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Classical light scattering quantitation of protein aggregates: off-line spectroscopy versus HPLC detection [1]

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Abstract

This paper describes the development and validation of a new off-line approach to quantitate both covalent and noncovalent, in-solution aggregates present in protein formulations and compares the new assay to established HPLC methods. This off-line analysis is well suited for use in QC release testing, formulation development and stability indicating applications. An inexpensive, continuous source HPLC fluorometer has been adapted with the addition of second order filters for use as a sensitive right-angle scatterometer which can determine the molecular weight of protein aggregates in solution. When used as an HPLC detector, right-angle light scattering is a sensitive method which can determine the molecular weight of peaks separable by HPLC, thus discriminating between monomers of different conformations and aggregates. The weight-averaged molecular weight of aggregate peaks can be calculated with system calibration, yielding the average number of monomers per aggregate. If the protein concentration is high enough for an adequate signal, the off-line technique of right-angle light scattering of protein formulations has advantages of convenience and speed over the HPLC approach. Samples are placed in standard fluorometer cuvettes and toluene is used as a calibrator. Data are presented which show the off-line (static) method to be extremely rapid, rugged and precise. The accuracy of this approach is demonstrated through cross-validation to traditional GPC analysis of protein aggregate distributions. This non-invasive light scattering approach is particularly useful when non-covalent protein aggregation is reversible and readily altered by chromatographic separations typically used for characterizing aggregates. © 1997 Elsevier Science B.V.

Keywords: Classical light scattering; Protein aggregates; Specific turbidity; HPLC light scattering; Protein weight-averaged molecular weight; Light scattering SEC analysis of proteins

1. Introduction

Noncovalent protein aggregation is a parameter of quaternary protein structure which is very difficult to quantitate. Traditionally, protein aggregation has been characterized by irreversible denaturation, or total loss of protein tertiary structure, leading to hydrophobic self association.

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With the advent of modern biotechnology and prevalence of large scale therapeutic protein manufacturing, it is common to also encounter reversible or irreversible protein aggregation, particularly as a result of lyophilization or during purification/concentration steps. Chromatographic analyses have been widely used to characterize these protein aggregates. In particular, the coupling of chromatographic separation of protein species with classical light scattering detection has proven very useful in characterizing protein aggregation phenomenon and other tertiary conformational changes [1]. By using a low angle laser light scattering HPLC detector, Karger et al. have shown that when β-lactoglobulin A is chromatographed on HIC that the multiple peak formation was due to distinct aggregates of tetramer, octamer and dodecamer formed by aggregation at the head of the column [2]. Krull et al. have also shown that a concentration detector (absorbance at 280 nm) may yield a nearly symmetrical sigmoidal shaped peak on ion exchange [3] or reversed-phase columns [4] at high protein loads, but that the LALLS-HPLC detector showed higher molecular weight aggregates eluting at the leading edge of the peak. Dollinger et al. have used right angle classical light scattering to demonstrate how light scattering is invariant to protein denaturation while size exclusion chromatography and dynamic light scattering show changes in the protein's hydrodynamic size [1]. These examples demonstrate that molecular weight information can be extremely valuable in identifying protein peaks on HPLC, particularly when distinguishing between tertiary protein conformers or aggregates. Even more importantly, classical light scattering detection for HPLC can quantitate transient protein aggregates with a lifetime comparable to that of the chromatographic elution processes, but which later dissociate during the time frame of peak collection and reanalysis.

HPLC with light scattering detection is a very powerful technique as the strength of each dimension is additive. Adsorptive HPLC recognizes the surface of a protein molecule and can readily discriminate changes to the protein's tertiary or quaternary structure. However, concentration detectors alone (such as UV absorbance) usually are insufficient to identify these structural changes. Light scattering detection yields the molecular weight of each peak and is a great help in discriminating between tertiary and quaternary structural changes. Adsorptive chromatography inherently changes the protein concentration through concentration at the head of the column and dilution during elution. Thus, quantitation of protein aggregates through their isolation is inherently invasive and may alter the aggregate equilibrium. It is possible to circumvent these protein concentration changes and perform light scattering on the sample solution directly. Classical light scattering measures the total amount of scattered light from a protein solution and is proportional to both protein concentration and the weight-averaged molecular weight of the scattering particles [5]. In the static light scattering measurement, protein concentration can also be measured off-line, in a spectrophotometer. Often, as in formulation development or stability experiments, the protein concentration is known and invariant between a series of samples and only the light scattering measurement needs to be performed to establish variation in the degree of aggregation. The weight-averaged molecular weight value which is proportional to the light scattering calculation (of specific turbidity), is extremely sensitive to changes in the larger aggregate species. Because there is no dilution of the protein mixture, the static light scattering methodology can more accurately characterize the in-solution protein aggregation than the HPLC-Light scattering combination. What is lost in the static approach is the separation of monomeric from aggregated protein species and the distribution of the protein aggregation. Similar to polymeric bulk properties, there is an infinite number of aggregate distributions which can yield an identical weight-averaged molecular weight.

This paper describes the procedure and validation of static classical light scattering analysis of protein aggregates which utilizes a standard fluorometer adapted as a right angle scatterometer. The rigorous theoretical treatment of this approach as an HPLC detector for proteins is described elsewhere [1]. However, for the mea-

surement of protein aggregation in protein formulations, the actual calibration of absolute molecular weight is not critical. Rather, a simpler calibration yielding 'specific turbidity' is described. This analysis is very useful as a comparative method to develop formulations with minimum aggregation and to establish formulation stability. Unfortunately, for proprietary reasons the identity of the therapeutic protein used in these experiments cannot be revealed. However, the therapeutic protein used is smaller than the human serum albumin (HA) used in the formulation, and the concentration of HA is in enormous excess compared to that of the therapeutic protein. For all practical purposes both the UV absorbance and light scattering output from these formulations is overwhelmingly dominated by the HA component, thus minimizing the relative importance of the identity of the therapeutic protein to this assay.

