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Absolute Quantification Method for Protein Concentration

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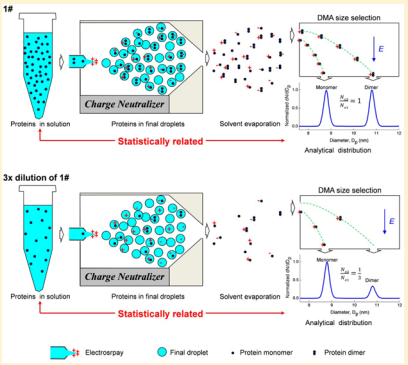
Supporting Information

ABSTRACT: A fast and accurate assay to determine the absolute concentration of proteins is described based on direct measurement of droplet entrapped oligomer formation in electrospray. Here we demonstrate the approach using electrospray differential mobility analysis (ES-DMA), which can distinguish monomers and dimers from higher order oligomers. A key feature of the method is that it allows determination of the absolute number concentration of proteins eliminating the need for protein-specific calibration. The method was demonstrated by measuring the concentration of a NIST Standard Reference Material 927e (bovine serum albumin), a high-purity immunoglobulin G 1κ, and a formulated Rituximab. The method may be applied to any electrospray source, regardless of diagnostic tool (e.g., MS or ion-mobility, etc.), provided the electrospray is operated in a droplet-fission mode.



Accurate quantification of protein concentration is essential to a wide variety of biochemical research, ranging from enzymatic to biopharmaceutical studies. Several methods are available for protein quantification including amino acid analysis,[1,2] ultraviolet−visible (UV) absorbance,[3,4] and colorimetric assays.[3,4] Spectrophotometric methods including UV absorbance, or colorimetric assays such as the Biuret, Lowry, and Bradford,[3,4] are the most frequently used methods because of their inherent simplicity and speed; however, these methods suffer from accuracy limitations.[5] The variation of amino acid components relative to a calibration protein, such as bovine serum albumin (BSA), can limit the accuracy of measured protein concentration, unless the concentration of a pure calibration protein similar in amino acid composition has been accurately determined. Furthermore, buffers and other substances in solution may interfere with color development or absorb in the detection wavelength region and thus lead to measurement bias. Amino acid analysis is an accurate method to determine protein concentrations by quantifying the free amino acid liberated from hydrolysis.[1,2] However, it is a time-consuming and laborious method for proteins.

Here, we describe a fast and accurate method to determine the absolute concentration of proteins in solution without the need for specific protein calibration. The method is based on a statistical analysis of droplet entrapped nonspecific aggregation during electrospray (ES) ionization[6] (Note 1 in the Supporting Information). ES generates aerosol dispersed material that, after solvent evaporation, leads to dried analytes, which can be analyzed by ion mobility (e.g., differential mobility analysis (DMA))[7−11] or mass spectrometry (MS).[12,13] It is reported that large proteins, which are the analytes of interest in this work, are produced as charged residues after complete droplet evaporation during ES,[14] thus following a charge residue mechanism.[15] This charge residue mechanism[15] indicates the existence of a droplet entrapped nonspecific aggregation effect,[6,16] i.e., two intrinsic monomers randomly entrapped in a droplet are observed as a dimer in the final analytical distribution after the droplet drying. The droplet entrapment effect is usually not as prevalent in MS analysis, where multiple charges result in serial fission and smaller final droplets,and protein aggregate thought to arise from large droplets in MS analysis was rarely reported.[17] To reduce serial fission in our studies, a charge neutralizer (Po-210(α) source) is placed immediately after the ES to rapidly drop the charge state.[7,18] In previous work, we demonstrated that the observed ratio of the droplet entrapped oligomers to monomers is statistically related to the particle concentration in solution, which enables determination of the absolute analyte particle concentration by measuring the dimer-to-monomer ratio.[19] Here we systematically study this approach and develop a robust protocol to determine the number concentration of protein particles without prior need for specific protein calibration (for example, UV−vis absorption requires knowledge of the extinction coefficient for each protein). This protocol is validated using NIST Standard Reference Material (SRM) 927e, (bovine serum albumin (BSA)), a high-purity immunoglobulin G 1κ (IgG), and a formulated Rituximab (Rmab). In addition, the merits and limitations of the method are discussed.

