Research Article

A Multi-Tiered Analytical Approach For the Analysis and Quantitation of High-Molecular-Weight Aggregates in a Recombinant Therapeutic Glycoprotein

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Abstract. In this study, we have investigated sedimentation velocity ultracentrifugation (AUC-SV), size exclusion chromatography (SEC), and circular dichroism (CD) methods for the detection and quantitation of protein aggregates using recombinant acid alpha-glucosidase (rhGAA) as a model. The results of this study showed that the formation and molecular weight distribution of rhGAA aggregated species were dependent upon the formulation conditions as well as the storage or stress conditions used to induce aggregation. The utility of CD as a probe for non-native, aggregated species was affirmed, as this method was sensitive to rhGAA aggregation levels of $\leq 4\%$. An extensive evaluation of AUC-SV variability was performed using nine levels of spiked rhGAA aggregate that were analyzed on six occasions. Based on our data, the precision of the AUC-SV results increased with increasing levels of aggregate, with a mean RSD of 37.2%. The limit of quantitation (LOQ) for the AUC-SV method, which was based on a Precision criterion of RSD $< 20\%$, was determined to be $\geq 3\%$ aggregated rhGAA. The Precision and LOQ of the SEC method, determined using the same rhGAA sample set, was found to be 3.8% and $\geq 0.2\%$, respectively. In general, there was good agreement between the levels of aggregated rhGAA determined using the AUC-SV and SEC methods, with a slight positive bias noted for the AUC-SV results. These studies emphasize the value of applying multiple, well-characterized analytical tools in the evaluation of therapeutic protein aggregation.

KEY WORDS: aggregation; analytical ultracentrifugation; circular dichroism; size exclusion chromatography.

INTRODUCTION

Self-association of therapeutic proteins into high-molecular-weight aggregates may result in loss of potency and decreased stability. High-molecular-weight protein aggregates, particularly those containing multivalent structures with molecular weights $> 100,000$ Da, may also elicit an immune response in patients (1,2). Antibodies that are raised against recombinant therapeutic protein aggregates can alter the pharmacokinetic behavior of the therapeutic protein as well as induce potentially severe adverse events (3). For these reasons, the analysis

and control of aggregated protein species in therapeutic protein preparations is critical. The analytical strategies employed in the detection, quantitation, and characterization of protein aggregates and aggregation typically evolve during the course of product development. Depending on the requirements for assay throughput and precision, methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and native PAGE may be appropriate (4). As product development progresses, high throughput methods that deliver precise and accurate data are required in order to support process validation, lot release testing, and stability studies. Size exclusion chromatography (SEC) methods meet these criteria and are routinely used in the analysis of protein aggregation in biopharmaceutical protein products. There are, however, limitations to SEC methods that include potential interactions between the sample and chromatographic matrix, a limited separation range, and running buffers that often differ from the product formulation buffer. Also, sample dilution, either before or during the SEC analysis, may result in the dissociation of concentration-dependent, reversible aggregate species (5,6).

Sedimentation velocity analytical ultracentrifugation (AUC-SV) is an established method that, with improvements in data analysis software, is finding increased application in

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ABBREVIATIONS: AUC-SV, Sedimentation velocity analytical ultracentrifugation; CD, Circular dichroism spectroscopy; c(S), Continuous size distribution; rhGAA, Recombinant human acid α -glucosidase; rhGAA-FP, Recombinant human acid α -glucosidase, finished product; RI, Radial invariant noise; TI, Time invariant noise.

the analysis of protein aggregation and protein–protein interactions (7). Unlike SEC, AUC-SV analyses may be performed in most formulation buffers without sample manipulation. Resolution of protein species occurs in solution, thereby removing the issues of potential column matrix interactions. AUC-SV methods have a broad dynamic range and also provide useful information on the hydrodynamic properties of proteins (6). Unlike SEC, however, AUC-SV methods are low-throughput and the initial cost of instrumentation is high. Although advances and improvements have been made in data analysis software (7,8), significant expertise is required in order to acquire and interpret high quality AUC-SV data (9).

