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# Is Any Measurement Method Optimal for All Aggregate Sizes and Types?

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## ABSTRACT

Protein-based pharmaceuticals exhibit a wide range of aggregation phenomena, making it virtually impossible to find any one analytical method that works well in all cases. Aggregate sizes cover a range from small oligomers to visible "snow" and precipitates, and generally only the smaller species are reversible. It is less widely recognized that aggregates also exhibit a broad spectrum of lifetimes, and the lifetime has important consequences for detection methods. The fact that the measurement itself may destroy or create aggregates poses a major analytical challenge and is a key determinant for method selection. Several examples of some interesting aggregation phenomena and the analytical approaches we have used are presented. In one case, an "aggregate" seen by SEC in stressed samples was shown to actually be a partially denatured monomer using both size-exclusion chromatography with online multiangle laser light scattering (SEC-MALLS) and sedimentation velocity. In a second case, freeze/thaw stress generates transient, metastable oligomers that are extremely sticky and difficult to measure by SEC. By using sedimentation velocity as the "gold standard" a much improved SEC method was developed and used to investigate the temperature-dependent dissociation of these oligomers. For problems with visible particulates, dynamic light scattering has been effective, in our hands, at detecting the precursors to the large, visible particles and tracking the source of stress or damage to particular manufacturing steps.

**KEYWORDS:** aggregate, oligomer, analytical ultracentrifugation, sedimentation velocity, light scattering

## INTRODUCTION

The measurement and control of aggregation continues to be a major concern for biopharmaceutical products. At Alliance Protein Laboratories it has been a privilege and a challenge to work on aggregation issues in peptides, proteins, and other macromolecules for more than 80 companies. Many clients come to us expecting that there should be one analytical method or approach that will give a complete answer and that will work in all situations and for all of their different products. I wish it was that easy!

In working with clients I have learned that many pharmaceutical scientists fail to appreciate the full range of aggregation phenomena that can and do occur and how that impacts the measurement approach and the proper interpretation of the data. Therefore, the first part of this article will highlight some common misunderstandings about aggregates and some of the analytical challenges we face. Then a few specific examples of some unusual or challenging aggregation problems will be presented, which we have approached using light-scattering and/or analytical ultracentrifugation techniques.

## THE WIDE SPECTRUM OF AGGREGATE SIZES AND TYPES

There is unfortunately no uniform terminology for aggregate sizes or types, but some of the different classes we should consider are (1) rapidly reversible noncovalent small oligomers (dimer, trimer, tetramer, and so forth); (2) irreversible noncovalent oligomers; (3) covalent oligomers (eg, disulfide-linked); (4) "large" aggregates (≥ 10-mer), which could be reversible if noncovalent; (5) "very large" aggregates (diameter ~50 nm to 3 μm), which could be reversible if noncovalent; and (6) visible particulates ("snow" or "floaters"), which are probably irreversible.

It is of course often the case that over time the aggregates in any particular sample evolve, typically becoming less reversible and larger. It is also important to remember that samples are likely to contain more than one of these types or classes.

Many pharmaceutical scientists like to divide aggregates into "soluble" and "insoluble" categories. However if you ask carefully you find that these words mean very different things to different people—to some scientists any aggregate big enough to elute in the void volume of an SEC column is "insoluble," to others "insoluble" means it forms a visible precipitate. Consequently this terminology can be far more misleading than helpful, and I would discourage the use of these terms.

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### *Reversibility*

With regard to reversibility, most scientists tend to think of "reversible" and "irreversible" as a permanent, black versus white distinction. In reality though there is a continuum of states between reversible and irreversible; an aggregate that is irreversible in one context can become reversible in another. Factors that can affect reversibility are (1) solvent components, including salts, sugars, and other excipients, as well as organic modifiers (alcohols, acetonitrile); (2) pH; (3) temperature; and (4) how long you wait.

### *The Time Dimension*

Perhaps the most overlooked property of reversible aggregates is that they have a wide spectrum of lifetimes. The rates of association and dissociation reactions vary enormously, so the lifetime of any one oligomer molecule may range from milliseconds to several days. Many analytical methods, especially those involving separation, will only detect the longer-lived species. Further, separation methods operate on different characteristic timescales; for example, separations by SEC typically occur in approximately 15 minutes, whereas sedimentation velocity (SV) experiments generally run for at least several hours. Thus, aggregate lifetimes can play a very major role in whether an aggregate will be resolved and detected by any particular analytical method.

When we apply a separation method to a protein exhibiting rapidly reversible self-association, there is a constant battle between separation and reequilibration because of the law of mass action. Under such circumstances the results often depend on the *rates* of the association-dissociation reactions as well as the equilibrium constants, and things can get surprisingly complex. Although we may actually resolve multiple peaks by SEC, SV, or flow field-flow fractionation (FFFF), generally each of those peaks does not represent a pure, individual oligomer, but rather a dynamic mixture of multiple oligomers. When the association-dissociation rates are slow (comparable to the rate of physical separation), in theory it is possible to resolve more peaks than there are species present[1,2]!

