Research Article

Theme: Aggregation and Interactions of Therapeutic Proteins

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Analyzing Subvisible Particles in Protein Drug Products: a Comparison of Dynamic Light Scattering (DLS) and Resonant Mass Measurement (RMM)

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Abstract. Aggregation is common in protein drug manufacture, and while the effects of protein particulates are under investigation, many techniques applicable for their characterization have been recently developed. Among the methods available to characterize and quantify protein aggregates, none is applicable over the full size range and different methods often give conflicting results. The studies presented here compare two such methods: dynamic light scattering (DLS) and resonant mass measurement (RMM). The performance of each method was first characterized using polystyrene particle size standards (20, 60, 100, 200, 400, and 1,000 nm) over a range of concentrations. Standard particles were measured both singly and in binary mixtures containing 20 nm particles at a fixed concentration (10^{14} particles/mL) and various concentrations of one of the other particle sizes (i.e., 60, 100, 200, 400, or 1,000 nm). DLS and RMM were then used to detect unknown aggregate content in stressed samples of IgG. Both instruments were shown to have a working range that depends on particle size and concentration. In binary mixtures and polydisperse solutions, DLS was able to resolve two species in a manner dependent on both concentration and particle size. RMM was able to resolve particles above 200 nm (150 nm for protein) at concentrations below 10^9 particles/mL. In addition, dilution was evaluated as a technique to confirm and quantify the number of particles in solution.

KEY WORDS: aggregates; biologics; dynamic light scattering (DLS); protein drugs; resonant mass measurement (RMM); subvisible particles.

INTRODUCTION

Detecting and quantifying subvisible particles in protein drug products is critical to ensuring the quality of this rapidly growing class of drugs. Though various kinds of particulate impurities may be present, protein aggregates are a significant concern since they may increase the potential for life-threatening immediate or delayed immune reactions and may reduce or even eliminate the effectiveness of the product (1–3). Current limitations in the analytical methods used to detect subvisible particles make meaningful measurements difficult and different methods often give conflicting results (4). In addition, the aggregate properties (e.g., size, morphology, surface chemistry) responsible for adverse immune responses are not known, so it is unclear which aggregates, if any, are “bad” and thus should be the focus of analytical attention (5–7). There also is a lack of clarity regarding the

integration of data from recently developed methods with that from more traditional, well-established techniques. Such limitations slow product development and complicate manufacturing and quality control for these important drugs (7).

In the studies presented here, we evaluated and compared two methods for analyzing particulates in the subvisible range, which recently has been described as a “gap range” for aggregates in protein drug products (8): dynamic light scattering (DLS) and resonant mass measurement (RMM). Biologics manufacturers are using DLS to characterize their products because the method is easy to perform, well-established and inexpensive, though DLS results can be difficult to interpret (9). RMM is a new method, which operates in a similar size range and can be considered complementary to DLS. DLS uses the scattering of light by particles in a fluid to measure their diffusion coefficients and hydrodynamic radius, detecting particles with hydrodynamic diameter of 0.3 nm to 10 μ m but without the ability to measure particle concentration (10). In RMM, a fluid containing the particles of interest flows through a microchannel. The microchannel is embedded in a resonator which resonates at a specific frequency. When a particle enters the channel, the resonant frequency shifts; this shift in frequency is used to measure particle mass with high precision (11). RMM can quantify both particle size and

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concentration, and detects 50 nm–5 μ m particles, depending upon their density.

To assess and compare the two methods, instrument performance was first evaluated using polystyrene particle size standards (20–1,000 nm), both singly and in binary mixtures. These studies established the working range for these standards for each instrument and provided information on the resolution of different species in mixtures and polydisperse samples. Partially aggregated IgG samples at low (5 mg/mL) and high (100 mg/mL) concentration were also analyzed, serving as examples of protein drug products with unknown aggregate content. Throughout, we studied the effects of sample dilution on instrument response and accuracy. The results provide a side-by-side comparison of the capabilities of the two instruments.

MATERIALS AND METHODS

Materials

NIST-traceable polystyrene particle standards (20, 60, 100, 200, 400, and 1,000 nm) were purchased from Thermo Scientific (Fremont, CA). Sodium chloride was obtained from Sigma-Aldrich Co. LLC (St. Louis, MO). Potassium chloride, dibasic sodium phosphate, and monobasic potassium phosphate were obtained from VWR International (Bridgeport, NJ). Deionized water (18 M Ω) was used and was obtained in-house. Disposable cuvettes and sterile 0.2 μ m Acrodisc® syringe filters with low protein binding were purchased from VWR. Human immunoglobulin G (IgG) from pooled plasma was purchased from Sigma-Aldrich (product # 14506). 100 mg/mL commercial IgG solution in glycine buffer (pH 4) was supplied by Dr. Scott's laboratory in the Center for Biologics Evaluation and Research (CBER, FDA) and used as received.

