



Lessons Learned

Some Lessons Learned From a Comparison Between Sedimentation Velocity Analytical Ultracentrifugation and Size Exclusion Chromatography to Characterize and Quantify Protein Aggregates

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ABSTRACT

There are numerous problems with size exclusion chromatography (SEC), which often lead to inaccuracies in protein aggregate characterization. Hence, this study tested sedimentation velocity analytical ultracentrifugation (SV-AUC) as an orthogonal tool to SEC for quantifying the monomer and aggregates in intravenous immunoglobulin (IVIg) formulations. IVIg samples were subjected to agitation stress and analyzed using SEC mobile phases composed of 200 mM sodium phosphate (pH 7.0) with 0, 50, 100, 200, or 400 mM of NaCl. Surprisingly, 400 mM of NaCl was required in the mobile phase to attain complete protein recovery from the SEC column. Significant discrepancies between SEC and SV-AUC were reported when SEC analysis was performed using suboptimal concentrations (e.g., 0, 50, 100, and 200 mM) of NaCl in the mobile phase. The continuous sedimentation coefficient distributions obtained with SV-AUC resolved the high molecular weight species, whereas with SEC the high molecular weight species eluted as a single peak. Only with the orthogonal use of SV-AUC, we were able to develop a robust SEC method for accurate quantitation of monomer and aggregates in unagitated and agitated IVIg samples. Additionally, this article describes a modification to an existing method of quantitating insoluble aggregates from SV-AUC boundary data.

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Introduction

The levels and sizes of protein aggregates are critical quality attributes for therapeutic protein products.^{1–3} Development of safe and effective therapeutic products requires sensitive and reliable analytical tools for aggregate quantitation and characterization. Size exclusion chromatography (SEC) is a long-standing industry standard as a routine method for measuring levels of aggregates at various steps of protein product development.^{4–6}

Ideally, SEC separates proteins and their aggregates based on their hydrodynamic volume. Permeation of protein species into the pores of stationary phase (e.g., porous silica beads) is inversely proportional to their size. However, numerous factors can cause problems with analysis of protein aggregates with SEC, and such problems have been recognized for more than 30 years.⁷

First, adsorption of protein to the solid-liquid interface (stationary phase) reduces protein mass recovery from the column,

thereby compromising the accuracy of protein monomer and aggregate quantitation. For example, with a monoclonal antibody (mAb) sample, it was found that stationary phase surfaces of a new column preferentially bound aggregates and thus a column passivation or preconditioning step was needed to minimize protein adsorption to new columns.^{8,9} Yu and He¹⁰ showed that by varying the mobile phase ionic strength and pH, adsorption to the stationary phase could be minimized, especially at pH values near the isoelectric point of the protein.

Second, during SEC analysis, protein samples are diluted in the mobile phase, which can have a composition that is very different from the product formulation. A number of review articles and commentaries have reported that greater than 10-fold dilution and exposure to solution conditions (e.g., high ionic strength) in the mobile phase can dissociate and create aggregates during SEC analysis.^{11–13}

Finally, physical filtration of protein aggregates by column frits is another potential limitation of SEC. This problem has been mentioned in some review articles. Aggregates larger than the size limit of frit pores may not pass into the column and may degrade the SEC method performance.^{12,14,15}

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The various problems that have been encountered with SEC analysis of protein aggregates supports the broad recommendation in the field that another method should be used to verify SEC results and the overall robustness of the method. Accordingly, a recent “Guidance for Industry” from the U.S. Food and Drug Administration discouraged the use of any single technique for assessment of aggregates in therapeutic protein products.¹⁶

Currently, sedimentation velocity analytical ultracentrifugation (SV-AUC) is the dominant orthogonal method used during development of accurate SEC methods. Although much has been written about the potential advantages of SV-AUC for validation of SEC methods, there are only a limited number of published results.^{17,18} Some of the important differences are illustrated in one published example,¹⁷ wherein a recombinant human mAb sample was aggregated by incubation at pH 2.5 for 1 h. The percent soluble aggregates measured by SEC (0.4%) were significantly lower than that observed by SV-AUC (4.2%), and there was incomplete protein recovery during SEC analysis due to adsorption of the protein to the column stationary phase. SV-AUC analysis was also used to show that there was dissociation of the mAb dimer upon dilution in the SEC mobile phase.

However, the quantitation of protein aggregates by SV-AUC is significantly dependent on variables that are controlled by the analyst. A review article by Gabrielson et al. lists a number of SV-AUC experimental and data analysis parameters that may potentially have an impact on the method precision and accuracy.^{19,20} A number of research studies have reported that variability in SV-AUC analyses can be attributed to hardware, software, and procedure-related factors such as sample cell alignment, temperature control, quality of the sample cell housing and centerpiece, and meniscus control.^{21–23} Also, the relatively low throughput of SV-AUC analyses makes it less suitable than SEC as a routine assay for protein aggregates.

Despite these issues, in addition to being used as an orthogonal method to SEC, SV-AUC can be used as an extended characterization test for supporting formulation/process development and comparability exercises.¹⁴ SV-AUC is a robust tool to quantify aggregates because matrices are not involved in the analysis and the aggregate content often can be analyzed in the original protein formulation. The separation of species in a sample is based on their sedimentation coefficients which are a function of molecular mass and molecular shape (described in the Svedberg equation).²⁴ A number of research studies and reviews have been published to date which describes the best practices and advances in SV-AUC methodology for accurate and precise quantitation of protein aggregates.^{19–21,23}

The complexity of proteins and sources of variability in both methods makes it impractical to generalize experimental conditions of analyses. Therefore, more than a comparison between both methods, it is important to optimize and qualify the method performance of SEC and SV-AUC to accurately quantitate and characterize trace levels of protein aggregates. Optimizing the SEC method to fully recover all the protein injected onto the column and accurately quantifying even trace levels of protein aggregates is crucial, without which the merits (high throughput and precision) of SEC may be compromised.