In this study, we have investigated multiple methods for detection and quantitation of aggregates using recombinant human acid α -glucosidase (rhGAA) as a model protein. RhGAA is an exo-1,4 and -1,6- α -glucosidase that hydrolyzes lysosomal glycogen to glucose. The precursor form of rhGAA used in these studies contains seven N-linked glycosylation sites and has an apparent molecular weight of 110 kDa (10,11). The sensitivity of circular dichroism (CD) to low levels of aggregated species was examined as were the performance characteristics, such as precision and limit of quantitation (LOQ), of SEC, and AUC-SV methods. We also evaluated aggregated rhGAA species formed under harsh conditions of elevated temperature and pH and compared those to aggregated species formed in finished product vials of rhGAA exposed to conditions of elevated temperature and humidity.

MATERIALS AND METHODS

Preparation of Forced rhGAA Aggregate

RhGAA was dialyzed into 25 mM sodium phosphate, pH 7.5, and incubated at 37°C for 18 h. This 100% aggregated sample was dialyzed into 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.2 SEC buffer, and then spiked into similarly dialyzed rhGAA, resulting in a series of samples containing 0%, 0.25%, 0.5%, 1.25%, 2.5%, 4%, 5%, 10%, and 20% rhGAA aggregate. The rhGAA starting material contained 0.2% aggregate by SEC. The final protein concentration of the samples in this series was 0.5 mg/mL.

Circular Dichroism Analyses

Circular dichroism analyses of the 100% aggregated sample and samples of a spiked aggregate series containing 0%, 1%, 2%, 4%, 8%, 12%, and 20% aggregated rhGAA were performed using a Jasco J-810 circular dichroism spectrophotometer. The circular dichroism analyses were performed using the following parameters: scanning speed 10 nm/min, band width 2.5 nm, data pitch 1 nm, response 8.0 s, wavelength range 250–350 nm, and pathlength 1.0 cm.

Sedimentation Velocity Analytical Ultracentrifugation

Six samples from each of the 0%, 0.25%, 0.5%, 1.25%, 2.5%, 4%, 5%, 10%, and 20% spiked aggregate series were analyzed in single AUC-SV runs. The 4% spiked aggregate sample was analyzed using the same cell and in the same

rotor position in all of the AUC-SV runs as an internal control. Samples were loaded at 0.5 mg/mL into two-sector, 1.2-cm Charcoal-Epon centerpieces (Beckman Coulter) and equilibrated at 20°C for at least 1 h in the centrifuge (Optima XL-A, Beckman Coulter). Reference sectors were filled with buffer matched to the sample. Sedimentation velocity (AUC-SV) analyses were performed at 40,000 rpm using an eight-hole rotor (Beckman An 50 Ti). Absorbance was monitored at 280 nm in continuous mode using a radial increment of 0.003 cm. At least 70 scans per cell were collected. For the AUC-SV data analysis, scans 1–40 were loaded into SEDFIT version 8.5 and an initial evaluation of the sedimentation coefficient distribution of the sample [c(s) analysis] was generated using a fixed meniscus and frictional ratio. These parameters were then floated during fitting to the Lamm equation (8). In general, data were fitted without time-independent (TI) or radially independent (RI) noise correction and regularization was set to 0.95. TI noise correction was performed on several samples to improve goodness of fit. All RMSD values were <0.00995 for samples where aggregate content was quantified. S-ranges of 0.1–20 were evaluated with a resolution of 200. The S-range was extended to 0.1–50S (resolution 500) when it appeared that species larger than 20S were present in the sample. Quantitation of aggregate was determined to be comparable when data were analyzed to either 20 or 50S. All data were analyzed using SEDFIT default values for density and viscosity. Measured percent aggregate was calculated by dividing the area under the aggregate peak (from 7.1 to 20S) by the total area under all the peaks from 0.3 to 20S (or 50S). To evaluate concentration-dependent reversibility, the 100% forced rhGAA aggregate sample was diluted to 0.5, 0.1 and 0.02 mg/mL and absorbance was monitored at 240, 231, and 211 nm using the AUC-SV method described above.