When the association-dissociation reactions are very slow compared with the time scale of the separation then our interacting system behaves like a true mixture and we can resolve individual oligomers. Surprisingly, such reversible but extremely slow association reactions, producing aggregates we call "metastable oligomers," seem to be fairly common. A good example in the literature for a monoclonal antibody was provided by Moore et al,[3] but I have seen similar phenomena in several other antibodies as well as smaller proteins.

The good news about the existence of such metastable oligomers is that their long lifetimes make them easy to detect. The bad news is that when a protein exhibits extremely slow association-dissociation that means every sample has a long "memory" of its prior history (concentration, temperature, solvent conditions) and may require hours or even days to reequilibrate to new conditions. This can easily lead to confusing differences from one measurement to another, as occurred in an Example 2.

## Our Measurements Are Inherently Perturbing

One of the most critical problems in analyzing aggregates is the simple fact that most measurement techniques at least potentially perturb the distribution of species we are trying to measure. Generally the problem is that the measurement destroys or loses some of the aggregates, but new aggregates can also be created by or during the measurement. Often this is the reason regulatory agencies may ask applicants to use multiple techniques (preferably orthogonal ones) to measure aggregation.

Table 1 summarizes some mechanisms that lead to loss or creation of aggregates and an indication of the relative size of that problem for SEC, SV, and FFFF. (Note the information regarding FFFF summarizes opinions given to me by expert users, I am not a practitioner of that technique.) Certainly it is true that different scientists might assign different weights to these issues, but the overall patterns should be correct.

Both SEC and FFFF produce a high dilution of the sample that will tend to dissociate the reversible aggregates; for SV the total dilution is only about 20%. For SV another difference is that the high molecular weight aggregates or

**Table 1.** Mechanisms for Loss or Creation of Aggregates by Several Separation Methods*

| **Dissociation or loss of aggregates can be caused by:** | **SEC** | **SV** | **FFFF** |
|---|---|---|---|
| dilution | +++ | + | +++ |
| change of solvent conditions | +++ | – | ++ |
| adsorption to surfaces | +++ | + | ++ |
| physical filtration (eg, column frit) | +++ | – | – |
| physical disruption (eg, shear forces) | ++ | – | – |
| **Creation of new aggregates can be caused by:** | | | |
| change of solvent conditions | +++ | – | ++ |
| surface or shear-induced denaturation | ++ | – | + |
| concentration on surface | – | – | + |

*The number of pluses indicates the relative size of the problem for that method.
SEC indicates size exclusion chromatography; SV, sedimentation velocity; FFFF, flow field-flow fractionation.

complexes are always sedimenting in the presence of the smaller, slowly sedimenting species, and that fact can have a significant impact on the distribution of species that is detected for reversibly interacting systems.[4]

SEC is also rather notorious for filtration effects and poor sample recovery, which often force the use of an elution buffer containing high levels of salts and/or organic modifiers, which in turn will modify the distribution of noncovalent aggregates. FFFF also can suffer from adsorption of proteins to the cross-flow membrane, and consequently some adjustment of elution solvent composition may be needed, but these issues are generally lower than for SEC. The ability to work with a wide range of solvent conditions is an important strength of sedimentation velocity, but it too has its limits and exhibits some interference from detergents and high levels of sugars.

With regard to creation of new aggregates, generally the biggest problem arises from changes in solvent composition. I have seen several cases where high ionic strength SEC elution buffers caused formation of aggregates that were not actually present in the formulated samples. Those problems are exacerbated by the common practice of prediluting the SEC samples with the elution buffer.

## Example 1: An "Aggregate" That Isn't Actually an Aggregate

A recombinant antigen being tested for vaccine applications exhibited some unusual and interesting behavior. When subjected to stress it developed new early-eluting SEC peaks, including a prominent peak near the elution position expected for a dimer (Figure 1). To confirm that this species is indeed a dimer I ran SEC with online multiangle classical light-scattering detection (SEC-MALLS). To my surprise, even before the chromatogram was complete the light-scattering data made it obvious that this alleged "dimer" peak was actually an altered conformation of monomer (Figure 2). Why do I say this is obvious? Remember that the true molecular mass for each peak is proportional to the *ratio* of the signals from the light-scattering (LS) and refractive index (RI) detectors.[5] Although the LS signal shows a strongly sloping background as a result of trailing of very large aggregates eluting near the void volume, it is nonetheless clear that the ratio of LS/RI peak heights for the putative dimer eluting at approximately 7.2 mL is about the same as the LS/RI ratio for the monomer peak eluting at approximately 8.3 mL. If this truly was a dimer, the LS/RI ratio would be twice as large as that for monomer.