The goal of this study is to investigate the effects of SEC mobile phase on recovery of protein monomer and aggregates from the column and to use SV-AUC as an orthogonal method to double check SEC results. For this part of the work, we analyzed samples of human intravenous immunoglobulin (IVIg) with various levels of aggregates, which were obtained by agitating IVIg solutions for various lengths of time. The main results confirmed the critical need to use SV-AUC during development of a robust SEC method for protein aggregation. In addition, this research presents a

modification to an existing method to quantitate insoluble aggregates using SV-AUC raw boundary data.

Experimental Methods

Materials

Human IVIg available as 100 mg/mL protein in 250 mM glycine buffer, pH 4.6 to 5.1, (Gammagard liquid; Baxter Healthcare) was purchased from the University of Colorado at Boulder's Wardenburg pharmacy. All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) and were of a reagent or higher grade. Unless otherwise noted, deionized Milli-Q[®] water was used to prepare all solutions.

IVIg Sample Preparation and Agitation

Prior to experiments, the IVIg formulation was dialyzed for 8 h in a Slide-A-Lyzer Dialysis Cassette with a 10-kDa molecular weight cut-off (Pierce, Rockford, IL) against 200 mM glycine buffer (pH 4.2) containing 25 mM NaCl. The dialyzed solution was then diluted to a final protein concentration of 0.5 mg/mL with 200 mM glycine buffer (pH 4.2) containing 25 mM NaCl.

Three polypropylene 15-mL conical tubes (Falcon[®], Corning, Inc.) were prepared as triplicate samples, each containing 12 mL of the 0.5 mg/mL IVIg solution. The tubes were rotated end-over-end at 20 rpm at room temperature using an RKSVD rotating mixer (Appropriate Technical, Laurel, MD). After 0, 90, 180, and 270 min of agitation, 2 mL aliquots were removed from each tube. Sample aliquots were transferred to 1.7 mL Eppendorf[®] tubes, and the tubes were centrifuged for 10 min using MiniSpin Plus (Eppendorf, Hauppauge, NY) at 14,000 rpm to pellet “insoluble” aggregates.²⁵ The supernatant was removed from the tubes, and aliquots were analyzed using UV spectroscopy, SEC, and SV-AUC. Unless otherwise noted, all dialysis, dilutions, agitations, and analyses were performed at room temperature (approximately 22°C).

UV Absorbance Spectroscopy

UV absorbance at 280 nm was measured using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, Santa Clara, CA). An extinction coefficient of 1.35 mL mg⁻¹ cm⁻¹ was used to determine protein concentration of IVIg solutions.²⁶

Size Exclusion Chromatography

An Agilent 1100 series chromatographic system equipped with an UV detector and ChemStation data analysis software was used for SEC analysis. To study the effects of mobile phase composition, the concentration of sodium chloride in the SEC mobile phase buffer was varied. The SEC mobile phase was composed of 200 mM sodium phosphate (pH 7.0) with 0, 50, 100, 200, or 400 mM NaCl. A TSKgel G3000SWXL column (TOSOH Bioscience, King of Prussia, PA) (7.8 × 300 mm²) was used, with a mobile phase flow rate of 1 mL/min and sample volume of 60 µL. UV absorbance was monitored at 280 nm. Peaks were integrated manually, and peaks that were not baseline separated were resolved by constructing lines at the point of peak intersection, perpendicular to the baseline. Total protein recovery of each sample was calculated using the following equation as described by Barnard et al.²⁷:

$$\text{Amount loaded} = \frac{\text{Total relevant area} \times \frac{\text{Flow rate}}{60}}{\epsilon \times \text{path length}} \quad (1)$$

where flow rate = 1 mL/min; total relevant area = integrated area under the curve (total peak area or species-specific peak area);

ϵ = extinction coefficient ($1.35 \text{ mL mg}^{-1} \text{ cm}^{-1}$); and path length = 1 cm. For all samples, percent total protein mass recovery and percent species were calculated using the following equation:

$$\text{Percent protein mass recovery} = \frac{\text{Concentration } (\mu\text{g/ml}) \text{ of protein recovered from SEC (from Eq. 1)}}{\text{Concentration } (\mu\text{g/ml}) \text{ of protein for 0 minute sample measured from benchtop UV absorbance}} \times 100 \quad (2)$$

In a separate experiment, the eluate from the SEC column was passed through 3 detectors in series: UV detector, then a VISCOTEK multiangle light scattering detector (SEC-MALS 20; Malvern Instruments, Worcestershire, UK) and finally a differential refractive index (RI) detector (VISCOTEK RI detector VE 3580; Malvern Instruments). For this experiment, a manual injector (Rheodyne Model 7725i) with a 100 μL fixed injection loop was used to load samples (100 μL) onto the column, and the mobile phase was composed of 200 mM sodium phosphate (pH 7.0) and 400 mM NaCl.