Size Exclusion Chromatography

Each of the nine spiked-aggregate samples was also analyzed in triplicate on three assay occasions by SEC using an Agilent 1100 HPLC with a Tosoh Biosep Super SW3000 column. The SEC analyses were performed in 25 mM sodium phosphate, 150 mM sodium chloride, pH 6.2, at a flow rate of 0.35 mL/min. Proteins were detected by UV absorbance at 280 nm; injections were 50 μ g each. The mean total peak area from triplicate injections of each spiked aggregate level was also determined without the SEC column in line, and the relative protein recovery based on peak area was calculated.

Preparation of Purified Aggregated rhGAA-FP

High-molecular-weight aggregates from lyophilized rhGAA finished product vials (rhGAA-FP) were obtained by incubating sealed vials for an extended period of time at 25°C, 60% relative humidity. Under these conditions, SEC analysis showed that the reconstituted rhGAA-FP contained approximately 5% aggregated species. The rhGAA-FP aggregates were purified by SEC and combined with purified rhGAA-FP monomer to yield a series of spiked purified aggregate samples with targeted aggregate percentages of 1%, 5%, and 10%. For this series, the final protein concentration was 0.5 mg/mL in 25 mM sodium phosphate,

2% mannitol, 0.005% polysorbate 80, pH 6.2. The SEC and AUC-SV analyses of this series of rhGAA-FP spiked aggregate samples were performed as previously described.

RESULTS AND DISCUSSION

Protein aggregation may occur when proteins are exposed to stress conditions that induce unfolding of the native protein structure. As a result of unfolding, exposed hydrophobic protein surfaces may interact with one another, resulting in aggregate formation. Changes in pH and temperature, freeze-thaw, exposure to light, and physical agitation are a few of the many external stress factors that can induce protein unfolding and subsequent aggregation (12). Protein modifications, such as oxidation, deglycosylation, deamidation, and glycation, may also cause proteins to unfold (13). In the studies described here, soluble aggregates were formed by incubation of rhGAA, a glycoprotein with an apparent molecular weight of 110 kDa, in sodium phosphate buffer, pH 7.5, for 18 h at 37°C. Subsequent analysis by SEC showed that under these conditions, conversion of rhGAA to a mixture of soluble, high-molecular-weight aggregated species was essentially complete (Fig. 1). AUC-SV analysis of the forced rhGAA aggregate preparation, shown in Fig. 2, confirmed that this pool contained very little monomeric rhGAA, but was comprised of a mixture of high-molecular-weight oligomers. AUC-SV analysis at concentrations of 0.02 to 0.5 mg/mL showed that the relative percent aggregate in the rhGAA aggregate pool remained unchanged regardless of dilution, ranging from 97.2% to 98.6% (data not shown). Overall, these results show that incubation of rhGAA at pH 7.5 for 18 h at 37°C resulted in the formation of irreversible high-molecular-weight rhGAA oligomers or aggregates.

CD spectroscopy is particularly sensitive to changes in protein secondary and tertiary structure (14) and is therefore ideal for monitoring protein unfolding under various time, pH buffer, and temperature conditions. The near-UV CD spectra, often used as a probe for native protein structure (15) of rhGAA, the purified rhGAA aggregate pool, and an rhGAA spiked aggregate sample series are shown in Fig. 3. There was a significant loss in the near-UV signal for the rhGAA aggregate pool compared to rhGAA, consistent with a loss of

Fig. 1. SEC analysis of rhGAA and rhGAA forced aggregate formed by incubation of rhGAA in phosphate buffer, pH 7.5, for 18 h at 37°C