Methods

Particle size was determined by DLS with 90° detection optics using a Zetasizer ZS90 (Malvern Instruments Ltd., Westborough, MA). Instrument performance was first evaluated using particle standards, singly and as binary mixtures. Particle size standards (20, 60, 100, 200, 400, and 1,000 nm), supplied in solution by the manufacturer, were diluted serially by volume using 10 mM freshly prepared and filtered NaCl solution, prepared using 0.2 μ m filters. Serial dilutions for all particle sizes were carried out and analyzed until particles were no longer detected. The samples were analyzed using the automatic mode (A), the automatic extended mode (AE), and the manual mode (M) settings of the instrument. The Z-average diameter, polydispersity index (PDI), and quality report generated by the instrument were analyzed for each particle standard to obtain a working range in terms of the number of particles/mL for each particle size. Samples were considered to be in the working range if: (i) the measured Z-average diameter at a given concentration was not significantly different than the nominal size reported by the manufacturer and (ii) the PDI value was <0.2, consistent with a monodisperse particle size distribution (12). In addition, the instrument quality report, which specifies instrument parameters such as signal counts and attenuation factor, was

considered as a categorical variable in defining the working range. In order to evaluate the repeatability of DLS, three measurements were performed for each particle size for all concentrations studied.

Particles were also analyzed in a similar fashion using a RMM system (Archimedes Particle Metrology System, Affinity Biosensors, Santa Barbara, CA) with a Hi-Q nanosensor. Polystyrene particles with sizes of 200 nm and below were not detected by RMM, so studies were performed on 400 and 1,000 nm particle size standards only. As with DLS, instrument performance was characterized over a range of sample dilutions. The size and concentration of the monodisperse particles in each solution were determined. Percent coincidence, which is defined as a measure of the frequency with which two particles pass before the sensor at the same time and thus are counted as one, was noted for all samples. In order to evaluate the repeatability of RMM, three measurements were performed for 400 and 1,000 nm samples at each of three different concentrations (10^9 – 10^7 particles/mL).

To assess the ability of DLS and RMM to detect large particles (e.g., aggregates) in the presence of smaller particles (e.g., monomeric protein), binary mixtures of standard particles were prepared and analyzed. Each mixture contained 20 nm particles at a fixed concentration (10^{14} particles/mL) and one of the other particle sizes (i.e., 60, 100, 200, 400, or 1,000 nm). A number of samples were prepared with different concentrations of the larger particles in order to cover the working range. The ability of the Zetasizer to detect and resolve the two particle sizes was evaluated using M, A, and AE modes.

To evaluate the performance of the two instruments for proteins, solutions of IgG were subjected to thermal treatment to produce varying aggregate content. Freeze-dried IgG, as received from the vendor, was dissolved in freshly prepared PBS buffer (10 mM, pH 7.4) and dialyzed with PBS overnight to remove impurities and unknown additives. The dialyzed solution was filtered through a 0.2 μ m filter and the concentration adjusted to 5 mg/mL. As preliminary studies have shown that aggregation can be induced *via* thermal stress, these solutions were then kept at 60°C for 2, 8, or 24 h to produce different aggregate content. In DLS analysis, the aggregated samples were then analyzed at reduced temperature (4°C) to inhibit further aggregation. Since the RMM instrument lacks temperature control, RMM analysis was conducted at ambient temperature. In both methods, the samples were diluted serially using the same buffer (PBS) and measured until the larger particles were not detected. A more concentrated IgG solution (100 mg/mL) was examined similarly, using dilutions with 25 mM glycine buffer (pH 4.2).

Viscosity measurements of protein solutions at various dilutions were performed using a Cannon-Manning Semi-Micro Viscometer (Cannon Instrument Co., State College, PA). In order to measure the viscosity of the protein solutions, 0.5 mL of sample was introduced into the wide arm of the viscometer and equilibrated for 5 min in a heated bath set at 25°C. The time (in second) required for the sample to flow between two preset points was noted and converted into viscosity using the calibration formula provided by the company. Each sample was measured three times to obtain a mean value of viscosity. Z-average diameters measured by DLS can be affected by viscosity. With the exception of

100 mg/mL IgG sample, the viscosities measured here were similar to that of buffer, so viscosity correction was not applied for any sample. Viscosity corrected values for the 100 mg/mL sample are noted, however.

Both DLS and RMM characterize particles irrespective of their nature and cannot differentiate between protein aggregates and foreign particles that may have been introduced during protein purification (e.g., by filters) or during the preparation of samples for analysis. Hereinafter, all particles observed in IgG samples will be referred to as aggregates for simplicity. In addition, when 10 mM NaCl solution, PBS, and glycine buffer were analyzed as blanks using both DLS and RMM, no particles were observed (data not shown). All samples were covered during analysis to minimize entry of any airborne particulates.

