Technical Decision-Making with Higher Order Structure Data: Detecting Reversible Concentration-Dependent Self-Association in a Monoclonal Antibody and a Preliminary Investigation to Eliminate It

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ABSTRACT: Protein self-association or aggregation is a property of significant concern for biopharmaceutical products due to the potential ability of aggregates to cause adverse toxicological and immunological effects. Thus, during the development of a protein biopharmaceutical, it is important to detect and quantify the level and nature of aggregate species as early as possible in order to make well-informed decisions and to mitigate and control potential risks. Although a deeper understanding of the mechanism of aggregation (i.e., protein-protein interactions) is desirable, such detailed assessment is not always necessary from a biopharmaceutical process development point of view. In fact, the scope of characterization efforts is often focused on achieving a well-controlled process, which generates a product that reliably meets established acceptance criteria for safety and efficacy. In this brief note, we evaluated the utility of size-exclusion chromatography, dynamic light scattering, and analytical ultracentrifugation in their simplest forms, to effectively reveal and confirm the presence of concentration-dependent reversible self-association (RSA) in a monoclonal antibody in the early stages of formulation development. Using these techniques, we also initiated preliminary work aimed at reducing the occurrence of this RSA behavior by varying the pH of the formulation buffer. J Pharm Sci 104:3984–3989, 2015
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INTRODUCTION

The propensity for a protein to aggregate with itself (or self-associate) is a function of its physicochemical properties (which is governed by its primary structure and three-dimensional folded structure), its chemical and physical environment (including solution matrix or formulation buffer, container closure, and storage conditions), and its prior history of production and purification. In most cases, the mechanisms associated with protein self-association are tied to the protein's colloidal stability and/or its conformational stability. Such self-association can be concentration-dependent involving covalent and/or non-covalent interactions. In the latter case, protein association can also be reversible or irreversible. Consequently, protein aggregation is a complex phenomenon.[1]

When placed in certain physicochemical conditions, protein biopharmaceuticals can exhibit intrinsic concentration-dependent reversible self-association (RSA) properties.[2] Such physicochemical characteristics have been observed in many biological molecules and can play a critical role across an array of biological activities.[3] However, in the case of recombinant monoclonal antibodies (mAbs), such behavior can give rise to irreversible aggregation that can lead to adverse effects.[4] At relatively low concentrations, mAbs are normally well-behaved and exhibit a low propensity to self-associate. However, at higher concentrations, molecular crowding and other effects can cause mAbs to aggregate and can lead to unwanted physical properties such as high viscosity, precipitation, poor manufacturability, lower bioactivity, and/or immunogenicity.[5,6] Because protein aggregation has been linked to adverse toxicological and immunological responses,[7,8] distinguishing the levels of various modes of aggregation in a protein biopharmaceutical drug is key to its successful development. Size-exclusion chromatography (SEC), the standard workhorse method used to measure protein aggregation, can easily miss the presence of RSA or erroneously invoke it.[9] A major reason for this is that SEC is often conducted under fairly low concentrations using mobile phase conditions that are different from the formulation of the biopharmaceutical product, in order to prevent adsorption of the protein to the chromatographic media. This difference in solution conditions can significantly weaken or eliminate RSA that is normally present when the biopharmaceutical is in its formulation buffer at vialed concentration (and under rare conditions might even induce RSA that was not present in the biopharmaceutical's formulation[9]. Another factor that can make the correct assessment of RSA difficult via SEC is the impact of increasing sample concentration on the retention time and shape of the eluting sample peak due to sample overloading. This factor can give rise to chromatographic effects (resulting in poor mass transfer between the SEC's mobile phase and its stationary phase) that can mimic the chromatographic behavior used to detect RSA. Hence, unless orthogonal characterization work is conducted, RSA behavior of proteins can easily go undetected or falsely implicated.[9]

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In assessing and characterizing aggregation of a protein biopharmaceutical, two key pieces of information are required. The first is to determine the levels of aggregation that are inherently present in the sample, and the second is to understand their nature. In the case of RSA, a native monomeric protein reversibly forms an oligomer or oligomers as a result of noncovalent intermolecular interactions. Oligomers may increase in quantity and size with increasing concentration. However, the long-term stability of such aggregates may not necessarily compromise the safety and efficacy of these drugs. Assessment of this question partly depends on the reversibility of protein–protein interactions during long-term storage, the nature of the final dosing solution, its mode of delivery, and its response within the *in vivo* environment. On the other hand, RSA may lead to non-reversible aggregation which is more problematic. Therefore, simple processes for detecting RSA behavior and simple approaches to minimize or eliminate it can be crucial to ensuring successful development of mAb-based therapies and expediting their development.