The MALS detector was equipped with a vertical flow cell aligned with 20 detectors at measurement angles from 12° to 164° . The raw data signals were processed using OmniSEC-Bio 5.10 software. The weight average molecular weight (M_w) of species was determined using the Rayleigh equation:

$$\frac{Kc}{R_\theta} = \left(\frac{1}{M_w} + 2A_2c \right) \frac{1}{P_\theta} \quad (3)$$

where K is the optical constant (directly proportional to square of dn/dc), R_θ is the Rayleigh ratio measured at angle θ , A_2 is the second osmotic virial coefficient, and $1/P_\theta$ defines the angular dependence of scattered light.

$$\frac{1}{P_\theta} = 1 + \left(\frac{16\pi^2 n_0^2 R_g^2}{3\lambda_0^2} \right) \sin^2 \left(\frac{\theta}{2} \right) \quad (4)$$

where n_0 is the RI of the solvent, R_g is the radius of gyration, λ_0 is the wavelength of laser in vacuum, and θ is the measurement angle. Measurements of light scattering intensity were made at the desired angle. With knowledge of the sample's concentration and other factors, the software can plot a partial Zimm plot. In this case, the Debye transformation (R_θ/Kc) was plotted as a function of $\sin^2(\theta/2)$. The y-intercept (which equates to an angle of 0°) is the molecular weight at that data slice. Combining the data from all the data slices within the peak allows M_w to be calculated.²⁸

Sedimentation Velocity Analytical Ultracentrifugation

SV-AUC analyses were conducted using an Optima XL-A analytical ultracentrifuge (Beckman Coulter) equipped with an UV absorbance optical system. Samples (390 μL) were loaded into 2-channel centerpieces, and IVIg formulation buffer (200 mM glycine buffer pH 4.2 containing 25 mM NaCl) was used as a reference (400 μL). The entire assembly was carefully aligned in an 8-hole An50Ti rotor. Sedimentation occurred at 40,000 rpm (4187

radians/s) and at a temperature of 22°C . Radial step size was 0.005 cm, and absorbance scans were collected at 280 nm between radial distances of 5.8–7.2 cm.

The raw data from sedimentation velocity experiments were analyzed using SEDFIT (version 14.6e).²⁹ Continuous sedimentation coefficient distributions, $c(s)$, were derived from data fitting, and the species resolved (monomer, dimer, and HMWS [high molecular weight species]) were quantified. At least 50 temporal absorbance scans capturing the entire sedimentation process were loaded into the program. The meniscus position was allowed to vary, and the cell bottom position was fixed at 7.2 cm. The analysis range was set from 6.15 to 7 cm. The frictional coefficient of 1.5 was allowed to vary, and a partial specific volume of 0.727 L/kg was used as suggested by Arthur et al.²¹ Buffer density and viscosity (corrected for temperature) of 0.99883 g/L and 0.00958 poise, respectively, were determined using the program SEDNTERP. A resolution of 200 increments between 0.2 and 20 Svedbergs was used for IVIg samples taken at 0 min of agitation. For IVIg samples taken after 90 min of agitation, a resolution of 350 increments between 0.2 and 100 Svedbergs was used, and for samples taken at 180 and 270 min of agitation, a resolution of 550 increments between 0.2 and 150 Svedbergs was used. All data fittings using SEDFIT were performed, while time-independent noise decomposition was enabled at a confidence interval set at 0.95.

For SV-AUC experiments, it is a common practice to collect a single absorbance scan at 3000 rpm as part of a leak check. Arakawa et al.³⁰ suggested that the value of absorbance plateau at the 3000 rpm scan can be used to estimate percent insoluble aggregates present in the sample. However, in this study, we found that with increase in time of agitation of IVIg from 0 to 270 min, an increase in the value of absorbance plateau at 3000 rpm was observed (data not shown). This increase in absorbance plateau may be interpreted as light scattering from agitation-induced large aggregates present in the sample which in-turn makes it unfeasible for quantitating insoluble aggregates. Therefore, in this study, we present a modification to the method, wherein precentrifuged agitated IVIg samples were loaded in SV-AUC experiments and the value of absorbance plateau of the first radial scan at 40,000 rpm, instead of 3000 rpm, was used to estimate percent insoluble aggregates. The centrifugation step performed before SEC and SV-AUC analysis (described in the sample preparation section above) presumably removes all the "insoluble aggregates," and therefore the decrease in absorbance plateau of the first scan at 40,000 rpm for agitated IVIg samples can be used to calculate percent insoluble aggregates. This determination is analogous to the loss of total peak area in an SEC chromatogram for the supernatant after insoluble aggregates are pelleted by prechromatography centrifugation. The difference between average plateau absorbance (APA) for IVIg samples agitated for 90, 180, and 270 min and the APA for unagitated IVIg samples was used to calculate percent insoluble aggregates.

$$\text{Percent insoluble aggregates} = \frac{\text{APA for samples agitated for time } t - \text{APA for 0 minute sample}}{\text{APA for 0 minute sample}} \times 100 \quad (5)$$

The concentration ($\mu\text{g/mL}$) of soluble protein was estimated from the APA of first scan using an extinction coefficient of $1.35 \text{ mL mg}^{-1} \text{ cm}^{-1}$ and a path length of 1.2 cm. The percent peak area for monomer, dimer, and HMWS obtained from $c(s)$ distributions was multiplied with the concentration of soluble protein obtained from APA of first scan to obtain concentrations of individual species (monomer, dimer, and HMWS). Percentage for each species was then calculated by dividing concentration of individual species from AUC with the concentration of protein present in 0 min sample measured from UV absorbance spectroscopy.

Results

To test the effect of mobile phase composition on total mass recovery of protein from the SEC column, IVIg solution agitated for 0, 90, 180, and 270 min was analyzed on SEC with different mobile phases composed of 0–400 mM NaCl. SEC chromatograms for IVIg samples agitated for 180 min are shown in Figure 1. The total percent mass recovery from SEC increased with increasing NaCl concentration in the mobile phase (Fig. 2a).