RESULTS

Particle Size Standards, Single Particle Size

DLS. For the particle size standards, the ability of DLS to accurately measure particle size varied with concentration (Fig. 1). For the 20 nm standard particles, DLS was able to accurately detect particle concentrations 10^{15} – 10^{13} particles/mL, and for these concentrations, the Z-average diameter was in good agreement with the particle diameter reported by the manufacturer (Figs. 1a and 2a). At concentrations of 20 nm particles $<10^{13}$ particles/mL, DLS showed a bimodal distribution of particle sizes and the Z-average diameter was greater than the true particle size (Figs. 1(a4) and 2a). For the 1,000 nm particles, the Z-average diameter was significantly less than the actual particle size at the highest concentration (10^{10} particles/mL) (Figs. 1(B1) and 2b). At a concentration of 10^8 particles/mL, the size of the 1,000-nm particles was correctly estimated (Fig. 2b). On further dilution to 10^7 particles/mL, the particle size was overestimated. Similar behavior was observed for all six particle size standards: at high concentrations, particle size was underestimated, while at low concentrations, particle size was overestimated (Fig. 2a, b). Values of the PDI were also affected by concentration for the particle size standards. At high concentrations for which the particle size was underestimated, polydispersity remained within the acceptable range (<0.2). At low concentrations for which particle size was overestimated, the PDI value was also overestimated. This effect was greater for larger particle sizes. Since different modes of operation did not have a significant influence on the measured particle size or PDI (data not shown), AE was used for subsequent analyses.

The pattern of underestimation of particle size at high concentration and overestimation at low concentration shows a dependence on the observed Z-average diameter. We defined the “working range” for DLS as the range of concentrations in which: (i) the observed Z-average diameter was equivalent to the actual size, (ii) the PDI values were less than 0.2 and (iii) acceptable count rate and instrument quality report were returned by the instrument (Fig. 2c). The working range depends on both particle size and particle concentration. As the particle size increased, the working range decreased with regard to both the breadth of the range and its upper and lower boundaries (Fig. 2c).

RMM. The measurement of particle size by RMM is based on the difference in density between the particle and the dispersion medium. The smallest size that can be detected depends on this difference in densities. Polystyrene particles have a density of 1.05 g/mL which is very close to the density of NaCl solution (0.99 g/mL, as measured by the instrument). The minimum measurable size for polystyrene (PS) particles as determined by the instrument was 220 nm. Thus, only the 400 and 1,000 nm PS particles were analyzed by RMM. Figure 3 shows the size distributions obtained for these two particle sizes. For 400 nm standards with a concentration of 10^9 particles/mL, the observed concentration was 3.5×10^8 particles/mL with 8% coincidence (Fig. 3(a1)). Upon 10-fold dilution, the observed concentration as reported by the instrument was 6.3×10^7 particles/mL with 4% coincidence (Fig. 3(a2)). When the same solution was diluted to a theoretical concentration of 10^7 particles/mL, the observed concentration was 8.9×10^6 particles/mL with 2% coincidence (Fig. 3(a3)). Similarly, when 10^9 , 10^8 , and 10^7 particles/mL samples of the 1,000 nm particles were analyzed, the observed concentrations were 5.5×10^8 , 5.9×10^7 , and 7.5×10^6 particles/mL and 25, 4, and 7% coincidence, respectively (Fig. 3b). The instrument was unable to accurately analyze 400 and 1,000 nm samples at concentrations $>10^9$ particles/mL. This shows that there is a concentration limit of $\sim 10^9$ particles/mL under these conditions, which is consistent with the instrument specifications. RMM was able to accurately estimate particle size for 400 and 1,000 nm particles at all concentrations $<10^9$ particles/mL, though the measurement time increased with decreasing particle concentration. For RMM, the working range can be defined based on the acceptable analysis time. Here, we restricted the analysis time to ≤ 30 min. With this constraint, the working range was 10^9 – 10^7 particles/mL for both 400 and 1,000 nm particles.

To evaluate repeatability, triplicate measurements were performed with both DLS and RMM and the mean and percent coefficient of variation (% CV) were calculated. The mean and % CV of replicate measurements obtained from RMM are compared with those from DLS in Table I. The % CV values are less than 2% for DLS and less than 0.5% for RMM, indicating good repeatability for both instruments. For RMM, measured concentrations also had % CV values less than 6%; percent coincidence values had % CV values of $\sim 0.01\%$ (data not shown).