Fig. 2. AUC-SV analyses of rhGAA and the rhGAA forced aggregate pool

tertiary structure. The CD analyses of the rhGAA spiked aggregate series also showed a loss in near-UV CD signal as the level of aggregated protein increased. Very little change was observed in the far-UV CD signal for the same sample

Fig. 3. Near-UV circular dichroism spectra of **a** rhGAA forced aggregate versus rhGAA and **b** the rhGAA forced aggregate dilution series. Spectra for 0%, 4%, and 20% rhGAA forced aggregate levels are shown

series (data not shown). This proportional loss in near-UV signal corresponds to the contribution of the partially unfolded, rhGAA soluble aggregates that were formed by incubation at neutral pH and elevated temperatures. Although the near-UV CD data provided in Fig. 3b are not quantitative, changes in the near-UV spectra were detected at low ($\leq 4\%$) levels of aggregated rhGAA, thus confirming the sensitivity of CD methods, in particular the near-UV spectra, to non-native rhGAA species. It is important to note that the ability of CD methods to detect changes in protein conformation is likely to be variable depending upon the protein sequence, secondary and tertiary structural features, and conditions of the analysis. There are cases in the literature where changes in both near- and far-UV CD spectra occur as a result of aggregation (16). Here, a change in the near-UV CD but not far-UV provides an interesting insight into the nature of the near-UV CD spectrum of rhGAA. This suggests that the near-UV CD spectrum may have significant contributions from chiral tertiary interactions that were either largely disrupted or randomly reoriented in the aggregated forms of rhGAA. In contrast, the far-UV CD spectrum was an insensitive probe for rhGAA aggregation, presumably because the overall secondary structure was not significantly altered in the aggregated forms. Importantly, CD analyses such as these have proven extremely useful in investigating root cause mechanisms for protein aggregation, evaluating degradation pathways, optimizing formulation conditions, and also for confirming protein aggregation results obtained using other methods, including SEC.

Aggregation analysis of therapeutic protein products is often performed at multiple process stages which may differ

in protein concentration, buffer pH, ionic strength, and storage condition. For these reasons, the rate at which high-molecular-weight aggregated species are formed and the biophysical and biochemical properties of those aggregated species may be process stage-dependent. In these studies, aggregated species of rhGAA were obtained using two approaches. As previously described, incubation of rhGAA in sodium phosphate buffer, pH 7.5, for 18 h at 37°C induced the nearly complete formation of irreversible, high-molecular-weight rhGAA soluble aggregates (forced aggregates). In a second approach, high-molecular-weight aggregated species were purified by SEC from rhGAA finished product vials (rhGAA-FP) exposed to 25°C, 60% humidity. This purified, high-molecular-weight rhGAA-FP aggregate pool was spiked into rhGAA-FP, creating a second spiked aggregate series that was compared to the forced aggregate series using SEC and AUC-SV methods.

The data in Fig. 4 show that the high-molecular-weight aggregated species in the forced aggregate series, formed by incubation at neutral pH and elevated temperature, were poorly resolved by SEC and eluted at or near the column void volume. These aggregated species were, however, well resolved by AUC-SV and appeared to be a mixture of rhGAA dimers, trimers, and other higher order oligomers based on both $c(M)$ and multi-angle light scattering data (not shown). While AUC-SV provides better peak resolution compared to SEC, the position of the higher molecular weight peaks does vary depending on the level of spiked aggregate, with a trend towards higher S values as the peak magnitude decreases. This is consistent with recent literature from Brown *et al.* (17) which suggests that the standard

Fig. 4. SEC and AUC-SV analyses of rhGAA and rhGAA forced aggregate. **a** SEC analysis of rhGAA forced aggregate. **b** AUC-SV analysis of rhGAA forced aggregate. **c** SEC analysis of rhGAA-FP purified aggregate. **d** AUC-SV analysis of rhGAA-FP purified aggregate