2. Theory

The intensity of scattered light can be expressed in terms of the Rayleigh ratio, R_θ, or, equivalently, in terms of turbidity, τ [5]. Turbidity can be related to the molecular properties of the particles interacting with the incident radiation by the following equation Eq. (1):

$$\frac{K}{\tau_{sp}^{\theta}} = \frac{1}{M_w P_{(\theta)}} + 2A_2C + 3A_3C^2... \quad (1)$$

where K is the optical constant, τ_{sp}^{θ} is the specific turbidity for the light scattered at angle θ, C is the weight concentration of the solute, $P_{(\theta)}$ is a size/shape scattering factor, and the expansion is in powers of C with virial coefficients. τ_{sp}^{θ} is also called the 'specific Rayleigh constant' or the 'specific Rayleigh ratio'. Since proteins are much smaller than the wavelength of light used for scattering measurements, a number of simplifications can be made over the popular method of low angle light scattering typically used for organic polymers. Adopting the three mathematical assumptions (1) that $P_{(\theta)} \approx 1$ for proteins $< 10^6$ Da, (2) that the virial expansion is negligible; and (3) that the refractive index for a class of protein formulations is constant, Eq. (1) can be simplified to:

$$\tau_{sp}^{\theta} = K\bar{M}_w \quad (2)$$

The optical constant K is a function of the refractive index of the solvent, the specific refractive index increment of the protein solution and the wavelength of light [5]. In this paper, calibration of the system was performed with the intrinsic Rayleigh scattering of toluene. Since the value of specific turbidity is proportional to the $\bar{M}_w$ of the sample, only specific turbidity (not $\bar{M}_w$) is determined which does not require the calculation of the optical constant K.

For polydisperse samples, the molecular weight is given as the weight-averaged molecular weight, $\bar{M}_w$. $\bar{M}_w$ is defined by Eq. (3):

$$\bar{M}_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} \quad (3)$$

Eq. (3) shows that since $\bar{M}_w$ is heavily weighted towards the higher mass components of the solution, τ_{sp}^{θ}, will also be heavily influenced by the more massive particles. This sensitivity to large aggregates is a desirable characteristic of an assay designed to quantitate aggregation.

For experiments described in this paper, the amount of light scattered at 90° was measured and the quantity of interest was specified as τ'_{sp} ($\tau'_{sp} = \tau_{sp}^{\theta}$ at 90°). Absolute specific turbidity of dilute protein solutions (concentrations up to 10–20 mg/ml) can be calculated using the following equation Eq. (4):

$$\tau'_{sp} = \frac{\tau'_{toluene}}{C_p} * \frac{i_{s(protein)} - i_{s(excipient)}}{i_{s(toluene)}} * \frac{n^2_{solution}}{n^2_{toluene}} \quad (4)$$

where $\tau'_{toluene}$ is the Rayleigh constant for toluene (pure solvent) and is equal to 39.6×10^{-6} cm for 488 nm light. Since $\tau'_{toluene}$ has only a weak dependence on wavelength in the visible region, its value at 488 nm can be used for measurements at 467 nm as well. As is apparent from Eq. (4), toluene was used for system calibration making it unnecessary to directly determine either i_0 or the various geometrical factors needed to calculate detector solid angle. Toluene is suited for this

purpose since its Rayleigh constant is well known and is easy to obtain toluene in clarified, stable, high purity solutions. C_p is the nominal protein concentration, i_s is the measured light scattering intensity of the protein solution, excipient, and toluene, and n is the index of refraction. The index of refraction for toluene is 1.494.

Instead of calculating or measuring the index of refraction for each protein sample, the index of refraction for water, 1.33, was used for all formulations. The refractive index, n, of a mixture is an additive function of the specific refractive index increments (dn/dc) of the individual components of the mixture weighted by their concentration. Since the buffer (25 mM) and protein (10–15 mg/ml protein or 1.0–1.5% solution) concentrations are much lower than the water concentration (~ 55 M) in these formulations, it can be safely assumed that contributions from these components will not change the index of refraction of water significantly.

The numerator in the second term of Eq. (4) is equal to the 'excess' scattering of the protein solution compared to the excipient. This procedure is similar to subtracting a 'blank' in an absorption measurement. The third term in Eq. (4) corrects for the different solution solid angles which the detector subtends for toluene and aqueous solutions. Substituting the $\tau'_{toluene}$, $n_{solution}$ and $n_{toluene}$ values into the specific turbidity equation simplifies it to:

$$\tau'_{sp} = \frac{0.0314}{C_p} * \frac{i_{s(protein)} - i_{s(excipient)}}{i_{s(toluene)}} \quad (5)$$

The absolute specific turbidity (τ'_{sp}, in cm^2/g) of a series of protein solutions, samples or formulation development samples where concentration is constant, can be determined by substituting the nominal protein concentration (C_p, in mg/ml) and the mean light scattering intensities measured ($i_{s(protein)}$, $i_{s(excipient)}$, $i_{s(toluene)}$, in cm^{-1}) into Eq. (5). The above equation was used as the working formula for calculating specific turbidity of protein formulations. The resulting units of τ'_{sp} were (cm^2/g) which relate to the normalized cross-sectional area of the scattering particles.

The turbidity of protein solutions can also be measured using nephelometry. However, since nephelometers typically use white light as the light source (tungsten lamp) and not monochromatic light and protein aggregates of different mass have different scattering relationships with wavelength, the linear relationship between turbidity and $\bar{M}_w$ expressed by Eq. (2) no longer holds, making the interpretation of data difficult.