Particle Size Standards, Binary Mixtures

DLS. Subvisible aggregates in protein drug products often present as a small number of large particles in a solution of a far greater number of small particles (i.e., native protein). To assess the ability of DLS to detect large particles in the presence of smaller ones, binary mixtures were prepared by adding different concentrations of 60, 100, 200, 400 and 1,000 nm standard particles to a solution of 20 nm standard particles. The 20 nm particles were kept at fixed concentration of 10^{14} particles/mL to represent protein monomer and larger particles representing protein aggregate of varying sizes were added over a range of concentrations spanning the working range. DLS was not

Fig. 1. Representative dynamic light scattering (DLS) traces for polystyrene (PS) particle size standards analyzed using AE mode as a function of dilution. 20 nm particles at 10^{15} (a1), 10^{14} (a2), 10^{13} (a3), and 10^{12} (a4) particles/mL. 1,000 nm particles at 10^{10} (b1), 10^9 (b2), 10^8 (b3), 10^7 (b4), and 10^6 (b5) particles/mL. (Note that the scale of y-axis is not identical among the sections and has been adjusted to allow comparison of peaks)

able to completely resolve 20 nm particles from particles <200 nm (i.e., 60 and 100 nm) (Fig. 4a, b), with the exception of a single concentration of 100 nm particles (Fig. 4(b3)), but was able to resolve 20 nm particles from particles with diameters ≥ 200 nm (e.g., Fig. 4c). This was consistent across all modes of measurement. In a mixture containing 20 nm particles at a concentration of 10^{14} particles/mL and 60 nm particles at a concentration of 10^{13} particles/mL, DLS was unable to resolve the two particles and only one peak at ~ 60 nm was observed (Fig. 4(a1)). As the 60 nm particle concentration decreased from 10^{13} to 10^{10} particles/mL, this peak shifted from 60 to 20 nm (Fig. 4(a4)). The 20 and 60 nm particles were not resolved at any concentration studied; a single peak was observed that varied from 20 to 60 nm Z-average diameter. Resolution was achieved for mixtures of 20 nm particles with particles ≥ 200 nm at concentrations within the working range of the larger particle (with the exception of 1,000 nm). For example, in a binary mixture containing 10^{10} particles/mL of 1,000 nm particles, DLS was unable to accurately determine the size of either particle and instead showed a peak at 10 nm (Fig. 4c) as expected because the concentration for 1,000 nm particles was out of range (Fig. 1b). As the concentration of 1,000 nm particles was

decreased to 10^9 and then to 10^8 particles/mL, DLS was able to detect only one peak at $\sim 1,000$ nm. At a concentration of 1,000 nm particles of 10^7 particles/mL, both 20 and 1,000 nm peaks were resolved. (Note that 10^7 particles/mL is out of the established range for 1,000 nm particles analyzed alone.) DLS was no longer able to detect 1,000 nm particles at concentrations $<10^6$ particles/mL and showed only a single peak at 20 nm (Fig. 4c).

RMM. In RMM, the size of each particle is measured individually as a result of the change in resonance due to the presence of the particle. Unlike DLS and spectroscopic methods, the measurement is not influenced by other particles in solution, so resolution of different species typically is not an issue (11). However, with concentrated samples, there is a possibility of more than one particle entering the resonator channel resulting in larger % coincidence values and higher particle size. When a mixture containing 10^{14} particles/mL of 20 nm and different concentrations of 400 nm particles (10^9 – 10^7 particles/mL) was analyzed using RMM, only a 400 nm peak was observed and the observed concentration was in good agreement with the actual concentration at all dilutions. The measured

Fig. 2. Concentration dependence of particle size and working range for polystyrene (PS) particle size standards as measured by dynamic light scattering (DLS). **a** Concentration dependence of particle size in the range: 20–200 nm. **b** Concentration dependence of particle size, 400 and 1,000 nm. **c** Working range for PS standard particles using DLS (bars) and RMM working range for polystyrene particles (cross-hatched area) and for protein (diagonal-slashed area); see text for discussion

particle size distribution, measured particle concentration, and average particle size did not change from those obtained in the monodisperse solution of 400 nm particles (data not shown). This indicates that particles below the limit of detection have no effect on the measurement of other particles, as expected. Similar results were obtained for mixtures of 200 and 1,000 nm particles.

IgG Samples, 5 mg/mL

DLS. 5 mg/mL samples of IgG in PBS buffer (pH 7.4) were analyzed after thermal treatment at 60°C. The effect of dilution on the ability of DLS to detect aggregates was also studied. An initial sample was analyzed by DLS to characterize the unstressed protein, which was detected at a Z-

average diameter of 10 nm. Following 10-fold dilution of the unstressed protein sample, no signal was observed by DLS, suggesting that the particle count was below the working range (data not shown). The viscosity of the unstressed protein sample was 0.979 ± 0.005 cP, approximately equal to that of water (~ 0.89 cP). After 2 h of thermal treatment, peaks were detected at 20, 60, and $>1,000$ nm (Fig. 5(a1)), indicating the presence of dimers and/or higher order aggregates. The monomer peak was not observed in these samples, which may be due to lack of resolution. Upon 10-fold dilution of this sample with PBS, the peak position did not change for peaks at 20 and 60 nm while the peak at over 1,000 nm disappeared (Fig. 5(a2)). This suggests that the population of high molecular weight species was below its limit of detection following dilution or that these species dissociate during dilution. Conversely, that the 20 and 60 nm peaks are detected following dilution suggests that the