One can of course calculate the true molecular mass for each peak from these data, and that molecular mass chromatogram is shown in Figure 3. The coelution of some sticky large

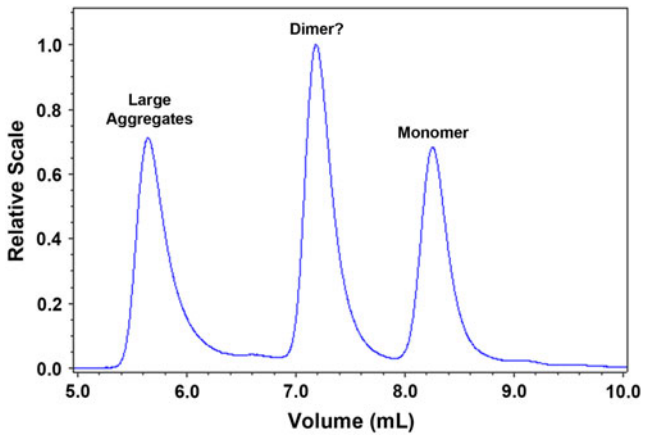


**Figure 1.** SEC chromatogram for a highly stressed sample of a test antigen.

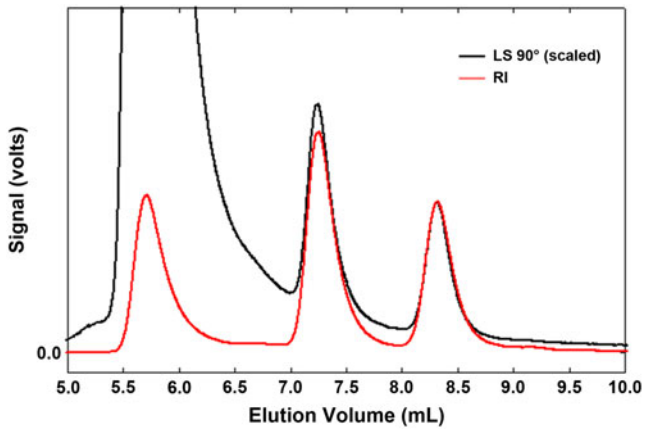


**Figure 2.** Overlay of the 90° light scattering (black) and RI (red) chromatograms for the sample in Figure 1 after scaling to match the peak heights for the monomer. Note that the sticky high molecular weight aggregates are tailing out to at least 10 mL, raising the light-scattering baseline substantially through that region.

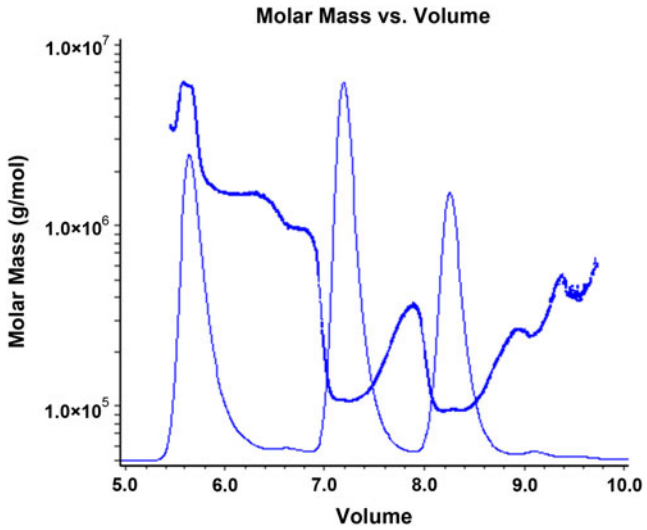


**Figure 3.** Overlay of the molecular mass (points) and RI (line) chromatograms calculated from the data in Figure 2.

aggregates with the peak at 7.2 mL raises its apparent mass to approximately 106 kDa, somewhat above the correct monomer mass of approximately 80 kDa, but nonetheless it is obvious that this species is indeed a monomer and not an aggregate. The early elution of this monomer at 7.2 mL implies this is a hydrodynamically expanded, partially denatured form of monomer.

That interpretation was confirmed by SV. The sedimentation coefficient distribution (Figure 4) shows the presence of both the normal native monomer at 4.60 Svedberg (S) and a large amount of a slowly sedimenting species at 3.62 S (the expanded monomer). This partially denatured monomer has higher hydrodynamic friction than the compact native monomer, but the same mass, and therefore it sediments more slowly. This sample also contains a low mass impurity at 2.43 S. It is unclear whether the minor peak at 4.04 S represents a true intermediate conformation or results from dynamic interconversion of the 3.62-S and 4.60-S conformations.

It is worth noting that altered conformations produce changes in opposite directions for SV and SEC. In SEC an expanded conformation elutes like a species of *higher* mass, whereas in SV expanded conformations sediment more slowly, like a species of *lower* mass. This is another way in which these methods are orthogonal, and that difference can be exploited to distinguish conformational differences from mass differences.