To cross-validate the relationship of τ'_{sp} with $\bar{M}_w$ expressed by Eq. (2), gel permeation chromatography was used as an independent measure of protein aggregation. For calculating the weight-averaged molecular weight ($\bar{M}_w$) of a peak by gel permeation chromatographic analysis, the method of Dollinger et al. [1] uses the following equation:

$$\bar{M}^s_w = k\frac{I^s_s}{RI^s} \quad (6)$$

where k is a system constant, RI is the output (peak area) of the refractometer, I_s is the output (peak area) of the light scattering photometer and the subscript 's' refers to the calculation of each slice or segment in a chromatogram. The weight-averaged molecular weight of the non-resolved chromatogram can then be determined using the following equation:

$$\bar{M}_w = \frac{\sum(RI^s \bar{M}^s_w)}{\sum RI^s} \quad (7)$$

where the summation is over all the slices after and including the void volume peak to before the included volume peak.

3. Experimental

3.1. Apparatus

3.1.1. Off-line classical light scattering

The use of a conventional fluorometer as a 90° light-scattering photometer requires that (1) both emission and excitation monochromators are set to the same wavelength, (2) a continuous (not pulsed) lamp is used as the detector light source, (3) cut-off filters are placed in front of the excitation and emission beams to absorb second-order

light, (i.e. 234 nm), and (4) the wavelength of the incident light is not absorbed by the scattering particle (protein).

Off-line (static) classical light scattering analysis (turbidimetric analysis) of protein aggregates in test formulations was performed in a conventional fluorometer which was adapted as a right angle (90°) light scattering photometer. The 90° refers to the angle between the excitation and measurement directions. A Hitachi Model F2000 fluorometer with a single position thermostatted cell holder was used with a yellow, sharp cut-off glass filter (Part # 03FCG059, Melles Griot, Irvine, CA) on the excitation beam and a 1.3 OD neutral density filter (Part # 03FNG019, Melles Griot, Irvine, CA) in addition to another yellow, sharp cut-off glass filter on the emission beam. The addition of the cut-off filters prevents passage of second-order light (234 nm). Use of a single cut-off filter on the excitation beam is sufficient, however, addition of the second cut-off filter to the emission beam increases sensitivity. A neutral density filter may be added to the emission beam to minimize detector over-ranging. For most of the experiments described in this paper, both excitation and emission monochromators were set at 488 nm. A few of the experiments used 467 nm, a convenient emission maximum of the xenon lamp and the recommended wavelength for specific turbidity measurements of protein aggregation. The 467 nm setting is recommended for all proteins not absorbing at this wavelength. The F2000 was equipped with a 1 ml fluorometer cell (10 mm light path) and was thermostatted at 25 ± 1°C using a circulating water bath (B Braun Thermomix 1420) and Thermostatic Circulator (LKB2219 Multitemp II).

Turbidity experiments conducted in this laboratory over several years using different fluorometers (Hitachi F2000, Hitachi F4500 and Spex Fluorolog II double-double monochromator) proved the turbidity assay to be valid and practical for a variety of fluorometer models with a continuous lamp source. The F4500 can be adapted for 90° light scattering by setting both the emission and excitation monochromators at the emission maximum of the xenon light source, 467 nm, and by inserting a GG420 yellow, sharp cut-off glass filter (Part # 03FCG059, Melles Griot, Irvine, CA, USA) at the entrance and exit slit windows.

3.1.2. On-line HPLC right angle light scattering

A form of size-exclusion chromatography data reduction, gel permeation chromatography (GPC), was used to cross-validate the accuracy of the turbidimetric analysis of the formulation samples. This form of GPC analysis requires a light scattering detector to determine molecular weight in addition to an RI or UV detector for protein concentration. For on-line size-exclusion chromatography (SEC-HPLC) analysis of protein aggregates, the chromatographic system used consisted of a Beckman Model 126 pump, a Waters Model 712 WISP injector, an Erma Model ERC-7512 RI detector set at 30°C, and a Hitachi Model F1050 fluorometer set up as a 90° light scattering detector. Light scattering detection was performed at either 450 or 467 nm. For 467 nm detection, a borosilicate UV filter (glass slide) was used on the excitation beam as a cut-off filter. The detectors were arranged in series, with the refractometer (as the concentration detector) ahead of the light scattering detector. The column used for SEC was a 30 cmx7.5 mm I.D. TSKgel G4000SW (TosoHaas, Montgomeryville, PA) and was thermostatted at 30°C using a Timberline temperature controller (Rainin Instrument, Emeryville, CA, USA). The isocratic aqueous mobile phase was 25 mM sodium phosphate—0.2 M NH_4SO_4 (pH 6.8) at a flow rate of 0.7 ml/min. Both samples and standards were analyzed in triplicates. An injection volume of 60 μl was used for all analytes. Data collection was performed with a Waters Maxima 820 system at a rate of one point/s. Files were translated into ASCII and GPC calculations of $\bar{M}_w$ were done with Microsoft Excel.

For on-line SEC-HPLC analysis, the use of the more current Hitachi Model L7480 HPLC fluorescence detector is recommended. The L7480 can be adapted with a Y-43 cut-off filter on the excitation beam and a L-39 cut-off filter on the emission beam. The optional filter holder and filter sets (Hitachi Part # 050-0890) can be purchased from Hitachi and provide a trouble-free adaptation of the HPLC fluorometric detection to a light scattering detector.

3.2. Materials

Size-exclusion protein standards (thyroglobulin, immunoglobulin G and ovalbumin) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Sodium sulfate was obtained from Fluka (Buchs, Switzerland), ACS grade NaH_2PO_4 from Chiron Technical Services and HPLC-grade toluene and methanol from Aldrich (Milwaukee, WI, USA). Glass-distilled water was used for the preparation of all samples and solutions. Samples consisted of HA-therapeutic protein test formulations at approximately 10–15 mg/ml. Test formulations were clear but appeared golden from HA. For static light scattering measurements, vials containing 1 ml of protein formulation were pipetted directly into the fluorometer cuvette, while for on-line HPLC analysis, a 60 μl of sample was injected onto the SEC column.