Recently, Esfandiary et al.[10] have combined dynamic light scattering (DLS), concentration-gradient multi-angle light scattering, and analytical ultracentrifugation (AUC) to comprehensively characterize protein RSA. The complementarity of these techniques allowed elucidation of detailed aspects of RSA behavior in order to develop a detailed model for aggregation. Here, we employ similar techniques (SEC, DLS, and AUC) to rapidly detect the presence of RSA behavior in an immunoglobulin G1 (IgG1) mAb (mAb1) when studied in its initial formulation buffer containing phosphate and sucrose at pH 7.2. Our objective was to approach the problem through the lens of product development, in order to accelerate formulation optimization rather than elucidating the detailed nature of the self-association mechanism. This tactic enabled us to simply and rapidly identify and confirm the occurrence of RSA, and initiate preliminary steps to reduce or eliminate it. In so doing, we were able to demonstrate our ability to support appropriate decision-making steps that could be taken early in the drug development process in a timely manner.

MATERIALS AND METHODS

Reagents and Antibody

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. The drug substance IgG1 antibody (mAb1) discussed in this note was expressed in mammalian cells and initially formulated in phosphate buffer containing sucrose at pH 7.2.

Size-Exclusion Chromatography

SEC analysis was performed on a Waters Alliance HPLC system (Milford, Massachusetts) using a TSKgel G3000SWXL column (TOSOH Bioscience, San Francisco, California) operating under isocratic conditions. Up to 100 μg of mAb1 was injected onto the column (200 μL injection at 0.5 mg/mL) with a flow rate of 1.0 mL/min. The SEC buffer was 50 mM sodium phosphate, 100 mM sodium chloride, pH 6.0.

Dynamic Light Scattering

Diffusion coefficients were measured as a function of protein concentration on a DynaPro PlateReader Plus (Wyatt, Santa Barbara, California) at a laser wavelength of 828.88 nm. Aliquots of the filtered (0.22 μm) mAb1 sample were transferred into sterile, 96-well, clear-bottom plate Greiner Sensoplates (Greiner Bio-One, Monroe, North Carolina). Wyatt Technology Dynamics software (v. 7.3.1) was used to schedule and automate 20 independent 60 s scans for each sample. Three replicates ($n = 3$) were averaged to reduce systematic error in the sample preparation and analysis. Measurements were performed at 20°C. The average mutual translational diffusion coefficients, D_m, determined for each mAb solution at protein concentrations between 0.5 and 10.0 mg/mL were plotted as a function of concentration. In these plots, the concentration-dependent interaction term, k_D, was obtained from the slope of a simple linear regression analysis, while the y-intercept provided the value for D_m at zero concentration.

Analytical Ultracentrifugation

AUC experiments were performed on a Beckman-Coulter XL-I centrifuge operated at 161,300 × g and 20°C. 12 or 3 mm double sector charcoal-filled epon centerpieces were used with sapphire windows. UV data were collected at 280 nm, and the data spacing was 0.003 cm (radius). Sedimentation coefficients were determined by processing the data with SEDFIT (v14.1).[11] AUC experiments were performed only once for each sample.

RESULTS AND DISCUSSION

Assessing Self-Association by SEC

Figure 1a shows the normalized SEC profiles (280 nm) of five different constant volume (10 μL) injections of mAb1 at concentrations of 0.5, 1, 2, 5, and 10 mg/mL in a mobile phase that is different from the mAb1 formulation buffer. The right-most chromatogram corresponding to mAb1 at 0.5 mg/mL shows an ideal monomer peak shape that broadens and appears less symmetrical with increasing concentration (from right to left) as a result of gradual tailing on the backside of the peak. In addition, as the mAb1 concentration increases, the peak maximum elutes at increasingly earlier retention times. However, no detectable high molecular weight species (as a peak or shoulder on the front side of the main peak) were observed for any of the concentrations analyzed.