## Example 2: Metastable Noncovalent Aggregates

The next example concerns a recombinant glycoprotein, Protein X. This protein is known to easily generate noncovalent aggregates, but it is also notorious for sticking to surfaces and therefore it is very difficult to measure those aggregates by SEC (because of poor sample recovery). In the course of trying to develop improved formulations for this protein we encountered wide variations in aggregate levels as measured by different labs and different methods. As you might expect, some of these differences did indeed turn out to be a result of analytical issues, but surprisingly we also found there were real aggregation differences because of an interesting transient aggregation phenomenon and differences in sample histories.

One of the issues we were facing was that this protein can be quite sensitive to freeze/thaw damage, making it difficult to hold or ship the bulk drug substance. Figure 5 shows sedimentation velocity data for a sample stressed by 4 freeze/thaw cycles, giving a total content of aggregates (species sedimenting faster than the main peak) of 19.9%, distributed over at least 9 different aggregate species. Dilution to different protein concentrations gave similar distributions (not shown), indicating these are long-lived species not in rapid equilibrium with the monomer.

These sedimentation velocity results were in sharp contrast to the values returned by the standard SEC method for this protein, which gave only approximately 4% aggregate. Why are the results so different? As mentioned previously, this protein is quite sticky and to get good recovery the standard SEC method uses an elution buffer that is fairly denaturing. To prove this was the source of the discrepancy, the same sample that was stressed 4 times was diluted into the SEC elution buffer and run by sedimentation velocity. Those results (Figure 6) clearly showed that the SEC buffer caused dissociation of most of the noncovalent aggregates, leaving only aggregates that are either covalent or highly irreversible. This is a good example of the point made earlier that

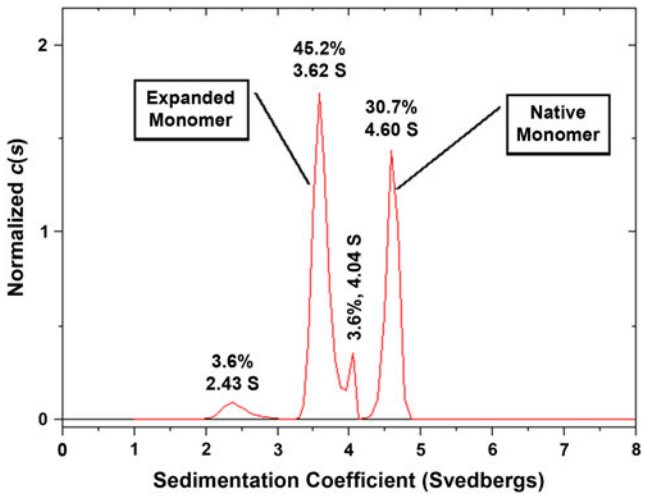


**Figure 4.** Sedimentation coefficient distribution for the stressed test antigen. The distribution has been normalized so the area under each peak gives the fraction of that species.

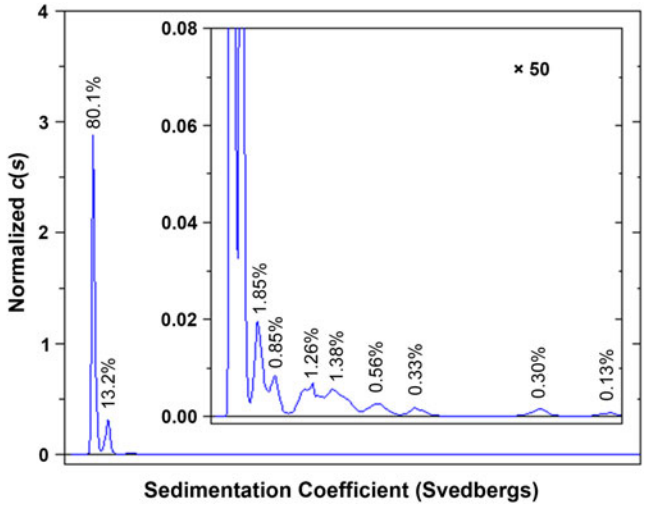


**Figure 5.** Normalized sedimentation coefficient distribution for Protein X drug substance stressed by 4 freeze/thaw cycles. The inset shows the same data vertically amplified ~50-fold so the minor peaks can be seen. The total aggregate content is 19.9%.