Fig. 3. Representative particle size distributions of 400 nm (a) and 1,000 nm (b) polystyrene (PS) particles determined by resonant mass measurement (RMM). 400 nm particles at 10^9 (a1), 10^8 (a2), and 10^7 (a3) particles/mL and 1,000 nm particles at 10^9 (b1), 10^8 (b2), and 10^7 (b3) particles/mL

concentration of these species remained within their working ranges and that any dissociation that may have occurred was incomplete. Further dilution did not produce a signal at any particle size, indicating that all particle concentrations are below the working range or that complete dissociation has occurred (data not shown). The observed viscosity of the 2 h-stressed 5 mg/mL IgG sample prior to dilution was $0.970 \pm$

0.002 cP, and after 10-fold dilution was 0.938 ± 0.002 cP; both values are comparable to the viscosity prior to thermal stress.

After 8 h of thermal treatment, two unresolved peaks at 110 and at 60 nm were observed (Fig. 5(b1)). Upon 10-fold dilution, the peak with highest intensity remained at 110 nm, the shoulder at 60 nm was not detected, and a new peak appeared at 30 nm (Fig. 5(b2)). When the sample was again

Table I. Repeatability of Particle Size Measurements by Dynamic Light Scattering (DLS) and Resonant Mass Measurement, Using Particle Size Standards at Concentrations Within the Working Range

	DLS ($n=3$)		RMM ($n=3$)	
	Mean Z ave. diameter (nm)	% CV	Mean (nm)	% CV
20 nm (10^{14} #/mL)	20.9	0.24	—	—
60 nm (10^{12} #/mL)	62.8	0.19	—	—
100 nm (10^{11} #/mL)	103	0.01	—	—
200 nm (10^9 #/mL)	200.7	0.47	—	—
400 nm (10^8 #/mL)	391.9	0.81	404.3	0.27
1000 nm (10^8 #/mL)	872.2	1.11	1,028.5	0.30

Fig. 4. Binary mixtures of polystyrene (PS) particle size standards as analyzed by dynamic light scattering (DLS). **a** Representative DLS traces for mixtures of 20 nm particles (fixed at 10^{14} particles/mL) and various concentrations of 60 nm particles; *a1*— 10^{13} ; *a2*— 10^{12} ; *a3*— 10^{11} ; *a4*— 10^{10} particles/mL. **b** Representative DLS traces for mixtures of 20 nm (fixed at 10^{14} particles/mL) and various concentrations of 100 nm particles; *b1*— 10^{12} ; *b2*— 10^{11} ; *b3*— 10^{10} ; *b4*— 10^9 ; *b5*— 10^8 . **c** Representative DLS traces for mixtures of 20 nm particles (fixed at 10^{14} particles/mL) and various concentrations of 1,000 nm particles; *c1*— 10^{10} ; *c2*— 10^9 ; *c3*— 10^8 ; *c4*— 10^7 ; *c5*— 10^6 particles/mL.

diluted to achieve 100-fold dilution of the original sample (Fig. 5(b3)), peaks were identical to those at 10-fold dilution suggesting that both the 10-fold and 100-fold dilutions are within the working range for these particles. Further dilution of the sample gave no signal (data not shown). The viscosity of the undiluted sample after 8 h of thermal treatment was 0.969 ± 0.006 cP and the value after 10-fold dilution or 100-fold dilution was 0.938 ± 0.002 cP.

After 24 h of thermal treatment, the undiluted sample gave a single peak at ~ 110 nm suggesting a high aggregate population (Fig. 5(c1)). The absence of a monomer peak at 24 h also suggests that any signal from unstressed protein may be masked by the signal from the aggregates. When this sample was diluted 10-fold, the peak was detected at a somewhat higher diameter than that of the undiluted sample (Fig. 5(c2)). When the sample was diluted 100-fold, a second peak was observed at 90 nm, indicating the presence of a different population (Fig. 5(c3)). At 1,000-fold dilution, a small peak appeared at 60 nm, the peak at 90 nm disappeared, while the dominant peak remained at 110 nm. These results are consistent with a large population of aggregates at 110 nm which may mask smaller aggregates. The undiluted sample with 24 h thermal treatment had a viscosity of 0.991 ± 0.002 cP which decreased to 0.938 ± 0.002 cP upon 10-fold dilution and remained at 0.938 ± 0.002 cP upon further dilution.