Although this type of chromatographic behavior is consistent with the presence of RSA, it can also be consistent with a chromatographic effect that is due to sample overloading, and/or concentration-dependent secondary interactions between the protein and the SEC stationary phase. The overloading effect arises when injecting samples of such high protein concentration that one may impact both the kinetics and thermodynamics of macromolecular transfer between the mobile and stationary phases in SEC. Such on-column behavior can result in artificially premature retention times, as well as peak tailing[9,12] that could effectively mimic the presence of RSA behavior, which would lead to erroneous conclusions about mAb1. As a result, SEC's ability to detect the presence of RSA may be limited, and the chromatographic behavior observed by SEC leaves unanswered questions.[13,14] Furthermore, differences between formulation buffer and SEC mobile phase composition, along with interactions between the injected protein sample and the surface of the chromatographic media, can perturb, exacerbate, or remove aggregates and render the SEC data unrepresentative of what was present in the initial sample. Therefore, techniques

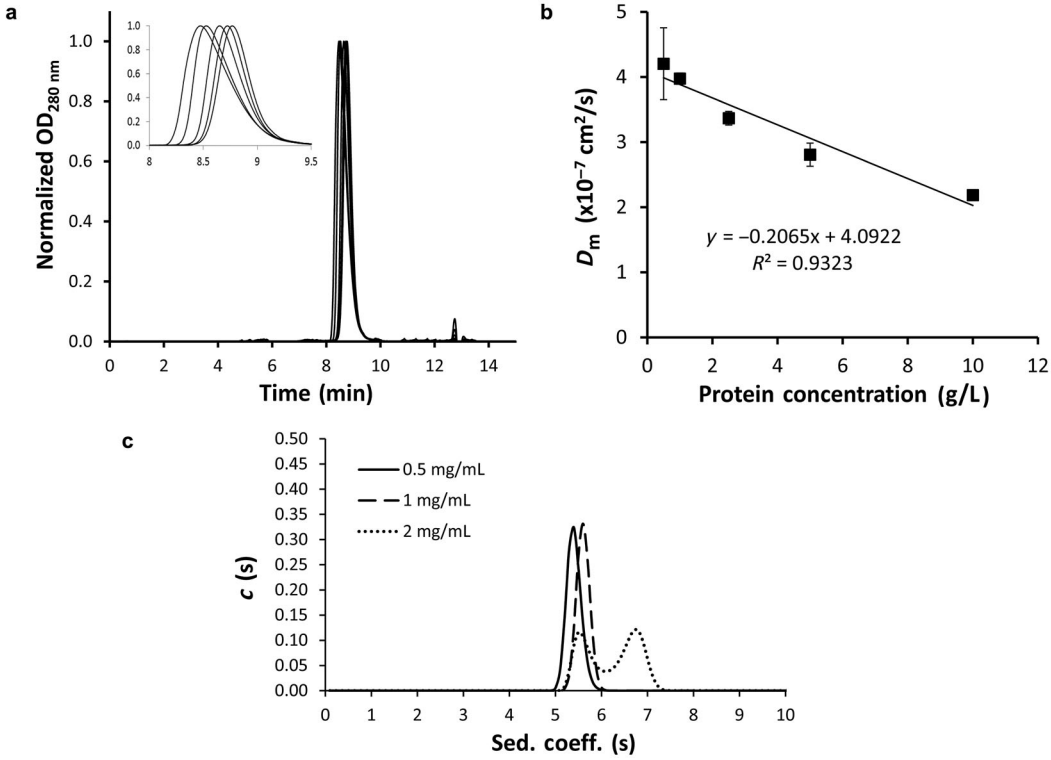

Figure 1. (a) SEC chromatogram of mAb1 injected at concentrations of 0.5, 1, 2, 5, and 10 mg/mL. The rightmost chromatogram corresponds to mAb1 at 0.5 mg/mL and displays an ideal monomer peak shape that broadens with increasing concentration (from right to left). (b) Mutual translational diffusion coefficient, D_m, as a function of protein concentration for mAb1 in its initial formulation buffer. (c) Distribution of sedimentation coefficients (c(s) vs. s) plots for mAb1 at 0.5, 1.0, and 2.0 mg/mL. The sedimentation coefficient values, s, for the individual peaks observed are labeled on the figure.

such as DLS and AUC are often needed to help elucidate the true presence of RSA.