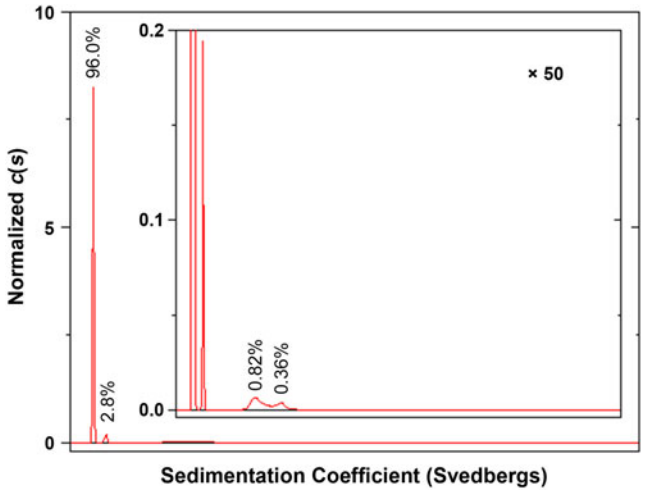


**Figure 6.** Normalized sedimentation coefficient for the freeze/thaw stressed stock in Figure 5 after dilution into the standard SEC buffer, giving 4.0% total aggregate.

reversibility depends on context—these noncovalent aggregates were effectively irreversible in the bulk drug substance buffer (they did not dissociate when the samples were diluted and run), but became reversible and dissociated quickly in the SEC elution buffer.

Using the SV data as the "gold standard" for the true aggregate levels, an improved SEC method was developed that preserves the noncovalent aggregates and still gives good recovery and reasonable resolution. Figure 7, A and B, shows parallel measurements of a sample subjected to many freeze/thaw cycles using this improved SEC method and SV. The methods agree quite well on the fraction monomer (63.4% by SEC, 63.2% by SV) and fairly well on the fraction dimer (12.2% by SEC, 11.4% by SV). It is clear the resolution of SV is much better for the larger aggregates, and indeed it seems likely that the "dimer" peak in SEC is partially contaminated by trimer, which may explain some of the quantitative differences. Overall though we should not really expect to get perfect quantitative agreement—what we are looking for is a high correlation between the methods.

### *The Freeze/Thaw-induced Dimer is Metastable*

Despite the improved SEC method there was still difficulty getting consistent aggregate levels for some samples. Finally we realized that the aggregate levels can change over time after the samples are thawed, and these changes are strongly dependent on temperature. Figure 8, A and B, shows the SEC and SV data for a freshly thawed sample of bulk drug substance. Both methods indicate levels of dimer similar to those for the sample subjected to many freeze/thaw cycles (Figure 7) but significantly lower levels of larger aggregates.

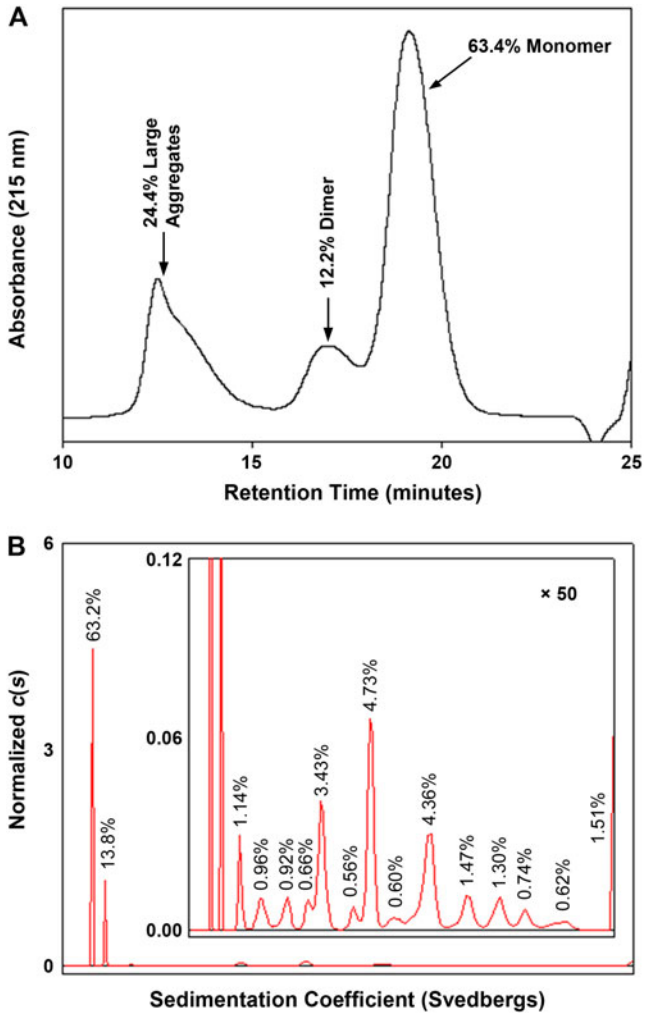


**Figure 7.** Comparison of the improved SEC method (A) with sedimentation velocity data (B) for a Protein X sample subjected to many freeze/thaw cycles.

If this stock is allowed to stand at warm temperatures for some time, however, there are dramatic shifts in the aggregate distribution. Figure 9, A and B, shows SEC and SV data for a sample thawed and then held at 29°C for 16 hours before the measurements. This incubation produces a dramatic drop in the dimer content, and some reduction in the larger aggregates, with a corresponding increase in monomer content. That is, these freeze/thaw-induced aggregates are metastable species that can dissociate and revert to monomer, but they do so only very slowly.