3.3. Methods

3.3.1. Specific turbidity measurements

The fluorometer and the circulating water bath were switched on and allowed to warm up for approximately 30 min prior to use. The fluorometer was first calibrated with toluene, which has a known Rayleigh constant. This calibration compensates for day-to-day and instrument-to-instrument variation, yielding assay results of high precision. A fluorometer cuvette was placed in the cuvette holder and rinsed with methanol twice, then aspirated to dryness. Without removing the cuvette from the fluorometer, 1.0 ml of toluene was directly pipetted into the cuvette and temperature was allowed to stabilize for 2 min before the intensity of the scatter, $i_{s(toluene)}$, was measured three times over 10 min. Similarly, scattering intensities for the excipient and formulation samples were also determined. Between measurements, the cuvette was cleaned thoroughly with methanol, water, and the appropriate excipient to prevent cross-contamination. To increase assay precision, three replicates of each sample were assayed and the values obtained were averaged. The absolute specific turbidities of the formulation samples were then calculated by substituting the known nominal protein concentration (C_p), as determined by A_{280}, and the mean light scattering intensities measured into Eq. (5).

In this laboratory, the $i_{s(excipient)}/i_{s(toluene)}$ ratio for a series of excipients was routinely determined from the mean intensities measured during formulation development. The measured light scattering intensity for the excipient used in this report was determined to be 98% that of toluene.

3.3.2. Gel permeation chromatographic analysis

The system constant k can be obtained from well-defined protein standards of known $\bar{M}_w$ on an empirical basis [1]. The k value in Eq. (6) was calculated for each one of the nine test formulations analyzed. This was done by taking a 4-s slice at the HA peak apex of each SEC chromatographic profile (Fig. 1) and using 67 000 Da as the weight-averaged molecular weight ($\bar{M}^s_w$) for monomeric HA. To adjust for detector delay volumes, the I^s_s/RI^s ratio for the HA peak was calculated using the highest signal from a 4-s segment of each profile containing the HA peak apex.

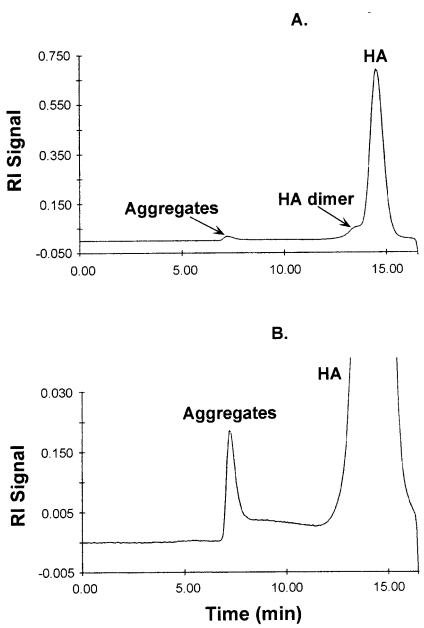

Fig. 1. SEC chromatogram of an HA-therapeutic protein test formulation. Column: TSKgel G4000SW, 30 cm × 7.5 mm I.D; mobile phase: 25 mM sodium phosphate—0.2 M NH_4SO_4 (pH 6.8); flow rate: 0.7 ml/min; detection: refractive index (RI). Panel A: normal scale; panel B: expanded scale.

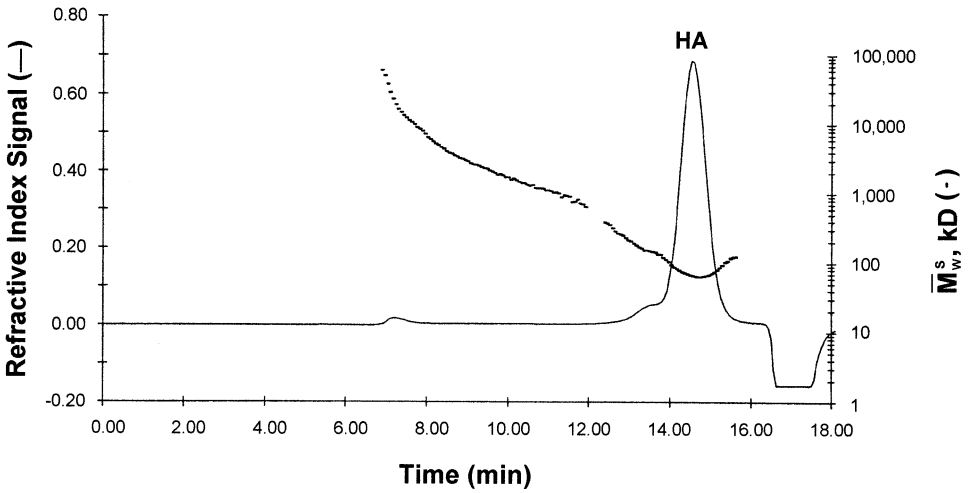

Fig. 2. Plot of weight-averaged molecular weight for each slice of the RI chromatogram from Fig. 1. The solid line is the RI signal and the dashed line is the slice weight-averaged molecular weight ($\bar{M}^s_w$).

Calculations from nine such analyses yielded consistent k values, with an R.S.D. of 0.66%.

