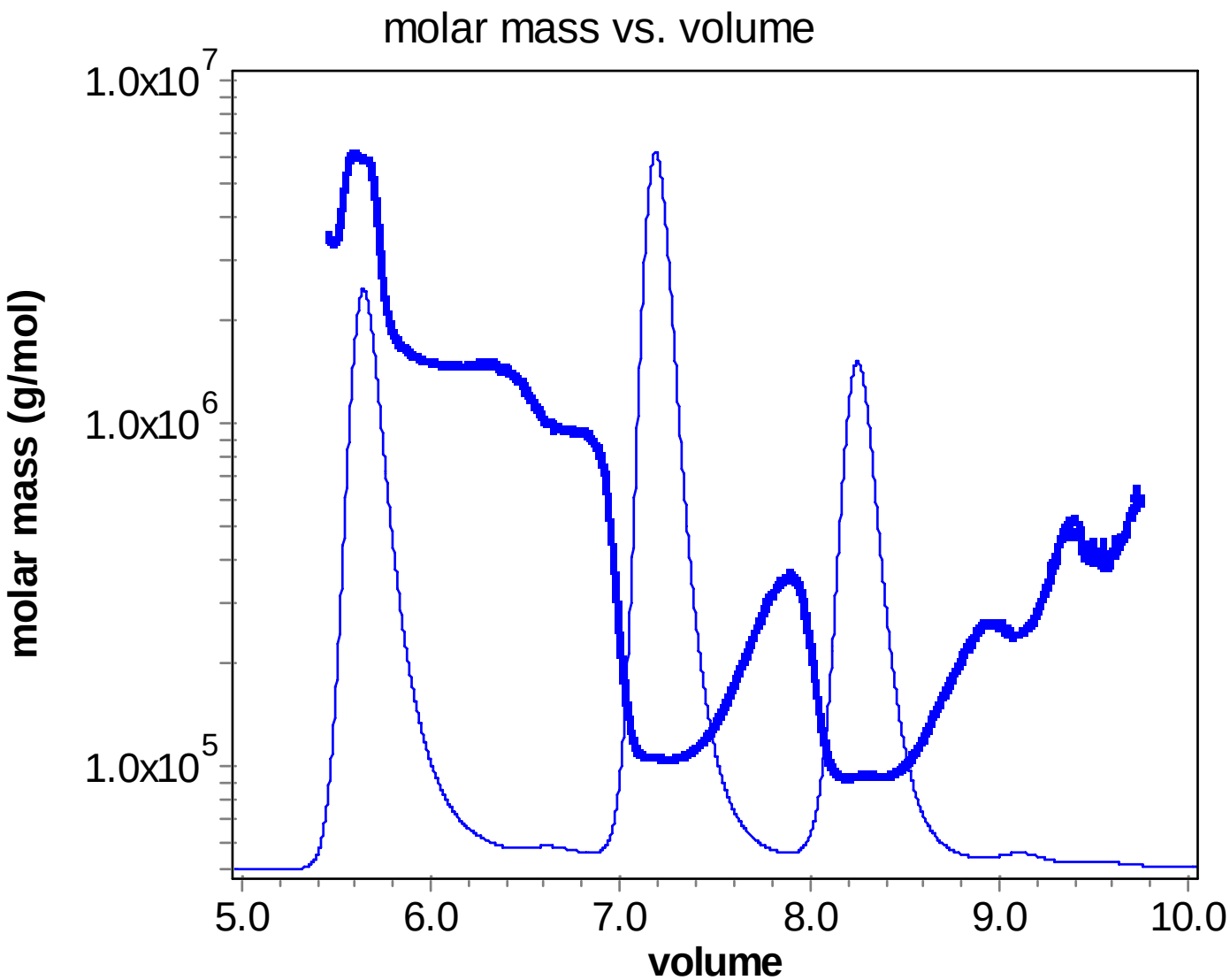


However SEC-MALLS immediately shows that alleged aggregate is actually an altered form of monomer!