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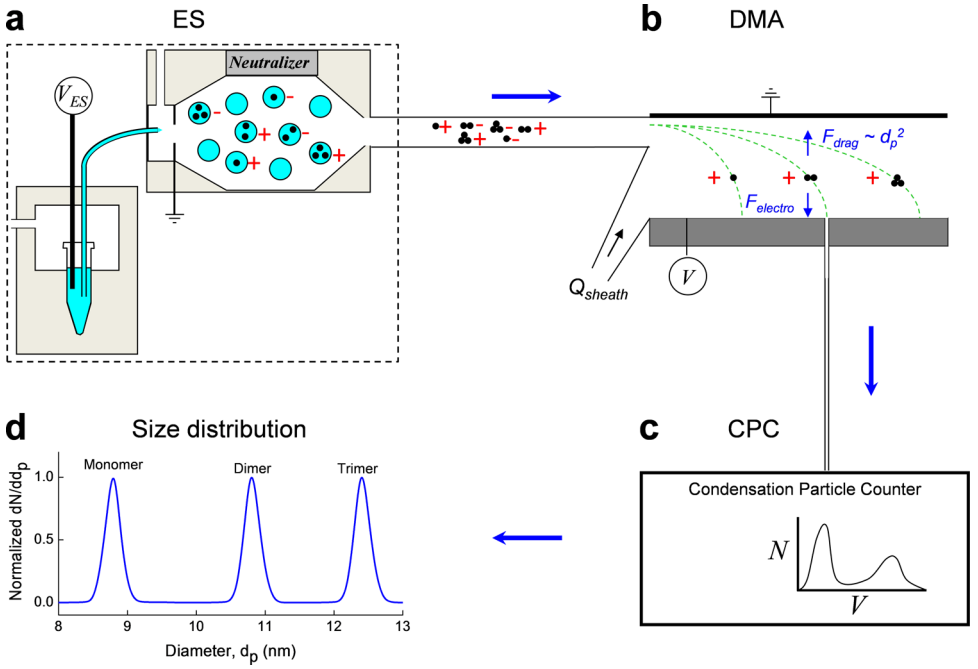

Figure 1. Schematic of the major components of the analysis system. (a) Electrospray (ES) with a charge neutralizer equipped to reduce charges on the droplets to a modified Boltzmann equilibrium charge distribution[20] which results in the majority of charged droplets possessing a single charge. (b) A differential mobility analyzer (DMA) to separate dry protein particles by their size-to-charge ratio determined trajectory for a fixed voltage. By scanning the operating voltage of the DMA, different size proteins can be selected. The flow of the proteins and the laminar sheath flow gas (Q_{sheath}) enter the annual region of the DMA, where the charged proteins move with electric force ($F_{electro}$) balanced by drag force (F_{drag}). (c) A condensation particle counter (CPC) to count particles selected by DMA to build a particle number versus operating voltage distribution. (d) A typical protein size distribution is shown after converting operating voltage to mobility size and correcting counts based on the known charge distribution.

EXPERIMENTAL SECTION

Experimental System. Protein oligomer size distribution measurements are performed with an electrospray aerosol generator (ES), a differential mobility analyzer (DMA), and a condensation particle counter (CPC) (Figure 1). Electrospray of the protein solution is passed over a charge neutralizer (radioactive Po-210(α) source) that reduces the charge on the droplets to a well-defined modified Boltzmann equilibrium charge distribution.[20] After complete solvent evaporation, the neutralized dry proteins enter a DMA for protein size selection, and are subsequently counted with a CPC. By scanning the operating voltage of the DMA (i.e., E-Field), different size proteins are selected to generate a size distribution for a given protein population.[8] Unlike a mass-spectrometer which selects proteins based on mass/charge ratio, the DMA selects proteins based on its equivalent spherical mobility diameter. With the known charge distribution for each selected size, the total number of protein can be obtained by simply counting the number of proteins bearing a single positive charge (detailed data analyses shown in the Excel file of the Supporting Information).

Bovine Serum Albumin (BSA) and Immunoglobulin G (IgG) Solution Preparation. NIST Standard Reference Material (SRM) 927e (bovine serum albumin (BSA) with certified concentration 67.38 g/L[21]) and a high-purity immunoglobulin G 1κ (IgG; a therapeutic-like monoclonal antibody of the IgG1 type produced from an industry-like monoclonal antibody purification platform process with a nominal concentration of 100 mg/mL ~ ±10%) were used.