To evaluate the accuracy of this method of calculating a system constant, the mean k value obtained from above experiments was used to determine the monomer molecular weights of three well-defined standard proteins, thyroglobulin (Tyr, 670 kD), immunoglobulin G (IgG, 158 kD) and ovalbumin (Oval, 44 kD) using Eq. (6). Sixty microliters of a protein standard mixture containing thyroglobulin, immunoglobulin G and ovalbumin was injected onto the column and SEC analyses using light scattering detection at 450 nm were performed as described under Section 3. Three replicate injections were made to generate the detector response values, I_s and RI peak areas, for each standard protein. Using the highest I_s and RI signals obtained from a 4-s segment of each profile containing the peak apex and the mean k value generated from above experiments, three $\bar{M}^s_w$ calculations were performed for each standard protein using Eq. (6). The mean $\bar{M}^s_w$ values calculated for Tyr (702 kD) and IgG (164 kD) agreed well with literature values of monomer molecular weights. However, the mean value calculated for Oval (55 kD) was higher than the literature value (44 kD), probably due to its high carbohydrate content. These results indicated that the method used for obtaining k and its application for measuring the $\bar{M}^s_w$ of known proteins is consistent and yields accurate results. Thus, the above method should be applicable for use in calculating the $\bar{M}_w$ of the protein species in test formulations.

A representative SEC chromatogram of a test formulation sample is shown in Fig. 1. To determine the weight-averaged molecular weight of each non-resolved sample chromatogram, the SEC chromatogram was divided into 4-s long segments and the area (and concentration) of the segment was assumed to be proportional to the height of the segment above a zero value baseline. The detector response ratio (I^s_s/RI^s) for each segment of the chromatogram was calculated taking into account detector delay volumes. Using HA monomer for internal calibration, the constant k was calculated for each chromatogram from Eq. (6), as described above. The weight-averaged molecular weight of each segment on a given sample chromatogram, $\bar{M}^s_w$, was then determined by substituting the calculated detector response ratio for the segment and the corresponding k value in Eq. (6). This is illustrated in Fig. 2 where the weight-averaged molecular weight of each slice is plotted along with the RI chromatogram. Next, the weight-averaged molecular weight ($\bar{M}_w$)

of each formulation sample was determined from its chromatogram using Eq. (7).

4. Results and discussion

Size-exclusion chromatography always dilutes the protein sample injected and may alter the aggregate equilibrium. Fortunately for this study, the protein aggregate distribution of the test formulations for HA and therapeutic proteins were not affected by the SEC conditions used (data not shown), allowing us to characterize the protein aggregates present in these test formulations by SEC. A representative size-exclusion chromatogram, with refractive index (RI) detection, of a HA-therapeutic protein test formulation containing relatively high concentrations of HA is shown in Fig. 1. The profile consists of a large HA peak eluting at approximately 14.5 min, an HA dimer peak at 13 min, a small aggregate peak at approximately 8 min, and a smaller amount of protein eluting across the range between these last two peaks (see enlarged profile of Fig. 1). As shown in Fig. 1, the concentration of aggregates in this formulation was quite low; 5% for the HA dimer and 4% for aggregates higher than HA dimer.

Since static classical light scattering (turbidity) is proportional to both protein concentration and the weight-averaged molecular weight of the scattering particle, the turbidity assay is sensitive to changes in this small percentage of protein which is aggregated.

4.1. Turbidity assay performance

Assay performance was evaluated using vials of protein test formulations containing relatively high concentrations of HA. Specific turbidity measurements were performed as described under Section 3.3.

4.1.1. Precision

Intra-assay, intraday and interday variability of the turbidimetric measurements were determined using a Hitachi Model F2000 fluorometer adapted as a right angle scatterometer. The intra-assay variability of the turbidity measurement for replicates of a single test formulation is presented in Table 1. Eight analyses, consisting of three replicates each, were performed and the average intensity, standard deviation (S.D.) and relative standard deviations (R.S.D.) were calculated for each set of three replicates. The results showed intra-assay R.S.D. values ranging from 0.3 to 3.7% with a mean of 1.3%. This mean R.S.D. value includes fluctuations inherent in vial-to-vial aggregation differences.

Intraday precision of the turbidity measurement was also determined for repeated analyses of the same test formulation in a single day. Eight tests consisting of three replicates each were performed in a single day and the specific turbidity was calculated for each assay. The data from this experiment are also presented in Table 1. The specific turbidity measurements varied from 0.57 to 0.59 cm^2/g with a mean value of 0.58 cm^2/g and an intraday R.S.D. of 1.2% for the eight assays.

Interday precision was evaluated through replicate analyses of the same test formulation on different days. Consistent results were obtained for twelve interday analyses of a single test formulation with an interday R.S.D. of 2.0% (Table 2). As expected, there was slightly higher variation within interday analyses compared to the intraday analyses. These results, taken together, show excellent precision for the turbidity assay.

Table 1
Intraday and intra-assay precision for replicate analyses of a single HA-therapeutic protein test formulation

Sample	Specific turbidity (cm^2/g)	% R.S.D. ($n = 3$)
1	0.59	0.3
2	0.57	0.3
3	0.58	3.7
4	0.58	1.6
5	0.58	1.4
6	0.57	2.5
7	0.58	1.8
8	0.57	2.1
Mean:	0.58	1.3
R.S.D.:	1.2%	—

Table 2
Interday precision for twelve analyses of a single HA-therapeutic protein test formulation

Date	Specific turbidity (cm^2/g)
June 2	0.56
June 3	0.57
June 4	0.56
June 7	0.57
June 8	0.59
June 9	0.57
June 11	0.57
June 14	0.55
June 21	0.59
June 23	0.58
June 28	0.57
July 8	0.57
Mean:	0.57
R.S.D.:	2.0%

While performing the interday precision experiments, a new xenon lamp was installed in the fluorometer. To prevent the increased output intensity from over-ranging the emission PMT, an additional 0.3 OD neutral density filter was added to the emission beam. To determine the constancy in calculation of specific turbidity using different amount neutral density filters on the emission beam, experiments were performed to measure the scattering intensities of two test protein formulations using a range of neutral density filters, from 1.0 OD to 1.6 OD. Toluene intensity was also determined with each filter set so that specific turbidity could be calculated for each of the various filter combinations. Within each test formulation, consistent results were obtained using the eight different filter combinations. For test formulation E, the specific turbidity measurements varied from 0.278 to 0.305 cm^2/g with a mean value of 0.298 cm^2/g, R.S.D. = 3.2%. For test formulation G, the specific turbidities ranged from 0.141 to 0.144 cm^2/g with a mean value of 0.144 cm^2/g, R.S.D. = 1.5%. These results demonstrate that as long as the PMT is not overranging, the measurement of specific turbidity is independent of the intensity of the incident light; this variation is compensated for by the toluene reference. Based on these data, a neutral density filter of 1.3 OD was used for turbidity measurements of test formulations.