Even for unagitated IVIg samples, total protein recovery with 0 mM NaCl was $89.4 \pm 0.5\%$, compared to $99.1 \pm 0.6\%$ recovery when 400 mM NaCl was present in the SEC mobile phase. Similarly, for all agitated samples, NaCl concentrations <400 mM in the mobile phase resulted in incomplete mass recovery of the protein. The effect of mobile phase composition on protein recovery was also observed for the individual species present in the sample (Figs. 2b and 2d), with percent HMWS showing the most substantial dependence on NaCl concentration in SEC mobile phase. However, there was not a clear trend with the mass recovery of dimer species. Because complete recovery of total protein was obtained with an SEC mobile phase buffer composed of 200 mM sodium phosphate (pH 7.0) and 400 mM NaCl, this composition was selected for the remainder of the studies. To be absolutely definitive of the effect of salt concentration on the percent recovery of IVIg from the SEC column, we also tested the effect of salt concentration on the UV extinction coefficient of IVIg. We found that the extinction coefficient of IVIg diluted in SEC buffer with 0 mM NaCl ($\epsilon = 1.39 \pm 0.05 \text{ mL mg}^{-1} \text{ cm}^{-1}$) was similar to IVIg diluted in SEC buffer with 400 mM NaCl ($\epsilon = 1.36 \pm 0.05 \text{ mL mg}^{-1} \text{ cm}^{-1}$).

To gain insight into the molecular weights of species separated by SEC, MALS analysis was used (Fig. 3). The molecular weight of monomer ($144.3 \pm 2.3 \text{ kDa}$) and dimer ($264.3 \pm 12.9 \text{ kDa}$) species were as expected for IgG molecules. The molecular weight for the species eluted in the peak for HMWS had a broad range from 500 kDa to 70 MDa, indicating a wide size range of aggregates coeluting in the void volume of the column. SEC was unable to separate these large species; hence all the large aggregates had to be quantified together as HMWS.

With SV-AUC analysis, $c(s)$ distributions (Fig. 4) for agitated IVIg samples showed a greater resolution in separation of HMWS when compared to the SEC. The sedimentation coefficients (corrected for temperature and buffer, $s_{20,w}$) for the monomer peaks in all samples was 6.2 and the root mean square deviation for all $c(s)$ data fittings was <0.0044.

Both SEC and SV-AUC showed an increase in percent HMWS up to 180 min of agitation, after which at 270 min, the levels of HMWS decreased (Figs. 2d and 4h). There was also a corresponding increase in the percent insoluble aggregates with increasing agitation time suggesting that extended exposure of IVIg to air-water interface during agitation may have damaged the structure of IVIg adsorbed to the hydrophobic interface. This structural damage may have further promoted aggregation via intermolecular contact of hydrophobic residues.^{31,32} Pathways to formation of

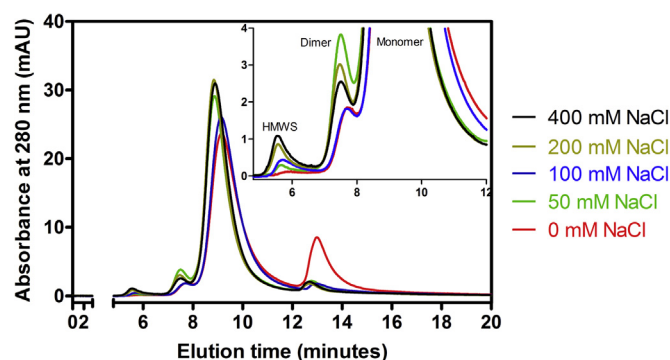


Figure 1. Size exclusion chromatograms for representative IVIg samples (agitated for 180 min) using different concentrations of NaCl in the mobile phase. Inset graph magnifies the details of the minor peaks. The chromatograms consistently show 3 distinct peaks as labeled in the inset graph. Increasing concentrations of NaCl in the mobile phase increased the recovery of protein (e.g., the peak height for HMWS is significantly higher for 400 mM NaCl when compared to 0 mM NaCl buffer conditions). For all samples, SEC mobile phase was 0.2 M sodium phosphate with “X” mM NaCl at pH 7, where X = 0, 50, 100, 200, or 400 mM NaCl.

insoluble aggregates may proceed through depletion of the soluble aggregates as well as the native protein.³³

In addition to quantitation of soluble monomer and aggregates, SV-AUC boundary data were used to quantify insoluble aggregates in agitated IVIg samples. An overlay of first radial scans at 40,000 rpm (Fig. 5) for agitated IVIg samples showed a systematic decrease in the absorbance plateau with increase in duration of agitation, indicating a loss in total soluble protein due to agitation-induced formation of insoluble aggregates. The percentage of insoluble aggregates was calculated using Equation 5 as described in the Methods section. It is also possible to estimate insoluble aggregates from the total area under the $c(s)$ distributions for each IVIg sample. As described in the Methods section, all IVIg samples were precentrifuged before SV-AUC analyses and with increase in agitation time, a decrease in the total area under the $c(s)$ distribution was observed. The percent decrease in this area can be equated to levels of insoluble aggregates present in the sample. The percent decrease in area under the $c(s)$ distribution for 0, 90, 180, and 270 min of agitation was found to be $0.8 \pm 0.7\%$, $0.4 \pm 0.2\%$, $2.4 \pm 0.2\%$, and $8.8 \pm 0.6\%$ correspondingly. The levels of insoluble aggregates obtained from SV-AUC (quantified using both methods; loss in absorbance plateau of the first radial scan at 40,000 rpm and loss in the total area under the $c(s)$ distribution) were comparable to those obtained from SEC analysis calculated from the loss of chromatogram peak area (Table 1).