Immediately prior to use in ES studies, a working BSA solution set was made by 140×, 350×, 701×, 1402×, 3505×, and 7010× dilution of the as-received SRM 927e BSA solution with a buffer of 20 mmol/L ammonium acetate at pH 7. The electrospray experiments were repeated over five different days, and in each day a new set of BSA protein samples was prepared following the protocol described above. The as-prepared BSA samples were then electrosprayed using an electrospray aerosol generator (Model 3480, TSI Inc., with a charge neutralizer mounted) into the differential mobility analyzer (DMA) (Model 3485 Nano DMA column, TSI Inc.)−ultrafine condensation particle counter (CPC) (Model 3025A TSI Inc.) system. Three different capillaries (outer diameter 150 μm, 40 μm inner diameter, and 24 cm length) were used for the experiments.

A sample set of working solutions for IgG was made by 100×, 200×, 500×, 1000×, 2000×, 4000×, 10 000×, and 20 000× dilution of the as-received high-purity IgG solution with the buffer of 20 mmol/L ammonium acetate at pH 7. The electrospray experiments were repeated six times in five different days with five different sets of samples. Four different capillaries (outer diameter 150 μm, 40 μm inner diameter, and 24 cm length) were used for the experiments.

Electrospray Particle Generation and Mobility Size Distribution Measurements. The analysis system is shown in Figure 1. The protein particles (BSA, IgG, etc.) are first aerosolized and charged in the electrospray (ES). The aerosolized highly charged droplets were generated using a 40 μm inner diameter capillary (outer diameter 150 μm, 24 cm length) mounted in an electrospray aerosol generator (Model 3480, TSI Inc.) where the liquid flow rates through the capillaries were ~433 nL/min.[22] The ES voltage was operated to have a stable Taylor cone at the tip of ES capillary, which was close to 2.5 kV in this work. A subsequent charge neutralizer, radioactive Po-210(α) source, which is mounted in the

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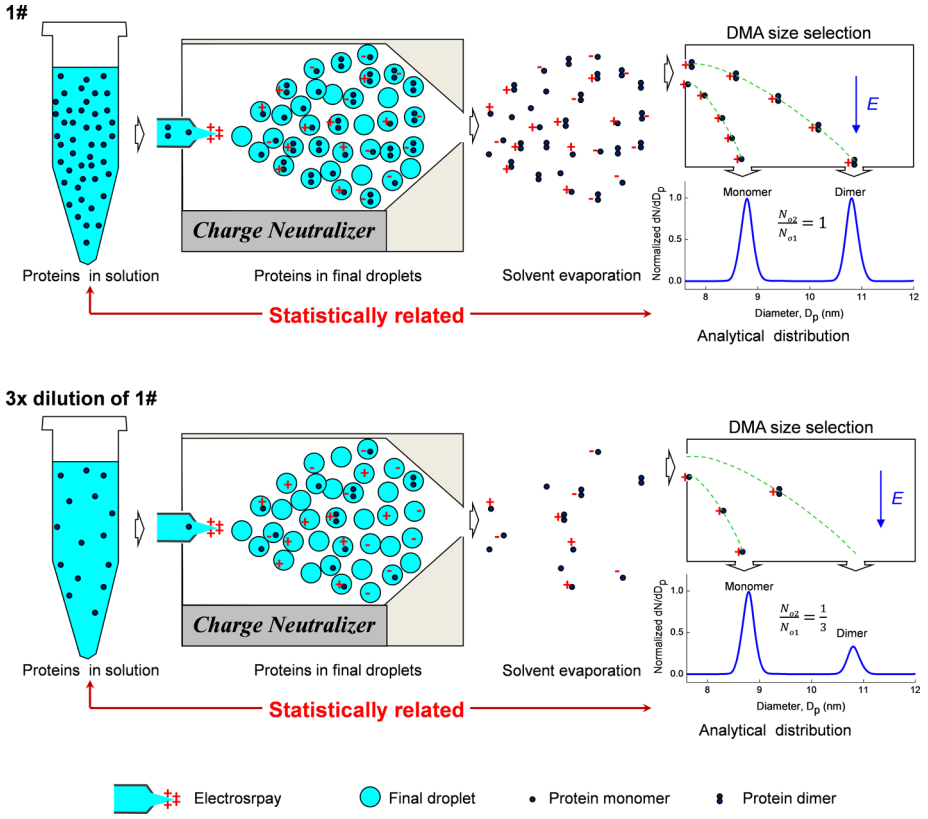