Figure 10 shows that the rate of dissociation of dimer to monomer is strongly dependent on temperature. Incubation for 24 hours at 4°C produces very little dissociation to monomer, but at 29°C most of the dimer is gone, and at 40°C dissociation is essentially complete. Thus, the overall picture is that freeze/thaw events create a nonequilibrium distribution of dimer and larger noncovalent aggregates, and with time and the right conditions those aggregates can dissociate to monomer. Because of these properties the aggregate content of each sample depends on its detailed

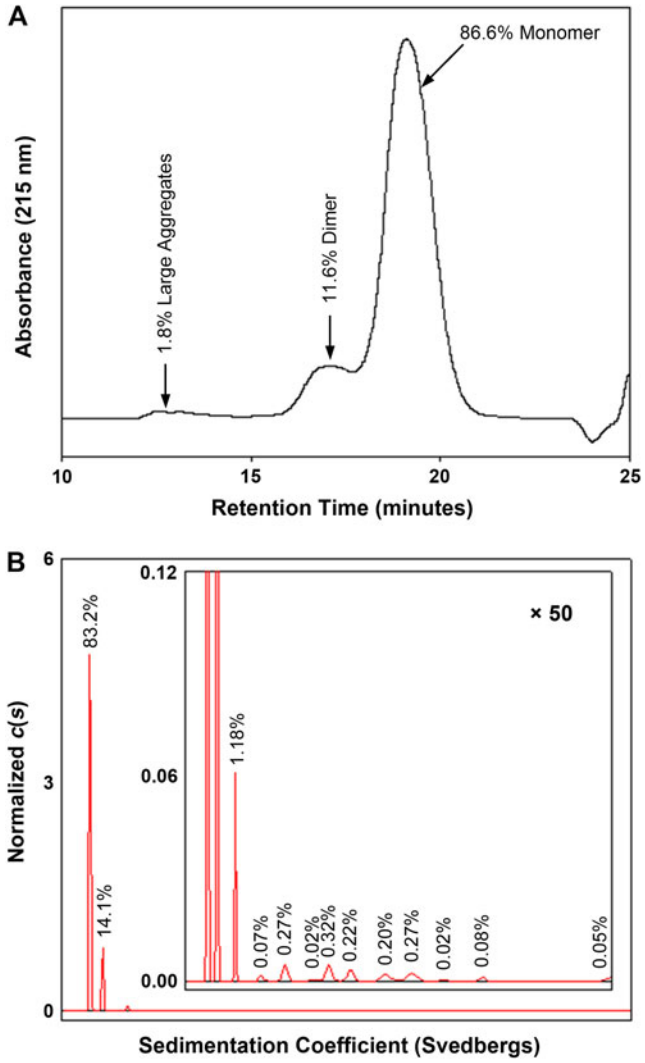


**Figure 8**. Aggregates in a freshly thawed sample of bulk drug substance measured by the improved SEC method (A) and by sedimentation velocity (B).

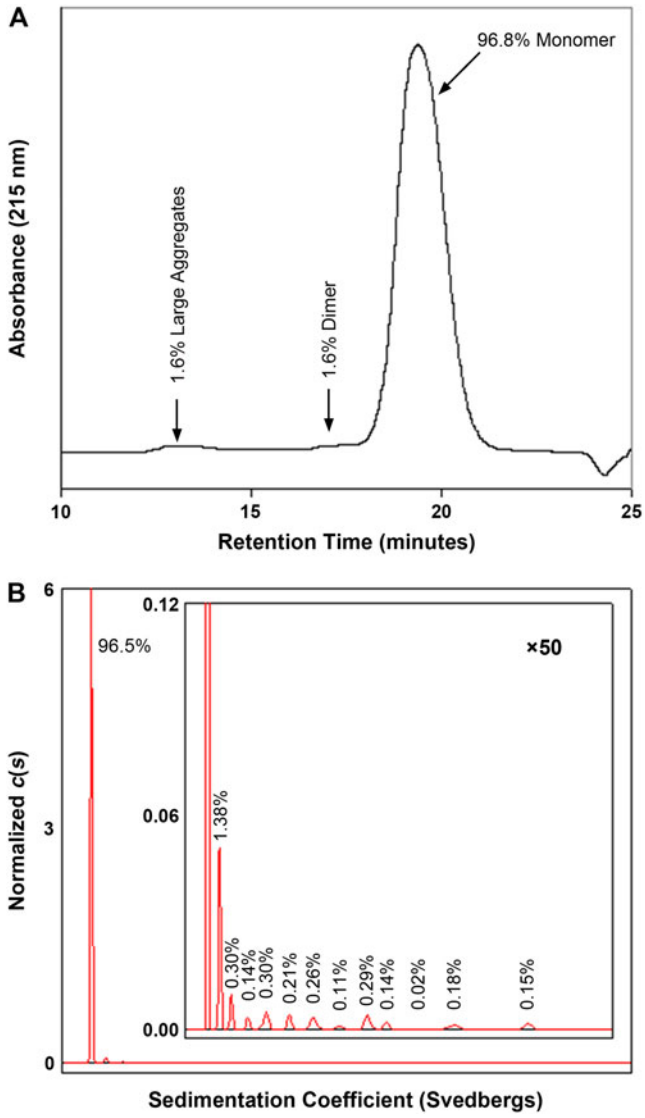


**Figure 9.** Aggregates in a sample of bulk drug substance that was thawed and incubated at 29°C for 16 hours prior to measurement. SEC data (A); sedimentation velocity (B).

history (time, temperature, and presumably other solvent conditions).