Table I. Analysis of rhGAA Forced Aggregate by AUC-SV and SEC

rhGAA forced aggregate (%)	0	0.25	0.5	1.25	2.5	4	5	10	20
% Aggregation (AUC), $N=6^a$	0.4	0.9	0.6	1.3	2.5	3.1	4.2	8.5	18.1
SD	0.3	0.7	0.4	0.5	1.1	0.3	0.4	0.7	1.3
RSD (%)	67.0	84.0	69.4	35.7	44.0	8.8	10.4	8.1	7.0
% Aggregation (SEC), $N=9$	0.2	0.3	0.5	1.0	1.9	3.0 ^b	4.1	8.4	17.5
SD	0.02	0.03	0.02	0.04	0.03	0.04	0.04	0.04	0.42
RSD (%)	9.2	10.8	3.7	3.7	1.6	1.2	1.0	0.5	2.4

^a For each spiked aggregate level, samples were analyzed in a single AUC run using six different cells. Data from cell A were excluded because this cell exhibited an unacceptably high level of variability

^b These SEC data were obtained on six samples (two runs $\times$ three replicates)

maximum entropy regularization used in the c(s) analysis performed here can cause peak location variability for trace species (and a small underestimation of peak area) and that the described Bayesian extension of c(s) method may correct these artifacts of the data analysis. Conversely, the purified rhGAA-FP aggregates, formed under less severe stress conditions using formulated rhGAA, eluted from the SEC column as a more resolved peak that did not contain the early-eluting species observed in the forced aggregate SEC chromatogram. The AUC-SV results provided enhanced resolution compared to the SEC data and showed that the purified rhGAA-FP aggregated species were predominantly dimeric forms of rhGAA. These data highlight the enhanced resolution and ability of AUC-SV methods to be performed directly on formulated protein products, without sample dilution or buffer exchange. It is important to note that recent studies have found that certain excipients may form density gradients during AUC-SV analyses. Under these conditions, accurate quantitation of aggregated species may be compromised (18). Equally as important, experiments such as those described here emphasize the impact of sample matrix and stress conditions on the formation and diversity of high-molecular-weight protein aggregates that may arise from a single protein product.

An extensive investigation into the reproducibility of the AUC-SV method was performed using the forced aggregate series. Six determinations from each of the nine levels of spiked aggregate were obtained from single AUC-SV runs. These results were compared to SEC results obtained on the same samples, which were analyzed in triplicate on three assay occasions. Although the AUC-SV method is not validated and is not suitable as a release test, estimates of the Precision and LOQ were determined from the AUC-SV data and compared to similar estimates obtained from the SEC results obtained from the same samples.

The overall Precision (% RSD) of the AUC-SV data for each of the nine spiked aggregate levels ranged from 7% to 67%, with an overall mean of 37.2% (Table I). The Precision with which the AUC-SV method measured the levels of forced rhGAA aggregates improved substantially with increasing levels of aggregate and at the 20% spiked aggregate level was 7%. Pekar and Sukumar (19), using purified antibodies as models, also noted decreases in the variability of AUC-SV results as the levels of aggregated species increased. The variability observed in aggregate measurements performed on samples containing very low levels of aggregated species or other low abundance species may be due to random noise in the AUC instrument optics, which affects the signal/noise ratio (6)

or to parameters related to the analysis of the data by SEDFIT, such as time-invariant noise (TI), radial-invariant noise (RI), and meniscus position (20). Not unexpectedly, the precision of the SEC method was significantly higher at all levels of spiked aggregate, with an overall mean RSD of 3.8%. Based on a precision criterion of $RSD \leq 20\%$, the LOQ for the AUC-SV method was estimated to be $\geq 3\%$ aggregated rhGAA species. Using the same criterion, the SEC method had adequate

Fig. 5. **a** The levels of rhGAA forced aggregate determined by SEC and AUC-SV. **b** Same as “A” with the 95% confidence intervals provided

precision throughout the spiked aggregate range, resulting in an LOQ of $\geq 0.2\%$. Because the LOQ of AUC-SV methods is dependent upon multiple factors, including the size and hydrodynamic shape of the aggregated species (20), this LOQ should not be universally applied to all AUC-SV analyses, but should be determined independently for each protein under investigation.