Assessing Self-Association by DLS

When performed in a 96-well plate format, DLS is a simple, high-throughput, low sample-consuming technique that can be used to obtain information on the size and mass of a macromolecule by measuring its D_m value based on the time-dependent fluctuation in scattered light. Such information helps in the identification and assessment of the propensity of a protein to exhibit concentration-dependent attractive or repulsive forces (non-ideality effects: e.g., excluded volume, electrostatic repulsion). In solutions containing approximately 0.5–20 mg/mL protein, a quantitative measure of the protein self-association behavior can be obtained from the interaction parameter, k_D. In general, a positive k_D value is indicative of repulsive interactions, whereas a negative k_D value points to attractive forces (i.e., self-association).[6,15,16]

Dynamic light scattering analysis of mAb1 in its formulation buffer at five different concentrations ranging from 0.5 to 10 mg/mL was performed. Figure 1b shows D_m as a function of protein concentration and the resulting k_D was determined to be -50.4 ± 5.6 mL/g. The large negative k_D value suggests that a strong attractive force exists between the mAb1 molecules across this concentration range. Although such behavior might be an indicator of the molecule's inherent propensity to exhibit RSA properties, this data alone cannot unambiguously confirm the presence of RSA. This is because of the possibility of concentration-dependent changes in the conformation of a macromolecule independent of any change in molecular weight, M (see Mori and Barth, 1999, chapter 5.2).[12] As a result, additional data through an orthogonal technique(s) that would directly show the increase in M with concentration is required.

Assessing Self-Association by SV-AUC

Sedimentation velocity (SV-AUC) is a classical method that effectively characterizes a molecule's size, shape, and mass by measuring its sedimentation coefficient (s). This information can also be helpful in identifying or assessing the propensity of a protein to exhibit RSA (similar to DLS) by conducting SV-AUC experiments at different concentrations. If RSA exists, a plot of the estimated distribution of sedimentation coefficient using the c(s) analysis in SEDFIT at increasing concentration should yield data showing a shift towards higher s values with increasing concentration, and this is indeed the case as seen in Figure 1c. However, using the common and standard form of c(s) analysis in SEDFIT, where a modeling approach is conducted that assumes the sedimentation of all species present are independent of concentration, the resulting c(s) versus s plots can be very inaccurate, resulting in the appearance of peaks that do not correspond to real species.[17] This is particularly true if the timescale of interconversion between different species (in response to the change in concentration during centrifugation) is within the time of AUC runs. To avoid this, a different c(s) modeling approach specific for the presence of RSA must be used.[18–20]

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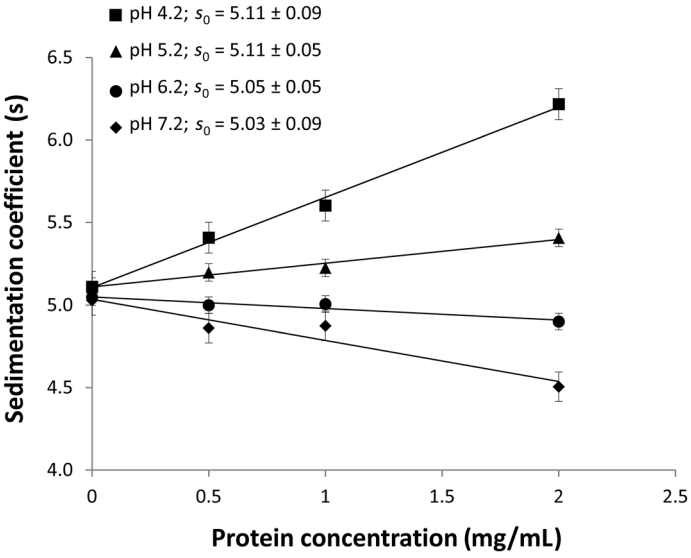

Figure 2. Weight-averaged sedimentation coefficient, s_w, as a function of protein concentration from pH 4.2 to 7.2. s_0 values were determined from linear regression analysis. Error bars are expressed as standard deviation, SD.