Overall, when 400 mM NaCl was used in the SEC mobile phase, there was good agreement for levels of insoluble aggregates and soluble species (monomer, dimer, and HMWS) measured by SEC and SV-AUC for unagitated and agitated IVIg samples (Table 1). However, significant discrepancies were observed between SEC and SV-AUC results when SEC analyses were performed using suboptimal concentrations of NaCl in the mobile phase buffer, as shown by an example comparison in Table 1 between SEC results obtained with 50 and 400 mM NaCl in the mobile phase and SV-AUC results.

Discussion

Incomplete Mass Recovery From SEC Column

Adsorption of protein molecules to the stationary phase is an inherent limitation of SEC.^{13,34} Nonspecific adsorption of protein to the column matrix has been previously shown to cause protein losses and changes in the chromatogram peak shape.^{7,35} The

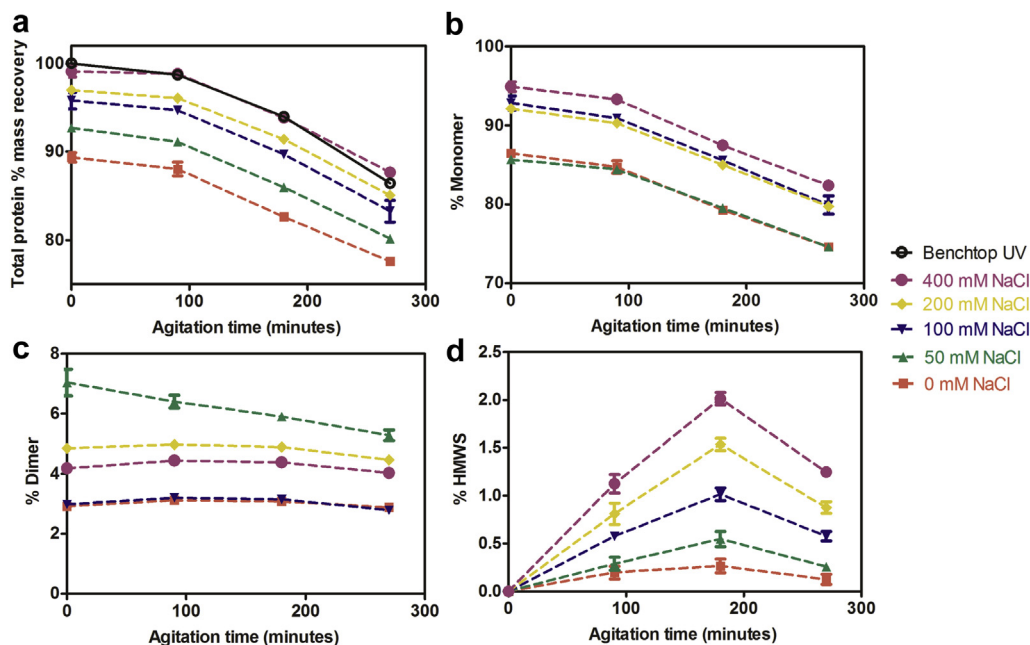


Figure 2. Effect of SEC mobile composition on percent mass recovery of (a) total protein and corresponding distribution of (b) monomer; (c) dimer and (d) HMWS for agitated IVIg samples. The IVIg samples were analyzed using mobile phases composed of 200 mM sodium phosphate (pH 7.0) with NaCl at 0 mM (solid red squares), 50 mM (solid green triangles), 100 mM (solid blue inverted triangles), 200 mM (solid yellow diamonds), or 400 mM (solid purple circles). The black circles in (a) represent the amount of protein present in each sample as measured by UV absorbance in spectrophotometer. Data points represent the mean $\pm$ SD for triplicate samples. Error bars for certain data points were smaller than symbols.

adsorption of IVIg to the column matrix observed in this study may be irreversible or at least very slowly reversible because the adsorbed protein did not appear to elute over the length of chromatographic run. The nature of interaction between IVIg and the silica-based column matrix may be electrostatic. The average

isoelectric point for protein molecules in IVIg is 8.13 and hence they may be slightly positively charged at the neutral pH of the SEC mobile phase.^{36,37} The TSK G3000SWXL column is packed with silica-based resin that presents negatively charged surface functional groups.^{38,39} The opposite charges between stationary phase