To further evaluate run to run consistency, the 4% forced aggregate sample was included in each AUC-SV analysis ($N=9$) as an internal control. This internal control was analyzed in the same rotor position using the same cell and centerpiece for each of the nine analyses. The mean level of observed aggregate for the internal control was 4.6% with an RSD of 21%. This level of variability was lower than the overall estimate of precision for the AUC-SV method (37.2%) derived from data obtained on multiple assay occasions using multiple AUC-SV cells. Interestingly, however, the intra-run variability of the internal control data, obtained on six replicates of the 4% forced aggregate sample analyzed in a single AUC-SV run, was higher than the estimate of inter-run variability (34% *vs.* 21%). It is likely that the intra-run variability seen in the analysis of the internal control is at least partially due to the fact that the data were obtained using six different cells and centerpieces rather than the single cell and centerpiece used in the evaluation of inter-run variability. It has been shown that the quality of AUC-SV results may be compromised by scratched, dirty, or old AUC-SV cells as well as by defective cell housings and centerpieces (19). Closer inspection of the data revealed that the AUC-SV results from a particular cell were consistently more variable than the data obtained from the other cells used in the study. Exclusion of the data obtained using this cell resulted in improved reproducibility of the overall results. Without this thorough evaluation, errors in the quantitation of aggregated species, particularly those errors related to the quality of the AUC-SV cells and cell housings, would go undetected.

AUC-SV data are particularly valuable in assuring that SEC chromatography conditions provide accurate information on the levels of high-molecular-weight species that may be present in therapeutic protein products. In the studies described here, the mean aggregate levels of the rhGAA spiked aggregate series determined by AUC-SV and SEC were statistically compared using the Student's *t* test. The results indicated that for each of the nine theoretical aggregate levels, the AUC-SV and SEC data were not statistically different, with $p > 0.08$. The levels of aggregated rhGAA determined by SEC and AUC-SV for each spiked aggregate sample were plotted and are shown in Fig. 5a, b. These data indicate that there is very good agreement between the AUC-SV and SEC results. Interestingly, it appears that the AUC-SV measurements show a consistent positive bias compared to the SEC results. One potential explanation for this bias is poor recovery of high-molecular-weight species from the SEC column, resulting in loss of signal at the absorbance detector. To evaluate total protein recovery, analyses of the spiked aggregate series were performed both with and without the SEC column in line. Although the results (not shown) indicated that the percentage of total peak area recovered at each level of spiked aggregate was $>97\%$, small losses of aggregated protein during the SEC analyses could influence the overall aggrega-

tion measurements. Previous studies using a monoclonal antibody have shown improved quantitation of aggregates using analytical systems with low solid-liquid interfacial area, such as AUC-SV (9). Another possible explanation for the bias in these results is dilution of reversible aggregate species during the SEC analysis, resulting in a decrease in the peak area of the aggregated species. This seems unlikely since dilution of the rhGAA aggregate pool to concentrations of 0.02 to 0.5 mg/mL did not show any appreciable loss in rhGAA aggregated species, as determined by AUC-SV.

CONCLUSIONS

In this study, CD, SEC, and AUC-SV methods have been used to characterize and, in the case of SEC and AUC-SV, quantitate aggregation in preparations of rhGAA. The near-UV CD spectra of rhGAA were sensitive to low levels of partially unfolded, aggregated rhGAA. This technique is particularly useful for identifying conditions which induce or prevent the formation of high-molecular-weight rhGAA aggregates. For quantitation of aggregated species, we expect that AUC-SV and SEC methods will continue to fulfill unique and complementary roles during therapeutic protein product development. A thorough evaluation of assay variability, which may be dependent upon the method, the instrument, the data analysis, and the protein, will enable consistent and appropriate application and interpretation of these methods throughout the product lifecycle.