As a result, a straightforward and more accurate representation of the experimental data is to simply determine the weight-average sedimentation coefficient, s_w, from plots as shown in Figure 1c. This approach is completely independent of any modeling. These s_w values calculated from the AUC experiments can then be plotted as a function of protein concentration, as shown in Figure 2 (filled diamonds). Results show that under the formulation conditions of mAb1, s_w increases with increasing protein concentration, providing further evidence that mAb1 is displaying RSA behavior. As with DLS, the possibility of concentration-dependent changes in the conformation of mAb1 without changes to M[12] (which in this case would require a reduction in hydrodynamic frictional effects with concentration) could call into question this data for supporting the presence of RSA behavior for mAb1 in its formulation buffer.

Combination of Diffusion and Sedimentation Coefficients to Confirm the Presence of RSA Behavior

Although the decreasing values of D_m and increasing values of s_w with concentration reveal the likely presence of RSA behavior via DLS and AUC analysis, definitive evidence of this phenomenon with mAb1 would be achieved by establishing directly that there is an absolute increase in M of the protein with increasing concentration. This can be readily calculated from the Svedberg equation[21–23] using the independently-determined values for D and s_w as shown in Eq. (1):

$$M = s(RT)/[D(1 - \nu\rho)] \quad (1)$$

In this equation, R is the gas constant and T is the temperature in degrees Kelvin. Upon substituting D with the z-averaged D_m value derived from the DLS experiment, and substituting s with the s_w value obtained from SV-AUC, the resulting M value represents a weight-averaged molecular weight, M_w.[23] It should be noted that all M_w values from this study are more accurately referred to as "apparent" M values, $(M)_{app}$, since they were acquired at finite concentrations. Using the solution density of the formulation buffer (ρ) and the partial specific volume of mAb1 (ν) calculated from mAb1's amino acid composition, $(M)_{app}$ values were calculated at each concentration (see Table 1) along with their estimated uncertainty. The resulting data shown in Table 1 exhibit an increase in $(M)_{app}$ as a function of concentration, which strongly suggests the presence of RSA behavior with mAb1 in its formulation buffer.

Table 1. Apparent Molecular Weight, $(M)_{app}$, Calculated from the Svedberg Equation at pH 6.2 and 7.2

Total Concentration (mg/mL)	$(M)_{app}$ at pH 6.2	SD	$(M)_{app}$ at pH 7.2	SD
0.5	141,000	18,000	149,000	20,000
1.0	148,000	5000	166,000	4000
2.0	151,000	5000	196,000	6000

Initial Attempt at Mitigating the Risk of RSA

Further improvement of formulation conditions can be highly effective at minimizing the effects of RSA. Among the attributes that affect the protein's physicochemical properties, and therefore its propensity to self-associate, pH is one of the most influential factors. Because DLS provides much higher throughput than SV-AUC and requires less material, we performed the same DLS experiment described above under five different pH conditions between 4.2 and 8.2, while keeping all other excipients and parameters identical to the initial formulation buffer. Figure 3a presents D_m values as a function of protein concentration for all five pHs, and Figure 3b shows the calculated k_D values at each of the pH conditions studied. It is evident that by decreasing the pH from 7.2, the k_D value becomes less negative at pH 6.2 (weaker attractive forces), slightly positive at pH 5.2 (weak repulsive forces), and more positive at pH 4.2 (stronger repulsive forces). Increasing the pH to 8.2 did not show any statistically significant improvement over pH 7.2.

To confirm the results of the DLS pH screen, we also performed the same SV-AUC experiment described above on mAb1 at pH 7.2, as well as at pH 4.2, 5.2, and 6.2 (see Fig. 2). We excluded pH 8.2 from the screen by SV-AUC because the DLS result showed no significant improvement over pH 7.2. Clearly, the concentration-dependent increase in s_w is much less pronounced at pH 6.2 compared with pH 7.2. Moreover, the decrease in $(M)_{app}$ when pH was dropped from 7.2 to 6.2 (at 2 mg/mL, see Table 1) supports the conclusion that RSA behavior could be substantially reduced at lower pH, consistent with the results obtained by DLS. Therefore, optimization of formulation pH presents an effective path forward for mitigating the presence of RSA behavior.