## Example 3: Dealing with Visible Particulates ("Snow")

The formation of visible particulates by protein samples can be a particularly vexing problem. Such particles may not appear until months after manufacturing, making it extremely difficult to track down the cause of the problem and to be certain it will not recur. Although the particles may be distinctly visible, the actual fraction of the protein they represent is typically quite small, often 0.01% or less of the total. The formation of these particles is often a nucleation-controlled reaction—no particles appear until the concentration of an intermediate-size particle (usually subvisible) reaches a critical point, and then large particles grow rapidly from these seeds. In some cases those critical nuclei are product aggregates (so-called homogeneous nucleation), but in other cases the critical nuclei are actually particulate contaminants introduced during manufacturing or from containers and closures (heterogeneous nucleation).

So the key to tracking down the source of these particulate problems and to predicting whether a change in formulation or the manufacturing process will cure them (rather than simply waiting to see if particles appear) is usually to detect these critical nuclei. However the fact that the critical nuclei are often present at levels well below 0.1% by weight creates a formidable analytical challenge. In my experience the most useful tool for detecting the precursors of the visible particles is dynamic light scattering (DLS), and this approach has helped to solve this type of problem in several cases.

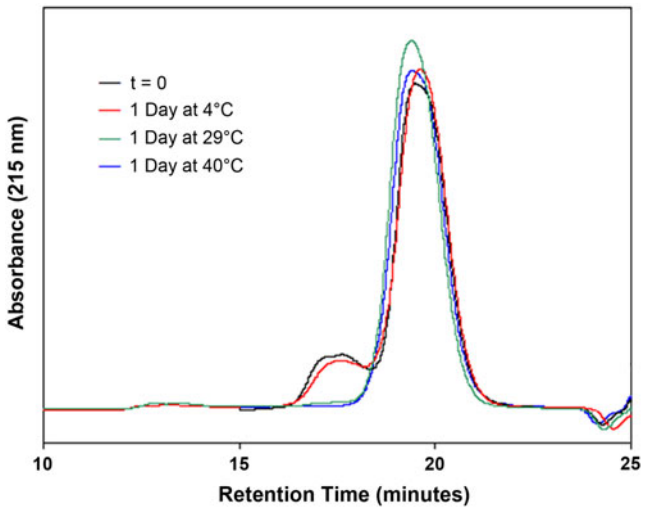


**Figure 10.** Effects of incubation temperature on dissociation of the aggregates in a thawed sample of Protein X over 24 hours, as measured by the improved SEC method.

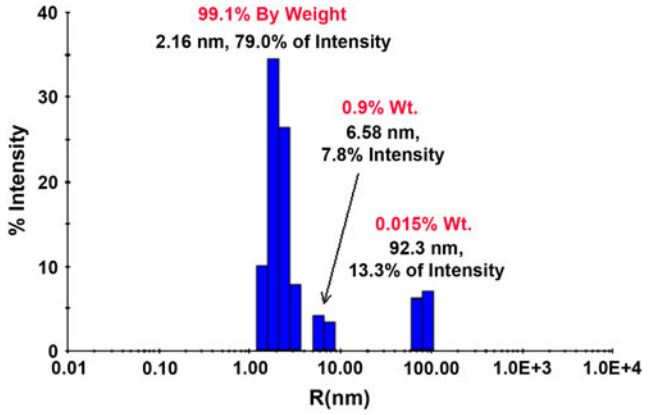


**Figure 11.** Example of a hydrodynamic radius distribution derived from DLS data. The plot is a histogram of scattering intensity (fraction of total scattering) as a function of hydrodynamic radius.

### *What is Dynamic Light Scattering?*

Dynamic light scattering is also known as quasi-elastic light scattering (QELS) and photon correlation spectroscopy (PCS). In DLS the fluctuations in light-scattering intensity as a function of time are measured, over time scales from approximately 100 ns to approximately 30 ms, rather than the time-averaged intensity that is studied in "classical" or "static" light scattering. Those fluctuations are due to the Brownian motion of the scattering particles. The time scale of the scattering fluctuations is directly related to the translational diffusion coefficient of the scattering particles, which in turn is related to their size. In DLS, as for classical LS, the scattering intensity for any species is proportional to the product of the weight concentration times its molecular mass. Thus, the large aggregates produce very strong scattering signals and the sensitivity for large species is very high.