RMM. The density of IgG (1.33 g/mL) is greater than that of PS particles (13). As a result, the *RMM* limit of detection decreased to ~ 150 nm for IgG samples as determined by the instrument. Figure 6a shows an *RMM* size analysis of a 5 mg/mL IgG sample after 2 h of thermal treatment. The undiluted sample has a measured concentration of 1.1×10^9 particles/mL and a narrow size distribution with 24% coincidence (Fig. 6(a1)). Upon 10-fold dilution, the average concentration decreased to 1.5×10^8 particles/mL with 12% coincidence (Fig. 6(a2)). Similarly, when an IgG sample after 8 h of thermal treatment was analyzed, the measured concentration was 1.2×10^9 with average size of 265 nm with 23% coincidence (Fig. 6(b1)). Upon 10-fold dilution, the measured aggregate concentration was 9.9×10^8 particles/mL with a mean size of 191 nm with 22% coincidence (Fig. 6(b2)). After 100-fold dilution, the observed concentration was 4.2×10^7 particles/mL with a mean size of 171 nm with 7% coincidence (Fig. 6(b3)).

After 24 h treatment, an undiluted sample showed an observed concentration of 1.2×10^9 particles/mL and a mean size of 549 nm with 22% coincidence (Fig. 6(c1)). On 10-fold dilution, the observed concentration decreased to 9.7×10^8 particles/mL with a mean size of 277 nm with 26% coincidence (Fig. 6(c2)). On further dilution, the sample showed an observed concentration of 2.4×10^8 particles/mL with a mean size of 226 nm with 4% coincidence (Fig. 6(c3)).

Fig. 5. Particle sizes in 5 mg/mL IgG samples by dynamic light scattering (DLS) following thermal treatment to induce aggregation. After 2 h of thermal treatment; *a1*—undiluted sample, *a2*—10-fold dilution. After 8 h thermal treatment; *b1*—undiluted sample, *b2*—10-fold dilution, *b3*—100-fold dilution. After 24 h thermal treatment; *c1*—undiluted sample, *c2*—10-fold dilution, *c3*—100-fold dilution, *c4*—1,000-fold dilution

After 1,000-fold dilution, the observed concentration decreased to 2.1×10^7 particles/mL with a mean size of 222 nm with 0% coincidence (Fig. 6(c4)). For highly concentrated samples, the initial decrease in particle size can be attributed to increased % coincidence values which overestimate measured particle size. Alternatively, given that larger aggregates are fewer in number than the monomer, dilution may decrease their concentration to below their working range. Dissociation of these aggregates may also occur. Also, the number of particles/mL did not decrease proportionally with dilution. This may be due to the fact that RMM has a concentration cutoff $\sim 1.2 \times 10^9$ particles/mL and samples with concentration near this value may be miscalculated.

IgG Samples, 100 mg/mL

DLS. In an undiluted 100 mg/mL sample of IgG, DLS showed two peaks at 4 and 57 nm (Fig. 7(a1)). The 4 nm peak was assigned to the monomeric protein while the 57 nm peak

was attributed to aggregates. With 10-fold dilution, the aggregate peak disappeared while the monomer peak shifted slightly towards greater size (Fig. 7(A2)). At higher dilution, the count rate decreased and the instrument was unable to report values. The viscosities of the undiluted sample and after 10-fold dilution were 2.7 ± 0.003 and 1.045 ± 0.002 cP, respectively. Applying a viscosity correction shifts the peak values for the undiluted sample from 4 and 57 nm to 2 and 32.5 nm, respectively.

RMM. When an undiluted 100 mg/mL IgG sample was characterized by RMM, an aggregate concentration of 3.4×10^8 particles/mL was observed with a mean particle size of 153 nm and 20% coincidence (Fig. 7(b1)). This suggests that in DLS these ~ 150 nm particles are masked by the larger population of monomeric protein which is below RMM's limit of detection, or that DLS is affected by the high concentration of the particles in the solution and is underestimating the particle size. Upon 10-fold dilution, the observed concentration decreased to 2.6×10^7 particles/mL with a mean particle size of 154 nm and 10% coincidence (Fig. 7(b2)). Further dilution decreased the observed concentration to 3.8×10^6 particles/mL with a mean

Fig. 6. Particle sizes in 5 mg/mL IgG samples by resonant mass measurement (RMM) following thermal treatment to induce aggregation. After 2 h thermal treatment; *a1*—undiluted sample, *a2*—10-fold dilution. After 8 h thermal treatment; *b1*—undiluted sample, *b2*—10-fold dilution, *b3*—100-fold dilution. After 24 h thermal treatment; *c1*—undiluted sample, *c2*—10-fold dilution, *c3*—100-fold dilution, *c4*—1,000-fold dilution

particle size of 125 nm and 19% coincidence (Fig. 7(b3)). The observed particle concentration decreases linearly with dilution, suggesting that the particles are not dissociated by dilution.