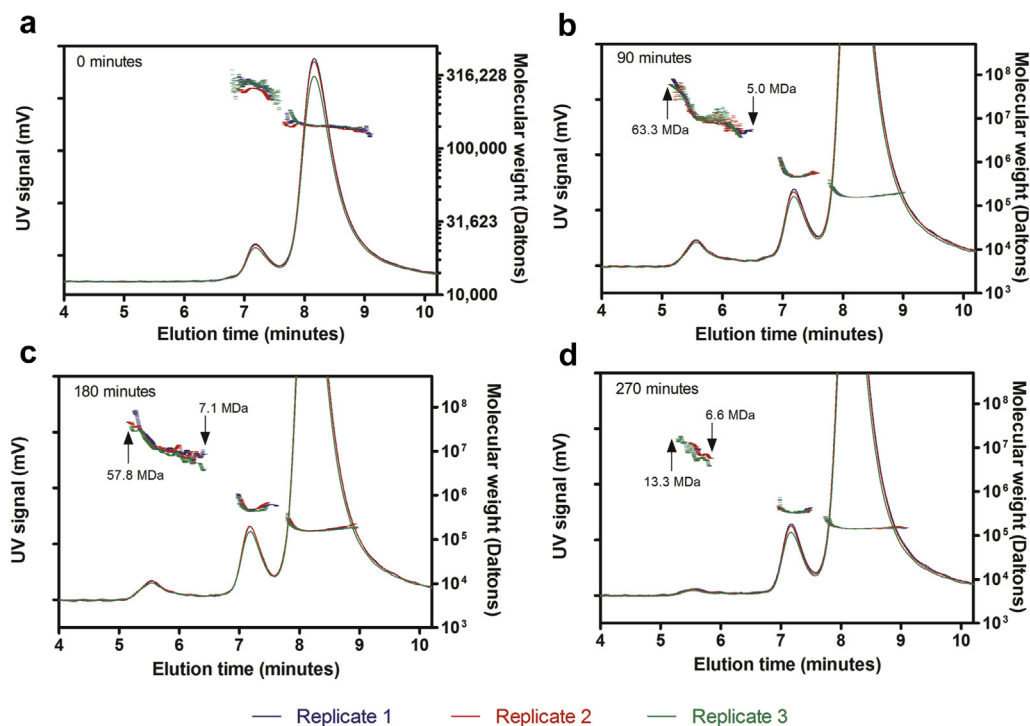


Figure 3. SEC-MALS chromatogram and weight averaged molecular weight (M_w) for IVIg samples agitated for (a) 0, (b) 90, (c) 180, and (d) 270 min. The monomer peak with an elution time of 8.16 min corresponds to M_w of ~144 kDa and the dimer peak with an elution time of 7.18 min corresponds to M_w of ~264 kDa. The MALS reported a wide range of M_w for the soluble aggregates eluting between 5 and 6.5 min.

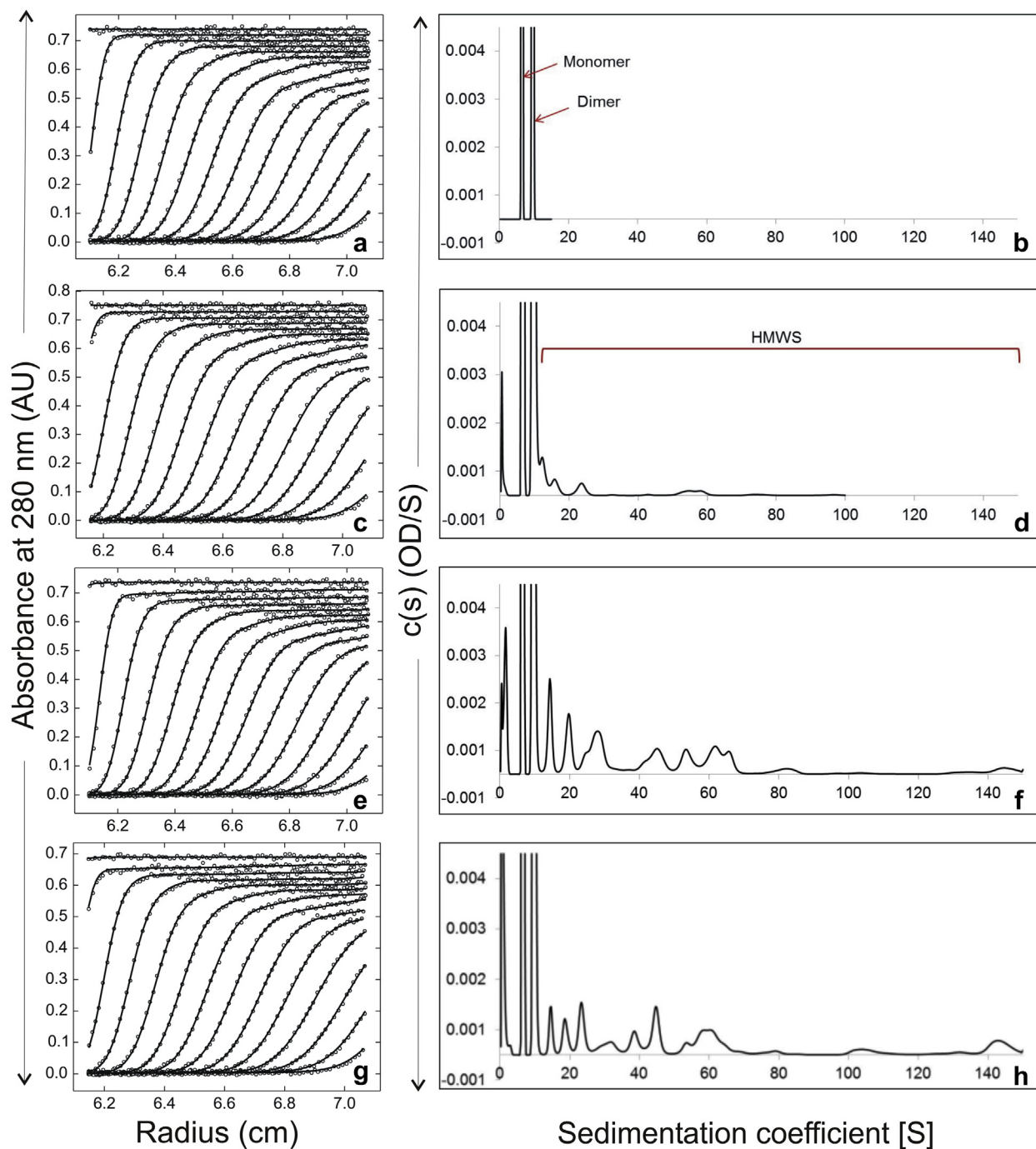


Figure 4. Sedimentation velocity analysis of unagitated and agitated IVIg samples. Boundary data and $c(s)$ distributions for IVIg samples agitated for 0 (a, b), 90 (c, d), 180 (e, f), and 270 min (g, h). Open circles in the boundary data represent absorbance data collected at 280 nm as a function of radial distance and time. For clarity, only every sixth scan and every third data point are displayed. Continuous solid lines represent the best fit from $c(s)$ analysis implemented in the SEDFIT program.