Figure 2. Schematic of electrospray process showing the resulting measured particle size distribution due to droplet entrapped aggregation. In this example there are no intrinsic dimers in the original protein solution. However, downstream of the electrospray, droplet entrapped dimers are observed in the analyte mobility spectrum. The observed dimer to monomer ratio is proportional to the protein concentration in solution. Therefore, by measuring the observed dimer to monomer ratio and the droplet volume, the absolute number concentration of protein particles in solution can be determined. The *y*-axis label "dN/dD_p" is defined by the total number of proteins in the range (D_p, $D_p + dD_p$) divided by the size interval, dD_p.

commercial Electrospray Aerosol Generator, was applied immediately, which reduces the charge on the droplets to a well-known modified Boltzmann distribution.[20] As a result, after the droplets dry, the majority of positively charged protein particles that enter the DMA possess a single positive charge. With the known charge distribution, the total number of particles can be obtained by only counting the number of single positively charged particles (detailed data analyses shown in Excel file of the Supporting Information). The electrospray was operated with a carrier gas of 1 L/min purified air, and 0.2 L/min carbon dioxide to stabilize the Taylor cone. A stable Taylor cone is necessary to quantitatively determine the absolute particle number concentration of analytes and the generated droplet size distribution.

The neutralized dry particles after ES then entered a DMA (Model 3485 Nano DMA column, TSI Inc.) for particle size selection followed by counting with a CPC (Model 3025A TSI Inc.). The DMA consists of a grounded cylinder and an inner negative high voltage rod with a slit for the particles to exit. For a fixed voltage applied to DMA, only the particles with a corresponding mobility size can exit, and be counted by the CPC. By scanning the center rod voltage, different size particles can be extracted to build up a size distribution for a given particle population.

Unlike a mass-spectrometer, which operates under vacuum and selects particles based on mass/charge ratio, the DMA operates near atmospheric pressure and selects particles based on its mobility or equivalent mobility diameter that is roughly proportional to the square root of averaged projected area for particles less than ∼50 nm and operates near atmospheric pressure. The nano-DMA was operated with a sheath flow of 30 L/min and an aerosol flow 1.2 L/min, which enables an operating range of between 2 and 45 nm by scanning the rod voltage ranging from −20 V to −10 kV. A different choice of flow rates and/or the use of a different length DMA enables a different size range to be analyzed. Because this is an ion-mobility measurement, the composition of the analyte particle is not relevant to the operational principles of the instrument.

Droplet Size Measurements. To apply our method for protein quantification, knowledge of the droplet size produced by the ES source is needed. The statistical analysis of droplet entrapped nonspecific aggregation requires measurement of a droplet size distribution. The droplet size distribution was determined by electrospraying a known volume to volume (V/V) concentration of sucrose (Sigma Life Science, >99.5%) solution, and measuring the resultant dry particle size distribution. Using this procedure, the original droplet size distribution can be determined directly. For droplet size evaluation for this paper, sucrose solution concentration of 0.063% V/V, was used. At low concentrations of sucrose and protein particles, the viscosity of solutions is governed by the buffer conditions, and the droplet size of these solutions with the same buffer condition is expected to be the same and can be evaluated by[22,23]

$$D_d = \frac{1}{C_s^{1/3}} D_s \quad (1)$$

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where D_d is the droplet diameter, D_s is the dried sucrose particle diameter (measured by DMA-CPC), and C_s is the known sucrose concentration (V/V) in solution (C_s = 0.063% in this paper; detailed data analyses shown in the Excel file of the Supporting Information).