DISCUSSION

DLS has been widely used to characterize micron and sub-micron sized particles in solution (14), using light scattered by suspended particles to estimate their size. The fluctuations in the intensity of scattered light depend on the Brownian movement of the particles, with larger particles moving more slowly than smaller ones (15). This enables DLS to have a hydrodynamic measurement range from 0.3 nm to a few microns. DLS can provide reasonably accurate size measurement for monodisperse solutions but does not provide any information on particle concentration (16). The results presented here demonstrate that the ability of DLS to correctly measure particle

size in a monodisperse solution depends on particle concentration (Figs. 1 and 3), with a working range that depends on particle size. That DLS underestimates particle size at higher concentrations may be attributed to lack of ideal Brownian motion resulting from increased inter-particle interactions, number fluctuations, multiple scattering, and measurement angle (90°). DLS instruments with back scatter capability have been shown to perform better with concentrated samples (17). The overestimation of particle size at lower concentration is more difficult to explain, but may depend on instrument configuration, sample impurities, and solution properties. When samples with unknown particle size and concentration (such as aggregated protein solutions) are subjected to DLS analysis, it is possible that inaccurate particle size measurements will be obtained if the sample concentration is outside the working range for that particle size. The protein samples used in the present studies have viscosities close to that of buffer, suggesting a minimum effect of viscosity on the measurements.

Fig. 7. Particle sizes in 100 mg/mL IgG samples by dynamic light scattering (DLS) (a) and resonant mass measurement (RMM) (b). a1—undiluted sample, DLS; a2—10-fold dilution, DLS; b1—undiluted sample, RMM; b2—10-fold dilution, RMM; b3—100-fold dilution, RMM

In RMM, as long as the particle concentration is within the working range and only one particle is in the resonator channel at any given time, the mass of each particle is measured independently and thus is not affected by other particles in the solution. In samples with high particle concentration, the instrument detects two or more particles in the sensor and automatically rejects the signal from the calculation. Also, the flow of particles is adjusted by the fluidics of the instrument in an attempt to alleviate this problem. Hence, instrument software and fluidics allow relatively good measurement of particles below a certain concentration making RMM attractive for measuring particle size in polydisperse solutions. As with DLS, there is a concentration and particle size cutoff (Fig. 2) for RMM and a practical working range for the instrument can be defined within which accurate particle size and concentration measurements can be obtained. The working range depends on particle size, the relative specific gravities of the particle and fluid (Fig. 3), and the time allowed for analysis. The percent coincidence values can be helpful in defining the working range for particle concentration, since percent coincidence decreases with dilution and a value of 10% or less is recommended by the manufacturer. As a general rule, we found that percent coincidence decreased with dilution. Undiluted samples had percent coincidence values of 20%

or more, which decreased to 10% or less with dilution. In RMM, the maximum particle concentration that provided meaningful size results was 10^9 particles/mL for both polystyrene standard particle sizes, 400 and 1,000 nm, consistent with the instrument specification. For the 400 nm particles, the determined number of particles changed from ~30% of the theoretical value for a concentration of 10^9 particles/mL to ~60% of 10^8 particles/mL and ~90% for the theoretical concentration of 10^7 particles per mL, suggesting that the best accuracy is achieved for particle concentrations of 10^7 particles/mL or lower. A similar trend was observed for 1,000 nm particles (i.e., an increasing number of particles detected with increasing dilution), although the number of particles detected for the same concentration was different for different particle sizes. In this case, the percent coincidence value for the undiluted sample was 25% which dropped below 10% with 10-fold dilution.

For polydisperse solutions, particle size estimation by DLS is affected by the light scattered from the surrounding particles and the average particle size may be skewed towards larger particles (18,19). Here, DLS was unable to resolve particles in binary mixtures in which the particle sizes differed by less than a factor of 10 (Fig. 4). This bias, together with the concentration dependence, makes interpretation of particle size measurement by DLS difficult in polydisperse samples. In mixtures of 20 and 1,000 nm particle standards, DLS was able to resolve the two species only at one concentration ratio (Fig. 4(c4)). For all other concentration ratios, only one particle size was detected. In contrast, particle size determination by RMM is unaffected by other particles in the solution with sizes less than the detection limit, as verified for mixtures containing 20 nm and 400 or 1,000 nm particles. For RMM, the addition of 20 nm particles had no effect on particle size estimation or on the measured concentration of 400 and 1,000 nm particles. The number of particles detected and their particle size depended on the total particle concentration in the range studied (10^7 – 10^9 particle/mL). The order of magnitude of the number of particles was correct, though values varied with concentration.