Another important advantage of DLS is that it can be done as a batch-mode measurement. In that mode the samples are at thermodynamic equilibrium (there is no dilution or physical separation) and there is no column matrix on which aggregates can be lost. In batch mode when multiple species (multiple hydrodynamic sizes) are present each species has its own characteristic time scale for fluctuations. Thus in principle it is possible to mathematically resolve and separate the contributions from each species, and when this is done one can generate a distribution of hydrodynamic sizes (like a chromatogram). However, the resolution of this mathematical separation is fairly poor.

To illustrate, Figure 11 shows the hydrodynamic radius distribution for a sample that contains some large aggregates. This histogram plots the fraction of scattering intensity versus the hydrodynamic radius (by definition the hydrodynamic radius of a molecule is the radius of a spherical particle that has the same diffusion coefficient as that molecule). Note that the radius scale is logarithmic, and this measurement covers an enormous range of sizes from approximately 0.1 to approximately 3000 nm (covering species differing in molecular mass by a factor of approximately $3 \times 10^{13}$!).

The size histogram for this sample shows 3 peaks at mean radii of 2.16, 6.58, and 92.3 nm, representing 79.0%, 7.8%, and 13.3% of the total scattering intensity, respectively. Although the aggregate peaks represent a large fraction of the scattering intensity, because the sensitivity increases proportionally to the radius cubed those peaks correspond to a very small fraction by weight (estimated as 0.9% for the 6.58-nm peak, and only 0.015% for the 92.3-nm peak). So this approach can easily detect possible precursors of visible particles even though they represent less than 0.01% by weight!

Figure 12 shows data for a small peptide that forms visible, threadlike particles. The prominent peak near a radius of

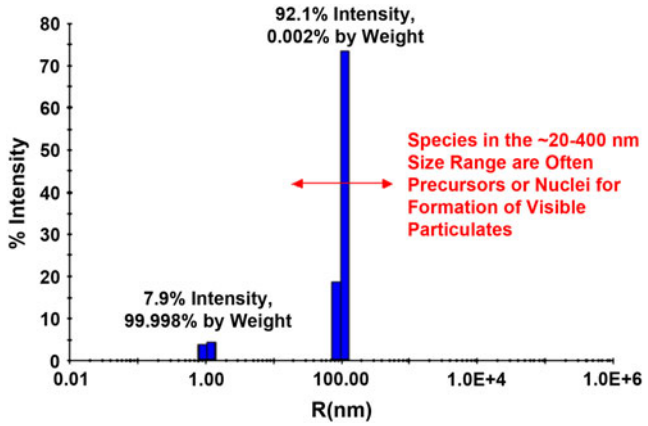


**Figure 12.** Hydrodynamic radius distribution for a small peptide that tends to form visible threadlike particles.

100 nm is a precursor to those particles (its presence correlates with manufacturing lots that eventually form the visible threads). Even though this peak represents over 90% of the scattering intensity, its estimated weight fraction is only 0.002%. In several other cases we similarly have found critical precursors of visible particulates that had radii in the range from approximately 20 to approximately 400 nm. In 2 cases that I can disclose we have been able to trace the formation of those precursors to damage that occurred at specific manufacturing steps (the viral clearance filter in one case, a specific pump in another).

### *Some Drawbacks of DLS*

On the other hand, DLS does have some important weaknesses that should be mentioned. The first is the low resolution. In general, 2 species are not resolved as separate peaks unless their radii differ by a factor of approximately 2 (a factor of ~8 in molecular mass). Thus, DLS is usually a poor tool for studying small oligomers; its best applications are for very large aggregates. A second weakness is that conversion of the intensity distribution (what the instrument directly measures) into fractions by weight is subject to many assumptions and sources of error; it is common to see variations in weight fraction of a factor of 2 or more among different aliquots of the same stock. Many useful applications of DLS, such as comparison of different formulations or "good" versus "bad" lots, are fairly qualitative so this relatively poor quantitation of weight fractions is fortunately often not a significant problem. Last, because DLS does not distinguish the chemical nature of the scattering species, it is not always easy to tell whether large particles are really product aggregates or some other type of contaminating particle.

## Can SEC be Replaced?

This discussion and the real-world examples have pointed out several drawbacks and issues with SEC, and the regulatory agencies are well aware of these concerns. Nonetheless it seems likely that SEC will continue to be the workhorse tool for measuring aggregation for quite some time. The alternative technologies discussed here still have one or more of these major drawbacks: (1) they are not sufficiently robust and easy to use to validate for lot release, (2) they have low throughput, (3) they require expensive equipment and highly trained personnel, and (4) the software may be very far from being 21 CFR part 11 compliant.

What certainly can be done today, however, is to use these alternate methods to help determine whether SEC is telling the whole story. Doing such cross-validation usually does not require a great deal of time or expense. When it does appear the SEC method is missing important species, these alternate methods can help guide the development of an improved SEC method.

## Acknowledgments

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