4.2. Inter-laboratory ruggedness

The inter-laboratory ruggedness of this assay was evaluated by comparing results of assays performed by three different laboratories on the same eight test formulations. Turbidity measurements in each laboratory were performed using a different fluorometer (Labs. 1, 2, and 3 used Hitachi Models F2000, F4500 and F2000, respectively). The inter-laboratory results for a given test formulation generated R.S.D. values ranging from 0.0 to 5.4%, with a mean R.S.D. of 2.3% for eight test formulations (Table 3). These results establish good correspondence among the turbidity data form the three laboratories, demonstrating the excellent laboratory-to-laboratory ruggedness of the assay.

4.3. Linearity

To test if the turbidity assay is linear over the range of specific turbidities that one can expect to encounter, linearity experiments were performed using mixtures of two test formulations; formulation O which was the most turbid and formula-

Table 3
Inter-laboratory ruggedness for eight HA-therapeutic protein test formulations analyzed in three different laboratories

Test formulation	Specific turbidity (cm^2/g)			% R.S.D.
	Lab. 1	Lab. 2	Lab. 3	
A	0.57	0.58	0.63[a]	5.4
B	0.58[b]	0.59	0.63	4.4
C	0.35	0.35	0.36	1.6
D	0.34	0.34	0.35	1.7
E	0.24	0.24	0.24	0.0
F	0.11	0.11	0.11	0.0
G	0.12	0.12	0.12	0.0
H	0.11	0.11	0.10	5.4
Mean:				2.3

[a]Mean of 6 assays.
[b]Mean of 8 assays.

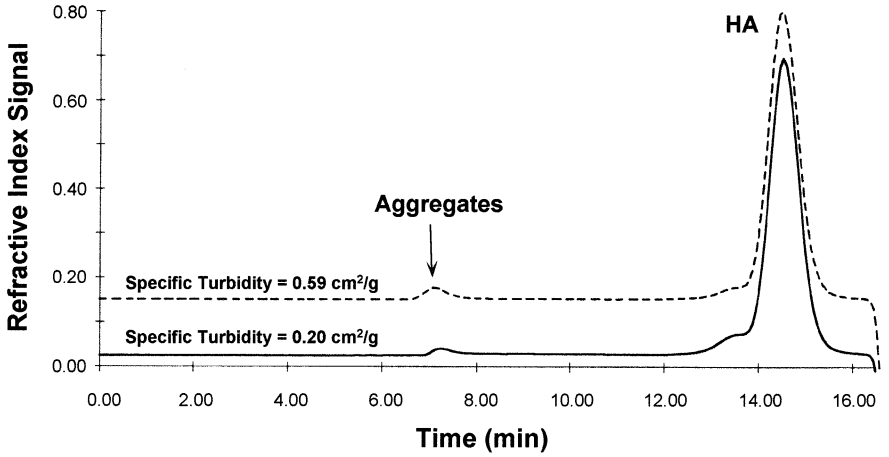

Fig. 3. SEC-HPLC analysis of two HA-therapeutic protein test formulation samples with different specific turbidities using refractive index (RI) detection. The solid and dashed lines represent the signals from low- and high-turbidity formulations, respectively.

tion H which was the least turbid test sample of that formulation assayed at that date. Measured amounts of test formulation O (turbidity value of 0.62 cm^2/g) were added to measured amounts of low turbidity formulation H (turbidity value of 0.11 cm^2/g) in the ratios of 1:0, 1:3, 1:1, 3:1 and 0:1. The specific turbidity values of the mixtures were then measured and compared to the expected turbidity values based on the measured specific turbidity of the two formulations and the assumption that turbidities are additive. The measured specific turbidity was plotted against the expected value for each sample, which resulted in a straight line with a slope of 0.995 and a correlation coefficient (R) of 0.998. These results demonstrate the linearity over the range of observed specific turbidities (0.1–0.6 cm^2/g) and the excellent correlation of measured turbidity with the predicted values. These results also show that changes in protein aggregation can be reliably detected by this assay.

4.4. Comparison of turbidity to SEC analysis

To compare the turbidity assay with SEC for detecting changes in protein aggregation, two test formulations with proportionally different measured specific turbidities (0.2 vs. 0.59 cm^2/g) were analyzed by classical light-scattering SEC using an RI detector for protein concentration and a 90° light scattering detector for determining molecular weight and the results obtained were compared with the measured turbidities. Fig. 3 compares the SEC chromatograms of the high and low turbidity test formulations. The upper RI trace (specific turbidity = 0.59 cm^2/g) has more aggregate (3.1 area%) at the void volume (7 min) than that of the lower trace (specific turbidity = 0.20 cm^2/g, aggregate = 1.7 area%). However, both the light scattering SEC analysis and turbidity analysis show nearly a threefold increase due to the large increase in mass, as opposed to refractive index concentration, of protein aggregates at the column void volume (Fig. 4). This example shows that even a modest change in concentration of protein aggregates, as seen by traditional SEC, is reflected a dramatic change in the specific turbidity since the mass of protein aggregates at the void volume is much larger. In addition, the high degree of precision of the turbidimetric analysis (R.S.D. = 1–2%) assures that extremely subtle changes in protein aggregate distribution will be reliably detected. These results demonstrate that traditional SEC, as performed with a single concentration detector (RI or UV), is not as sensitive as classical light scattering in detecting changes in larger protein aggregates.