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REFERENCES

1. Hermeling S, Schellekens H, Maas C, Gebbink MFBG, Crommelin DJA, Jiskoot W. Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. *J Pharm Sci.* 2006;95:1084–96.
2. Rosenberg A. Effects of protein aggregates: an immunologic perspective. *AAPS J.* 2006;8:E501–7.
3. De Groot AS, Scott DW. Immunogenicity of protein therapeutics. *Trends Immunol.* 2007;28:482–90.
4. Ribarska JT, Jolevska ST, Panovska AP, Dimitrovska A. Studying the formation of aggregates in recombinant human granulocyte-colony stimulating factor (rHuG-CSF), lenograstim, using size exclusion chromatography and SDS-PAGE. *Acta Pharm.* 2008;58:199–206.
5. Berkowitz SA. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J.* 2006;8: E590–605.
6. Liu J, Andya JD, Shire SJ. A critical review of analytical ultracentrifugation and field flow fractionation methods for measuring protein aggregation. *AAPS J.* 2006;8:E580–9.
7. Lebowitz J, Lewis MS, Schuck P. Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Sci.* 2002;11:2067–79.
8. Schuck P. Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and lamm equation modeling. *Biophys J.* 2000;78:1606–19.

9. Gabrielson JP, Brader ML, Pekar AH, Mathis KB, Winter G, Carpenter JF. Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity. *J Pharm Sci* 2007;96:268–79.
10. McVie-Wylie AJ, Lee KL, Qiu H, Jin X, Do H, Gotschall R. Biochemical and pharmacological characterization of different recombinant acid alpha-glucosidase preparations evaluated for the treatment of Pompe disease. *Molec Genet Metab* 2008;94:448–55.
11. Moreland RJ, Jin X, Zhang XK, Decker RW, Albee KL, Lee KL. Lysosomal acid alpha glucosidase consists of four different peptides processed from a single chain precursor. *J Biol Chem* 2005;280:6780–91.
12. Kueltzo LA, Wang W, Randolph TW, Carpenter JF. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing. *J Pharm Sci* 2008;97:1801–12.
13. Maas C, Hermeling S, Bouma B, Jiskoot W, Gebbink MFBG. A role for protein misfolding in immunogenicity of biopharmaceuticals. *J Biol Chem* 2006;282:2229–36.
14. Friefelder D. *Physical biochemistry, applications to biochemistry and molecular biology*. San Francisco: Freeman; 1976.
15. Schmid FX. *Spectral methods of characterizing protein conformation and conformational changes*. Oxford: IRL Press at Oxford University Press; 1989.
16. Kendrick BS, Cleland JL, Lam X, Nguyen T, Randolph TW, Manning MC. Aggregation of recombinant human interferon gamma: kinetics and structural transitions. *J Pharm Sci* 1998;87:1069–76.
17. Brown PH, Balbo A, Schuck P. A Bayesian approach for quantifying trace amounts of antibody aggregates by sedimentation velocity analytical ultracentrifugation. *AAPS J* 2008;10:481–93.
18. Gabrielson JP, Arthur KK, Kendrick BS, Randolph TW, Stoner MR. Common excipients impair detection of protein aggregates during sedimentation velocity analytical ultracentrifugation. *J Pharm Sci* 2009;50–62.
19. Pekar A, Sukumar M. Quantitation of aggregates in therapeutic proteins using sedimentation velocity analytical ultracentrifugation: practical considerations that affect precision and accuracy. *Anal Biochem* 2007;367:225–37.
20. Gabrielson JP, Randolph TW, Kendrick BS, Stoner MR. Sedimentation velocity analytical ultracentrifugation and SED-FIT/c(s): limits of quantitation for a monoclonal antibody system. *Anal Biochem* 2007;361:24–30.