AUC data can provide further assurance that optimizing the formulation pH will not cause unintended effects to protein higher order structure (HOS). This was achieved by comparing the resulting sedimentation coefficients at zero concentration (s-$_0$) over the pH range studied (Fig. 2). The analysis showed a high level of comparability ($s_0 = 5.07 \pm 0.04$ S) between the s_0 values across the various pH conditions, thus indicating that the overall shape of the protein, and thus its HOS, is likely not disturbed by the pH drop from 7.2 to 4.2. One would expect that similar comparability should be observed between D_m values at zero concentration across the same pH range; however, the precision of AUC is superior to DLS in this respect, and the

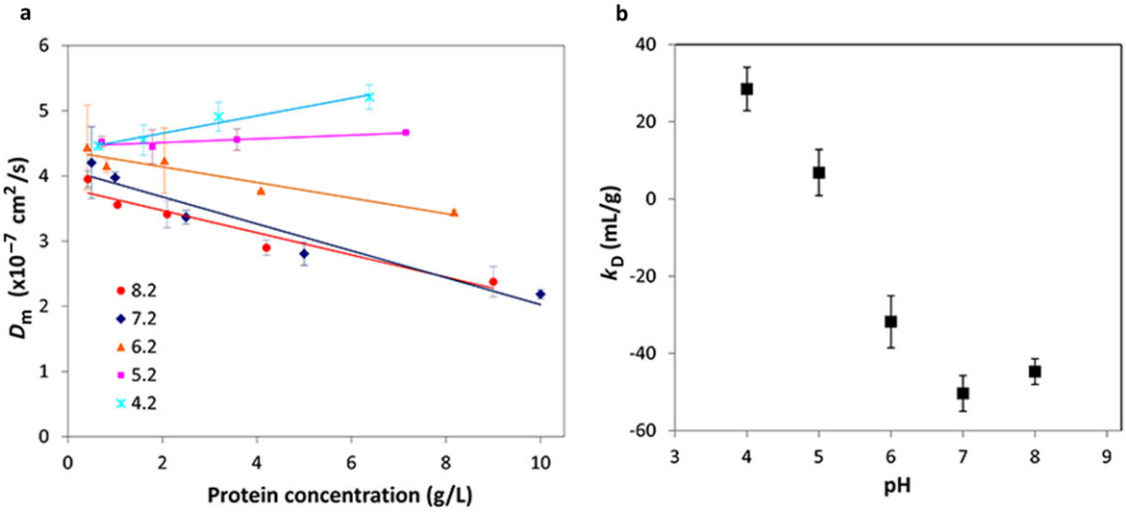

Figure 3. (a) Mutual translational diffusion coefficient, D_m, as a function of mAb1 concentration measured at five different pHs. (b) The interaction parameter, k_D, was determined from the slope and the y-intercept as a function of pH. Error bars represent 1 standard deviation.

inherent error of D_m measurements preclude any firm conclusions on the basis of DLS data alone. Further analysis by circular dichroism and fluorescence spectroscopy supported the AUC results, indicating that the secondary and tertiary structure of mAb1 remained intact after this pH change (data not shown). Thus, we conclude that the observed RSA behavior, which is easily reduced by lowering the formulation pH, is likely due to the charge surface properties of mAb1 and not changes to the protein's HOS.

It should be noted that when changing formulation conditions in an attempt to reduce or eliminate RSA, the potential impact to long-term pharmaceutical stability attributes must be evaluated to ensure that formulation optimization is performed holistically, with all critical attributes considered. In this particular case study, subsequent stability studies with mAb1 indicated that a formulation pH of 6.2 did provide the optimal balance of colloidal and chemical stability for the product.

CONCLUSION

In this study, we have demonstrated the utility of combining DLS and SV-AUC data in their simplest forms to quickly ascertain whether a mAb exhibits RSA behavior in its formulation buffer and identify a means to mitigate it. This was achieved by the combined use of diffusion and sedimentation data and applying these data to generate accurate M values (using the Svedberg equation) as a function of protein concentration. These data enabled us to avoid issues concerning possible artifacts that could impact SEC behavior, which can eliminate or mimic the presence of RSA and thus lead to erroneous conclusions. Although both DLS and SV-AUC may be used in other ways, and the data may be analyzed differently to gather a deeper in-depth understanding of the RSA mechanism,[2,10] we found these techniques can also be used simply, quickly, reliably, and robustly to expedite the decision-making process concerning RSA, as well as reduce the time and cost of developing biopharmaceuticals.

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