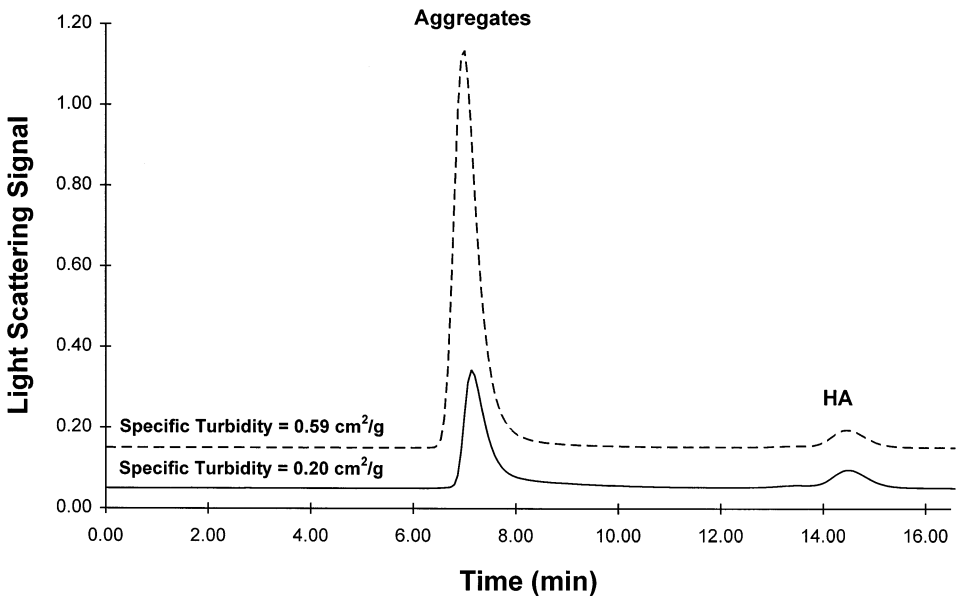

Fig. 4. SEC-HPLC analysis of two HA-therapeutic protein test formulation samples with different specific turbidities using right-angle light scattering detection. The lines are the same as defined in Fig. 3.

4.5. Comparison of turbidity to GPC analysis

To establish the appropriateness of the turbidity assay for measuring protein aggregation, nine different HA-therapeutic protein test formulation preparations were analyzed by the turbidity assay and also by GPC analysis, as described under Section 3, and the turbidity results obtained were compared to those of GPC. The method of Dollinger et al. [1] was modified to determine the weight-averaged molecular weight ($\bar{M}_w$) of the chromatogram. This characterization of a continuous polymeric distribution yields a single value for $\bar{M}_w$ which is extremely sensitive to changes in distribution of the larger species. Using HA monomer for internal calibration, a standard GPC calculation was made on each non-resolved sample chromatogram, as described under Section 3.3. Eq. (6) and Eq. (7) were used to calculate the weight-averaged molecular weight of the species in nine test formulations. Each test formulation was analyzed in triplicate and the $\bar{M}_w$ values obtained were averaged. The mean $\bar{M}_w$ values ranged from 397 to 1086 kD between formulations, with a mean R.S.D. of 4.2% for the triplicate analyses.

The same nine test formulations were also analyzed in the turbidity assay and the accuracy of the turbidity assay was assessed by comparing turbidity results to those of GPC. The comparison of GPC-derived $\bar{M}_w$ and measured specific turbidity values for the nine test formulations is shown in Fig. 5. Plotted against each other, the two sets of results yielded a straight line with a slope of 1651, an R^2-value of 0.9838 and a y-intercept of 70704. The extrapolated value for the y-intercept is consistent with the $\bar{M}_w$ of monomeric HA (67 000 Da) at infinite dilution. Since turbidity is proportional to both protein concentration and the weight-averaged molecular weight of the scattering particles, the sensitivity of the analysis will be a product of these two variables. Thus, the specific turbidity value obtained from very dilute solutions will approach zero, limiting the sensitivity of the method to protein solutions of moderate concentration or to dilute protein samples of larger molecular weight. These data of Fig. 5 demonstrate that the GPC calculation correlates linearly with specific turbidity, as expected from Eq. (7). This relationship provides additional vali-

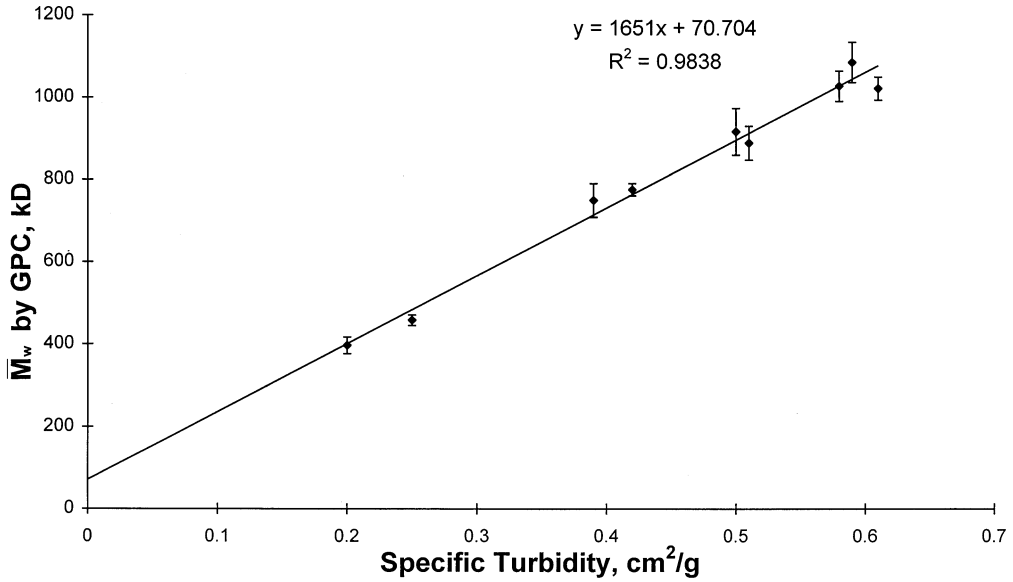

Fig. 5. Comparison of measured specific turbidity with GPC-derived $\bar{M}_w$.

dation to the suitability of specific turbidity for measuring protein aggregation in formulated protein.