■ ABSOLUTE CONCENTRATION QUANTIFICATION METHOD

Droplet Entrapped Aggregation as a Useful Diagnostic. When a protein colloidal solution is transformed to small solution droplets by electrospray (ES), it is reported that large multiply charged proteins are generated as charged residues after complete droplet evaporation.[14] Even though the manner by which ES ionizes proteins is not as settled as one might have expected, with some observations[24] seemingly incompatible with the charged residue mechanism for multiply charged protein ions, this debate fortunately does not affect the results presented in this work because the charge neutralization in this work ensures that the protein ions will be created by a charge residue mechanism. Based on the charge residue mechanism,[15] evaporation of the solvent in those droplets leaves behind protein particles that can be passed to an analyzer, such as a differential mobility analyzer (DMA) or mass spectrometer. When two or more protein particles exist in a final droplet, and the droplet eventually dries, the detector measures an oligomer in the spectrum. Because this oligomer is not intrinsic to the solution, but rather two or more monomers that were entrapped in one droplet, we refer to this effect as droplet entrapped (induced) aggregation.[6] A final droplet is defined here as the droplet after all fission processes. This effect was investigated through a statistical model by Li et al.[6] that indicates that the observed oligomer to monomer ratio is statistically related to the original protein concentration in solution. The power of this method to obtain the original protein concentration is that absolute concentration can be determined by measurement of the relative concentrations of dimer to monomer, thereby eliminating the need for instrument calibration for protein concentration due to loss, which is a function of the type of protein. The basic concept is illustrated in Figure 2. In this simplest of cases, only protein monomers exist in solution; however, following ES and solvent evaporation, droplet entrapped protein dimers are observed. The relative observed dimer-to-monomer ratio measured by the DMA is proportional to the protein monomer concentration originally in solution, but can be converted to an absolute measurement if the droplet volume is known. Therefore, by measuring the observed dimer to monomer ratio and the final droplet volume, the absolute number concentration of protein in solution can be extracted, without the need for a specific calibration on that or any protein.

Application of Poisson statistics[25] to this problem[6] leads to a characteristic parameter λ (Note 1 in the Supporting Information), where $\lambda = V_d C_p$, which physically represents the mean number of particles per final droplet. Here V_d = final droplet volume and C_p = protein number concentration (our unknown). Thus, the larger the value of λ is, the greater the entrapment effect. Because most mass spectrometry (MS) protein characterization is conducted at relatively low concentration and, without the charge neutralizer (i.e., more droplet fissions and smaller final droplet volume), droplet entrapped aggregation is likely not as significant in MS. Thus, in our case the use of a charge neutralizer diminishes droplet fission, creating larger stable drops that have a significantly higher probability of entrapped multiple proteins (see Figure 2). The employment of a neutralizer results in the majority of charged droplets possessing a single charge, which could make the charge residue mechanism valid for even smaller proteins than literature reported, 6.5 kDa and up, for multiply charged proteins[14] because the singly charged droplets would not encounter electric fields as high as those multiply charged ones present at the Rayleigh limit.

Absolute Quantification Method of the Protein Number Concentration. The approach which we describe can be applied to quantify the absolute concentrations of any order of oligomer. Because only intrinsic monomers, dimers and trimers were observed in this work, we only show the concentration calculations up to trimers. An intrinsic dimer or trimer is defined here as the dimer or trimer formed in solution before ES, thus not from droplet-entrapped effect.

A work-flowchart to determine the absolute number concentration of a protein solution is presented in Figure 3. If one considers the simple case, whereby the ES generator produces monodisperse droplets with volume V_d, an analysis of the Poisson distribution leads to a remarkably simple linear relationship between the observed dimer number (N_{o2}) to