Aggregated protein samples typically contain a large number of monomeric protein molecules together with a relatively small number of aggregates of various sizes (20). Analysis of aggregated 5 mg/mL IgG solutions using DLS yielded both a 20 nm peak and an aggregate peak (Fig. 5). After 2 h of thermal treatment, 5 mg/mL IgG samples showed both a 20 nm peak and aggregate content (Fig. 5a). While the 20-nm peak was not detected after 8 and 24 h of thermal treatment, particles with sizes smaller than the large aggregates present in undiluted samples were observed upon dilution (Fig. 5b, c). This suggests that dilution can be used in DLS to detect smaller particles that may be masked by larger species, as observed (Fig. 4(c4)). However, this inference assumes that the aggregates are not altered by dilution, and the absence of peaks in diluted samples cannot be used to confirm the absence of smaller particles.

When 5 mg/mL IgG samples were analyzed by RMM, a heterogeneous particle size distribution was observed (Fig. 6). Particles smaller than 150 nm were not detected by the instrument. RMM also provides an approximate concentration of particles in the solution. Although the concentration of aggregates in the samples is not known *a priori*, the percent

coincidence value along with the observed concentration can be a good indication of whether the sample is within the working range. To further assess whether aggregate concentrations were within the working range for RMM, dilutions were carried out. For 5 mg/mL IgG treated for 24 h, the concentration of an undiluted sample of 1.2×10^9 particles/mL did decrease linearly upon 10-fold and 100-fold dilution (Fig. 6(c1, c2)). Upon further dilution, the measured particle concentration decreased in proportion with dilution (Fig. 6(c3, c4)). Percent coincidence value also decreased from 22% for the undiluted sample to 0% for the 1,000-fold diluted sample. Hence, the concentration of aggregate in the initial, undiluted sample appears to be $\sim 2 \times 10^{10}$ particles/mL, assuming that aggregate size and number are unaffected by dilution. This is similar to the results for the particle standards. Thus, it appears that dilution affects the count in the 10^8 – 10^9 particle/mL concentration range. We also note that the percent coincidence values can provide additional information on the correctness of the measured concentration, since at higher percent coincidence values ($\sim 20\%$ or more) the instrument reaches its concentration cutoff.

There have been numerous studies comparing DLS with other methods and demonstrating its strengths and weaknesses. Filipe *et al.* compared DLS with nanoparticle tracking analysis (NTA) at concentrations of 10^8 – 10^{12} particles/mL. They found that DLS was unable to estimate particle size for polydisperse solutions like mixtures of particle standards and proteins (21), an observation similar to our findings. Mahl *et al.* characterized binary mixtures of gold and silver nanoparticles, and showed that different instruments like scanning electron microscopy, DLS, analytical disc centrifugation, and NTA gave different average particle sizes for the same mixture (22), which is again consistent with our findings using DLS and RMM.

RMM is a relatively new technique, so there are few published reports. Recently, Weinbuch *et al.* compared micro-flow imaging (MFI) with RMM using oil droplets and protein aggregates of 0.5–4 μm and suggested complementary use of both methods (23). Zolls *et al.* compared MFI, RMM, and light obstruction using various monoclonal antibodies, underscoring the importance of refractive index in particle size measurement for light-based techniques (24).

DLS and RMM have different working principles and can be used as orthogonal techniques to improve on the individual shortcomings of each technique. It is evident from the findings that a comprehensive understanding of the instruments and their limitations is necessary to interpret the results. Hence, for each technique, establishing critical parameters like working range and resolution is of utmost importance. These parameters are both instrument-specific and sample-specific. For DLS, changing measurement angle can affect the working range and resolution of particles. Similarly, for RMM, increasing the allowed measurement time can expand the working range and decrease the lower concentration limit. DLS measures particles in a small volume window; however, this sample fragment is in equilibrium with a much larger volume limited only by the size of the cuvette. RMM may not be able to accurately capture the particle size distribution due to the small volume tested, a limitation that

can be mitigated by multiple samples and longer analysis time. Applying both methods to samples of interest may aid in identifying inaccuracies introduced by these practical limitations that would not be evident if either were used independently.

In summary, the studies reported here compared DLS with RMM using solutions of monodisperse particle size standards, binary mixtures of particle size standards, and aggregated protein solutions to evaluate the effect of concentration and particle size on their measurement ability. Working ranges were established for both instruments using PS particle standards. DLS was able to accurately measure particle sizes over a wider range than RMM and could resolve particles with diameters differing by at least a factor of 10, but does not measure particle concentration. In contrast, RMM measures particle concentration and provided more accurate particle size measurements in binary mixtures. Sample dilution was shown to be useful in confirming particle concentration (or concentration range), if aggregates could be assumed to be unaffected by the dilution process. For aggregates which dissociate upon dilution, obtaining true measurements can become challenging and can limit the use of measurement of samples by dilution. The effect of dilution on aggregate stability must be determined on case-by-case basis. Confirming particle concentration when dilution is not a tenable option should be the focus of future studies.

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