surface and protein molecules may facilitate nonspecific protein binding to the resin. A common approach to minimize such interactions is by including salts in the SEC mobile phase.^{40–42} NaCl, for example, suppresses such electrostatic interactions by screening charges on the protein and the resin surfaces.⁴³

Results from this study showed that 0–200 mM NaCl in the mobile phase may not completely suppress the electrostatic interactions, therefore leading to protein adsorption to the column matrix and incomplete recovery of protein injected in SEC. With 400 mM NaCl in the mobile phase, the total amount of protein that was injected was recovered from SEC. Similarly, Herold⁴⁴ studied

the binding of chymotrypsinogen to TSK 3000SWXL column while systematically varying the NaCl concentrations from 0 to 2000 mM. Two main inferences were drawn: (1) at least 400 mM of NaCl was required to completely suppress protein adsorption and (2) electrostatic interactions dominate at low NaCl concentrations whereas hydrophobic interactions dominate at high salt concentrations.^{42,44}

Underestimating levels of HMWS in a protein product solely from SEC analyses poses a risk to product quality and may compromise patient safety. At suboptimal concentrations of NaCl, HMWS may preferentially bind to the column packing because soluble aggregates may have a shape that is more aspherical with a

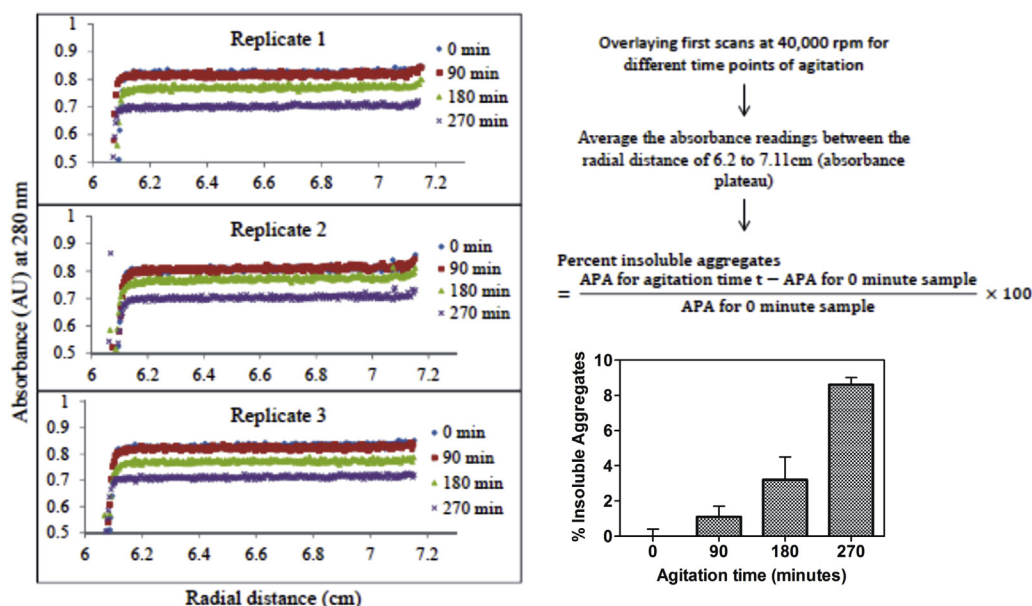


Figure 5. Estimating percent insoluble aggregates from the average plateau absorbance of the first SV-AUC absorbance scan at 40,000 rpm.

greater asymmetry in charge distribution than monomeric species. In a recent study, 4 oligomeric species of an mAb (found to preferentially bind the column resin) were modeled, and the assembly state was inferred to be more rod-like rather than spherical.¹⁷ In that particular case, they found that end-to-end assembly mechanism for large oligomers results in a shape of the oligomer that is more rod-like, wherein the interaction sites on each oligomer subunit lacks rigidity when compared to small oligomers. The lack of rigidity of large rod-like oligomers imparts rotational freedom to the interaction sites (hydrophobic and electrostatic) on the oligomers, which in turn could result in preferential binding of asymmetric oligomers to the solid phase of the column.¹⁷

As described in a comprehensive review by Arakawa et al.,⁴² the mechanism of protein binding to SEC resin involves 3 steps: (1) binding of the protein to the resin surface with one or multiple sites of contact; (2) protein conformational changes which may increase the number of contact sites; and (3) irreversible or only slowly reversible protein binding. Addition of NaCl (or other salts) in the SEC mobile phase may help to inhibit the step 1 in this mechanism,

thus minimizing protein adsorption to the column packing material.⁴²

Importance of SV-AUC for Orthogonal Analysis

SV-AUC can serve as a tool for quantitating species present in the sample as well as to validate the SEC methods. Over the years, SV-AUC evolved as the most popular orthogonal technique to double check SEC results because of several advantages.^{11–13,15,17,45–47} SV-AUC can analyze samples in the original formulation buffer, as is without any change of solvent conditions. Solid matrices are not involved in the separation process and therefore, unlike SEC, there is a negligible risk of reduction in protein recovery during analysis. Moreover, the *c(s)* model has an advantage of applying sophisticated numerical solutions to the SV-AUC data analyses, resulting in much improved resolution of HMWS over a range of sedimentation coefficients.