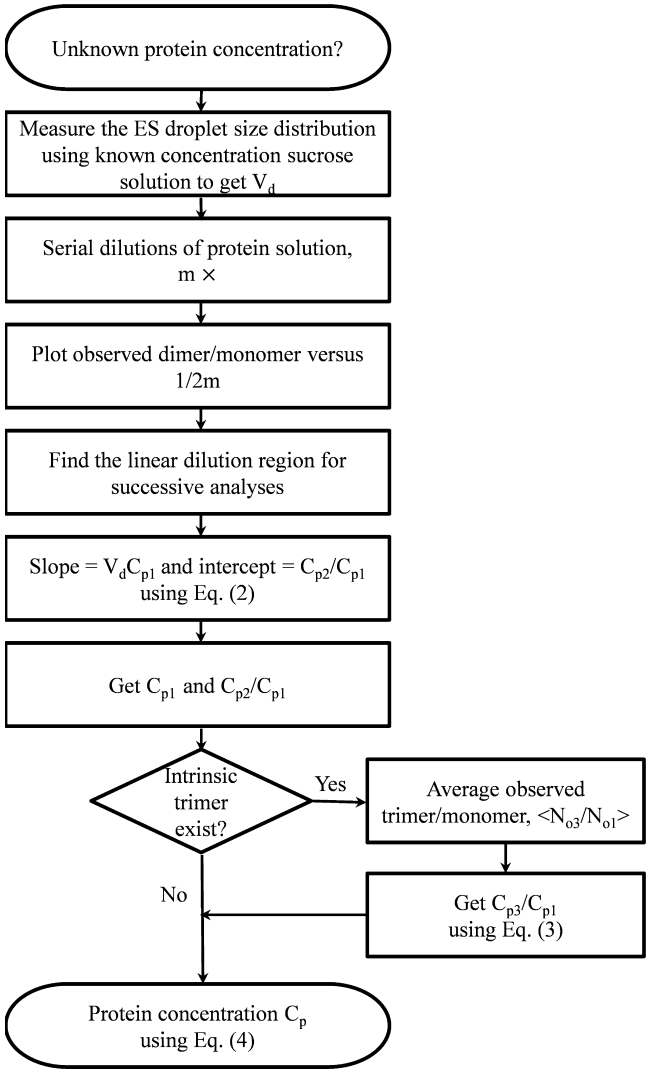

Figure 3. Work-flow to determine the absolute number concentration of a protein solution.

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monomer number (N_{o1}) ratio and the protein concentrations in solution (Note 2 in the Supporting Information).

$$\frac{N_{o2}}{N_{o1}} = (V_d C_{p1})\frac{1}{2m} + \frac{C_{p2}}{C_{p1}} \quad (2)$$

Here m is the dilution factor; C_{p1} and C_{p2} are the intrinsic monomer and dimer protein number concentrations in solution, respectively. Given eq 2, it is evident that for a series of experiments varying dilution (m), a plot of the observed (N_{o2}/N_{o1}) versus ($1/2m$), yields a slope = $V_d C_{pl}$, and intercept = (C_{p2}/C_{p1}) . Thus, the slope directly leads to a measure of the protein monomer concentration in the original solution once the droplet size is known (see the Experimental Section above on determination of the droplet size). The dimer concentration is then extracted from the intercept. Once the monomer concentration and dimer concentration are determined, the intrinsic trimer concentration, C_{p3}, is calculated by the average of observed trimer number (N_{o3}) to monomer number (N_{o1}) ratio in each dilution, $\langle(N_{o3}/N_{o1})\rangle$, as (Note 2 in the Supporting Information)

$$\frac{C_{p3}}{C_{p1}} = \left\langle \frac{N_{o3}}{N_{o1}} \right\rangle - V_d C_{p2}\left\langle \frac{1}{m} \right\rangle - \frac{(V_d C_{p1})^2}{6}\left\langle \frac{1}{m^2} \right\rangle \quad (3)$$

Thus, from a measurement of the "observed" monomers and dimers (N_{o1}, N_{o2}) and a measurement of droplet size, the absolute number concentration of intrinsic monomers, C_{p1}, and of intrinsic dimers, C_{p2}, can be obtained through a linear regression fit based on eq 2. Then the intrinsic trimer concentration C_{p3} is determined using eq 3. The total concentration thus can be determined by summation

$$C_p = C_{p1} + 2C_{p2} + 3C_{p3} \quad (4)$$

The linear relationship (in eqs 2 and 3) is valid, assuming the droplet sizes emanating from the ES source are monodisperse. In reality, the measured droplet size is like the distribution displayed in Figure 4e. We then resort to a more general theory[6] where we expect a linear region to exist at low concentrations, an average droplet volume of the main peak is applied to this linear region, and eqs 2 and 3 can still be applied (Note 3 in the Supporting Information). This protocol is applied to determine the concentration of NIST SRM 927e (BSA), a high-purity IgG 1κ, and a formulated Rituximab in the next section.

RESULTS

Application to NIST Standard Reference Material 927e, Bovine Serum Albumin. We first apply the methodology to NIST Standard Reference Material (SRM) 927e, bovine serum albumin (BSA)[21] in Figure 4 (detailed data analyses shown in the Excel file of the Supporting Information). The original BSA solutions were diluted to m = 140×, 350×, 701×, 1402×, 3505×, and 7010× for the ES-DMA-CPC measurements. For each dilution, we repeated the measurement for three successive times (detailed data analyses shown in the Excel file of the Supporting Information). The analyte size distributions of the 701× and 7010× dilutions are shown in Figure 4a,b, respectively. For the low concentration case (7010× dilution of the original BSA) shown in Figure 4b, only monomers, dimers, and trimers were observed, and thus only those are used to evaluate the absolute concentration. The observed dimer to monomer ratios (N_{o2}/N_{o1}) versus ($1/2m$)