4.6. Effect of sample centrifugation and filtration on specific turbidity

Because of the experimental relationship of scattered light to particle mass, the turbidity measurement can be dramatically affected by the presence of even minute amounts of particles with dimensions much larger than that of the bulk of the protein in solution. These particulates (1 μm) of dust or precipitated protein aggregates can be removed from protein solutions by either filtration or centrifugation. Since large particulates can invalidate the specific turbidity measurement, experimental technique must carefully exclude particulate contamination. This can be accomplished by rinsing both the inner and outer walls of the cuvettes and the glassware used for the transfer of solutions with filtered solvents and making the turbidity measurements immediately after sample clarification.

If the protein solution to be measured contains visible aggregates which constitute only a very small portion ($< 1\%$) of the sample and monitoring changes in the bulk of the sample ($> 99\%$) is important, then either filtration or centrifugation can be used for sample clarification. In this case, the decrease in protein concentration after sample filtration (or centrifugation) will be imperceptible but still the measured turbidity will change considerably because even small quantities of very large particles affect the intensity of scattered light appreciably. These visible protein aggregates with dimensions larger than that of the filter pore size will also be lost in SEC via filtration by the HPLC support.

If the visible aggregates that are removed through filtration (or centrifugation) are a significant portion of the sample, the protein concentration of the remaining solution will have to be determined. The amount of protein that is removed by filtration or centrifugation can be determined from the difference in absorption at 280 nm between an unfiltered (or unspun) aliquot and the supernatant of a filtered (or spun) sample.

Alternatively, protein concentrations of the untreated and treated sample aliquots can be determined by measuring the intrinsic tryptophan fluorescence of the protein at 340 nm after excitation at 280 nm.

Sample turbidity can also decrease markedly upon filtration if a portion of the protein is retained by the filter through non-specific adsorption. Such non-specific adsorption loss of protein was not observed with HA-therapeutic protein test formulations filtered through 0.2 μm Acrodisc filters. Absorbance measurements (A_{280}) before and after filtration revealed $<$ 1% change in sample protein concentration. Centrifugation was also evaluated as a non-adsorptive approach to sample clarification. To test the effect of a short centrifugation ($14\,000 \times g$ for 3 min) on measured specific turbidity, twenty test protein formulations were analyzed by the turbidity assay, both with and without inclusion of the centrifugation step, prior to the actual turbidity measurement. The specific turbidity values obtained for unspun formulations ranged from 0.11 to 0.61 cm^2/g with a mean specific turbidity value of 0.35 cm^2/g. The calculated specific turbidity ratios (unspun $\div$ spun) ranged from 1.00 to 1.11 with a mean ratio of 1.03, R.S.D. = 3.3%. Since the turbidity results were not significantly affected by centrifugation, the sample centrifugation step was not deemed necessary for these test formulations.

As a general rule, when starting with a new test formulation, the most adequate clarification technique should be established by a systematic comparison of results obtained using different clarification procedures. Since the assumptions in the basic Eq. (1) for light scattering from dilute solutions do not hold for large particles ($> 10^6$ Daltons or $> \lambda/20$) if the scattering angle $\theta \neq 0°$, precise calculation of their contribution to the turbidity measurement is problematic. However, determining the change in specific turbidity and protein concentration before and after sample treatment will provide the researcher with an evaluation in the utility of the selected clarification procedure.

5. Conclusions

This paper describes the procedure and validation of the static classical light scattering assay for determining the degree of non-covalent aggregation in protein formulations using a standard fluorometer adapted as a right angle scatterometer. Adaptation of a fluorometer as a scatterometer is easy and inexpensive. The only fluorometer modifications required are two yellow filters, to eliminate second order light, and a neutral density filter for concentrated or heavily aggregated solutions. Protein solutions are pipetted directly into a standard fluorometer cuvette. Instrument calibration is performed with toluene, a well-characterized scatterer, and provides a very rugged method, invariant to fluorometer type. Since static classical light scattering measures the total amount of scattered light from a protein solution, it is proportional to both protein concentration and the weight-averaged molecular weight ($\bar{M}_w$) of the scattering particle. This convenient and quantitative analysis determines specific turbidity which is proportional to $\bar{M}_w$ and has the advantage of being non-invasive and much more rapid than the on-line HPLC method. It is particularly useful to optimize formulation development in stability studies. It can also be used to characterize in-process purification steps, determine the stability of protein formulations and as a QC release assay of formulated proteins.

The turbidity assay has been shown to be extremely rapid, rugged and precise. Through cross-validation to traditional GPC analysis of protein aggregate distributions, the assay has also been shown to be accurate. The high degree of precision of the turbidity measurements allows this method to sensitively and reliably detect changes in protein aggregate distribution. One limitation of the method is that the turbidity measurement can be dramatically affected by the presence of few very large aggregates and non-specific particulate matter (i.e. dust) in solution. For these specific test formulations studied, the use of a sample clarification step prior to the actual turbidity measurement was not found necessary. However, if visible precipitation occurs, sample clarification by centrifugation or filtration should be investigated.

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