The SEC chromatogram, on the other hand, has a resolution of aggregate species that is inversely related to the dynamic range of

Table 1
Species Concentrations (Monomer, Dimer, HMWS, and Insoluble aggregates) in Agitated IVIg Samples Determined Using SEC and SV-AUC

Agitation Time (min)	Species	% Species (Mean ± SD)		
		SEC (n = 9) Using 50 mM NaCl in the Mobile Phase	SEC (n = 9) Using 400 mM NaCl in the Mobile Phase	AUC (n = 3)
0	Monomer	85.6 ± 0.5	89.1 ± 0.1	88.0 ± 1.1
	Dimer	7.0 ± 0.4	10.2 ± 0.5	11.1 ± 0.7
	HMWS	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.8
	Insoluble	0.0 ± 0.0	0.7 ± 0.3	0.0 ± 0.4
90	Monomer	84.4 ± 0.5	88.6 ± 0.3	89.2 ± 0.6
	Dimer	6.4 ± 0.2	9.1 ± 0.3	9.0 ± 0.1
	HMWS	0.3 ± 0.1	1.0 ± 0.1	0.7 ± 0.2
	Insoluble	1.6 ± 0.3	1.3 ± 0.3	1.1 ± 0.6
180	Monomer	79.5 ± 0.4	84.4 ± 0.4	85.9 ± 0.9
	Dimer	5.9 ± 0.1	8.5 ± 0.2	7.5 ± 0.4
	HMWS	0.5 ± 0.1	4.0 ± 0.1	3.4 ± 0.3
	Insoluble	6.7 ± 0.4	3.1 ± 0.4	3.2 ± 1.3
270	Monomer	74.6 ± 0.2	79.7 ± 0.3	82.0 ± 0.7
	Dimer	5.3 ± 0.2	7.5 ± 0.1	6.7 ± 0.2
	HMWS	0.3 ± 0.0	3.5 ± 0.1	2.7 ± 0.2
	Insoluble	12.6 ± 0.3	9.4 ± 0.4	8.6 ± 0.4

Species concentrations determined from SEC while using 400 mM NaCl in the mobile phase were in good correlation to SV-AUC data. However, significant differences between SEC and SV-AUC data can be observed when using 50 mM NaCl in the SEC mobile phase. Values are reported as mean ± SD.

molecular weights that can be separated.⁴² The pore volume of the column packing dictates the separation of different-sized species in SEC. Small species penetrate into the resin pore, and hence access greater column volume and elute later in the chromatographic run than larger species. However, very large molecules (e.g., oligomers) only access the voids in the column packing and elute early as a broad unresolved peak. Multiangle light scattering analysis in conjunction to SEC can be used to gain important insights into the molecular weight distribution of species coeluting in the void volume. We found that aggregates in agitated IVlg samples ranged from 500kDa to 70 MDa which eluted under a single peak in the void volume. Similarly, Li et al. described an SEC-MALS method to analyze size distribution of aggregates in thermally stressed bovine α -chymotrypsinogen. The ratio of M_w and number averaged molecular weight (M_n) provided an apparent value of polydispersity for high molecular weight aggregate species. As a reference point, a monodisperse sample would have M_w/M_n of 1.⁴⁸ We used this approach and found that the M_w/M_n values for the IVlg monomer and dimer species for all time points of agitation were consistently below the empirical threshold value of ~1.2 indicating a fairly monodisperse population. On the contrary, the M_w/M_n values for the HMWS were >1.2 indicating a broad size distribution of aggregates. Additionally, SV-AUC $c(s)$ distributions (Fig. 4) confirmed the presence of a wide range of HMWS.

SEC fails to resolve HMWS eluting in the void volume. As a consequence, it is impossible to distinguish their type (e.g., dimer, trimer, or oligomers) and respective levels. A case study published by Philo¹¹ showed that a “dimer” peak in SEC was in reality a mixture of dimer and trimer of glycoprotein when analyzed using SV-AUC. If species eluting in the void volume during SEC analysis cannot be resolved, then there is a risk that important alterations in protein product quality would not be detected. For example, a manufacturing change (e.g., scale up from clinical to commercial manufacturing) may result in assembly of the smaller species that eluted in the SEC void volume into larger aggregates that coeluted. This redistribution of species into larger, and potentially more clinically unsafe, aggregates would not be resolved with SEC, unless MALS detection was also employed.

The orthogonality between SV-AUC and SEC methods can be extended to quantitating insoluble aggregates. The first radial scans of SV-AUC runs can be used to calculate percent insoluble aggregates in agitated IVlg samples. We observed that with increase in duration of agitation of the protein samples, the absorbance of the plateau region of the first radial scans decreased in reference to the 0 min samples. This decrease in absorbance plateau can be attributed to agitation-induced insoluble aggregates that were pelleted in the precentrifugation step during sample preparation. This method of quantitating insoluble aggregates presents an additional point of validation for information obtained from SEC, as well as maximizes the information that can be inferred from the raw SV boundary data. The levels of insoluble aggregates from SV-AUC were comparable to those obtained using SEC wherein insoluble aggregates were calculated using loss in the chromatogram peak areas with reference to 0 min samples.

Summary and Conclusions

The first step in accurate quantitation of trace levels of protein aggregates is to optimize the method performance of SEC and SV-AUC. A comparison of results between SEC and SV-AUC may be undermined by any number of variables involved in the methods. SEC may underestimate soluble protein aggregates because of adsorption of protein to the solid phase of the column. At least 400 mM of NaCl was required in the SEC mobile phase for complete recovery of IVlg from the SEC. The use of SV-AUC as the main

orthogonal tool to optimize and validate SEC method is crucial for accurate quantitation of protein monomer, soluble as well as insoluble aggregates. Additionally, the orthogonality between SV-AUC and MALS analyses can be extended to determine the quantity and type of protein aggregates present in the sample, especially because SEC fails to resolve HMWS eluting in the void volume.

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