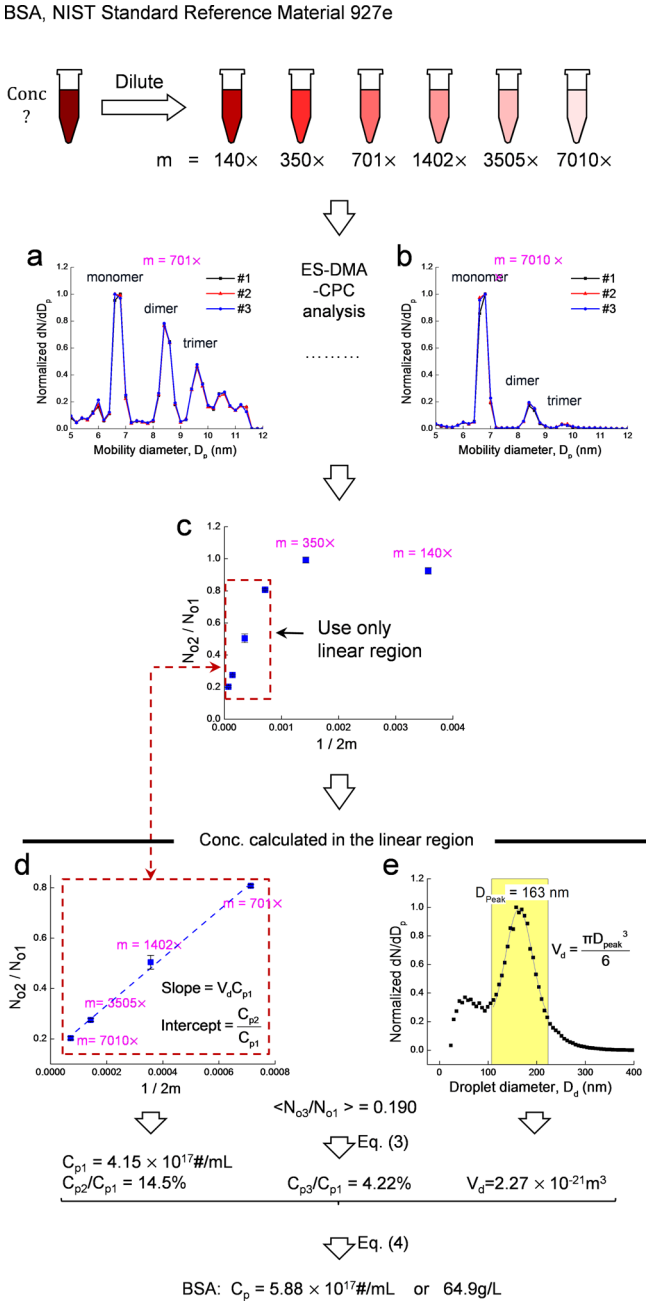

Figure 4. Applying our method to quantify the concentration of NIST standard reference material 927e, bovine serum albumin (BSA). The original BSA solutions were diluted to m = 140×, 350×, 701×, 1402×, 3505×, and 7010× times for ES-DMA-CPC measurements. (a) (b) Size distributions of the 701× and 7010× BSA dilutions, respectively. (c) Observed dimer to monomer ratios versus $1/2m$. Error bars based on three repeat experiments. (d) Linear region determined at the low concentrations 7010×, 3505×, 1402×, and 701×. Linear regression fit applied to this region to obtain the slope =$V_d C_{p1}$ and the intercept = $((C_{p2})/(C_{p1}))$ based on eq 2. (e) Droplet size distribution measured using 0.63% (volume to volume) sucrose solution with the same buffer as the protein solutions. Main peak dominates the contributions to the linear region for obtaining the protein concentration, and thus droplet volume, V_d, is calculated based on the Gaussian fit of the main peak. Finally, the total number concentration of BSA determined by summing C_{p1}, C_{p2}, and C_{p3} using eq 4, and the mass concentration calculated using the theoretical molecular weight of BSA, 66 398 Da.[21]

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a BSA, NIST standard reference material 927e

	Conc. (C_p)
Day 1. Sam. #1 Cap. #1	66.3 g/L
Day 2. Sam. #2 Cap. #1	67.6 g/L
Day 3. Sam. #3 Cap. #1	63.6 g/L
Day 4. Sam. #4 Cap. #2	66.5 g/L
Day 5. Sam. #5 Cap. #3	64.9 g/L