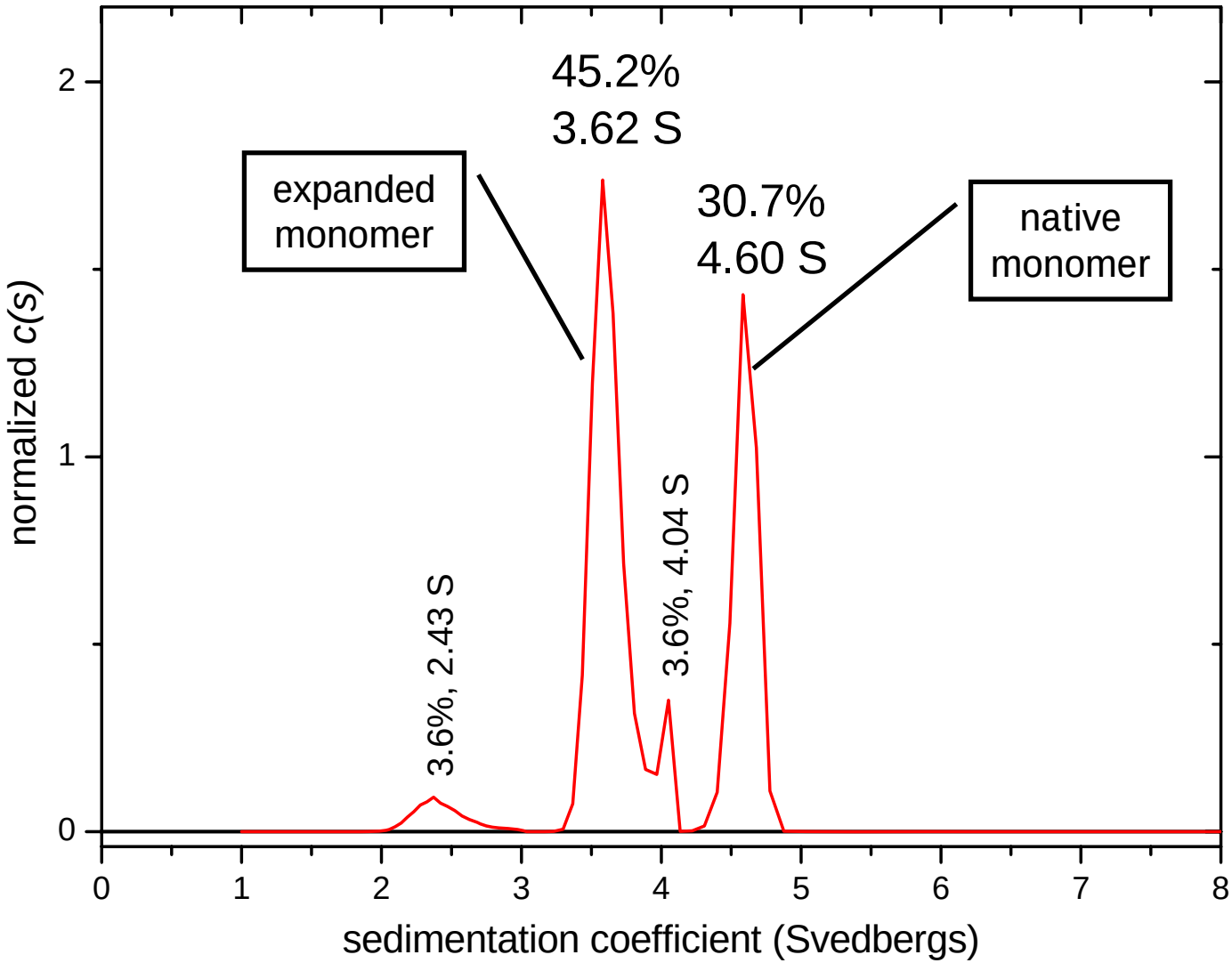


Here is the actual molecular mass chromatogram calculated from the light scattering data.

The apparent mass of the alleged aggregate is somewhat higher than the monomer mass due to co-elution of sticky large aggregates, but clearly this is a monomer.



Sedimentation velocity confirms formation of an expanded monomer that sediments slowly



Acknowledgements

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