Our results	NIST certified concentration
65.8 ±1.6 g/L	67.38 ±1.38 g/L

b IgG, a high-purity immunoglobulin G 1κ

	Conc. (C_p)	C_{p2}/C_{p1}
Day 1. Sam. #1 Cap. #1	114 g/L	0.6%
Day 2. Sam. #2 Cap. #2	116 g/L	-0.2%
Day 3. Sam. #3 Cap. #3	113 g/L	-0.1%
Day 4. Sam. #4 Cap. #3	114 g/L	0%
Day 5. Sam. #4 Cap. #4	111 g/L	0.2%
Day 5. Sam. #5 Cap. #4	114 g/L	0.3%

	Our results	Provider's estimates
C_p	113.7±1.6 g/L	~ 100± 10 g/L
C_{p2}/C_{p1}	0.1%	< 1%

c Rmab, a formulated Rituximab

	Conc. (C_p)
Day 1. Sam. #1 Cap. #1	1.12 g/L
Day 2. Sam. #2 Cap. #1	1.16 g/L

Our results	UV-Vis result
1.14 ±0.03 g/L	1.0 g/L

Figure 5. Quantifying concentration of NIST SRM 927e, BSA (Figure 4; detailed data analyses shown in the Excel file of the Supporting Information), a high-purity IgG 1κ (Figure S-1 in the Supporting Information), and a formulated Rituximab, Rmab (Figure S-2 in the Supporting Information). (a) The SRM 927e BSA concentration in the linear region of dilutions was repeated five times over five different days with three different capillaries. Our measured BSA concentration was 65.8 ± 1.6 g/L, in excellent agreement with the NIST certified BSA concentration 67.38 g/L based on ID-LC/MS/MS and amino acid analyzer measurements.[21] The theoretical molecular weight of BSA, 66398 Da, was used to obtain the mass concentration. (b) Concentration of the IgG in the linear region of dilutions was repeated six times over 5 days with four different capillaries and the measured mass concentration is 113.7 ± 1.6 g/L and the measured intrinsic dimer to monomer ratio is about 0.1%. The provider estimates, the concentration is 100 ± 10% g/L based on UV−vis absorbance at 280 nm and the dimer to monomer ratio is <1% based on SEC measurements. Theoretical molecular weight of this IgG1κ is 148 kDa. (c) Concentration of a formulate Rituximab, Rmab in the linear region of dilutions was repeated twice over 2 days and the measured mass concentration is 1.14 ± 0.03 g/L, which is consistent with the UV−vis absorbance result 1 g/L at 280 nm.

with error bars are plotted in Figure 4c. As expected (Note 3 in the Supporting Information), the data are linear at lower concentrations between 710× and 7010× and it is this region that is used to calculate concentration (see Figure 4c,d). Applying a linear regression fit to eq 2 and using the measured droplet size (a Gaussian fit to the main peak of the droplet size distribution), the concentrations of intrinsic monomer, C_{p1}, and dimer, C_{p2}, are obtained. And the intrinsic trimer, C_{p3}, is then obtained using eq 3. Finally, the total number concentration of BSA is determined by a sum of C_{p1}, C_{p2}, and C_{p3} using eq 4. The mass concentration is calculated using the theoretical molecular weight of BSA, 66 398 Da.[21]

The above procedure to determine the BSA concentration in the linear dilution region was repeated five times over 5 days,

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with three different capillaries, and the results are summarized in Figure 5a. For each of the experiments, the samples were prepared freshly from the original BSA SRM 927e solution. The average measured BSA concentration from the five repeat experiments is 65.8 ± 1.6 g/L, which is in excellent agreement with the NIST certified BSA concentration 67.38 g/L.[21] The NIST certified value was determined using isotope dilution liquid chromatography/tandem mass spectrometry (ID-LC/MS/MS) and a commercial amino acid analyzer.[21]

Application to a High-Purity Immunoglobulin G 1κ. We also applied the quantification method to a high-purity immunoglobulin G 1κ (IgG), using the similar procedure described above (Figure S-1 in the Supporting Information). The original IgG solution was diluted to m = 100×, 200×, 500×, 1000×, 2000×, 4000×, 10 000×, and 20 000× and measured by ES-DMA-CPC. Because at the low concentration 10 000× no trimers were observed (Figure S-1b in the Supporting Information), we only consider monomers and dimers for concentration calculations. To determine the linear region, the observed dimer to monomer ratios versus $1/2m$ was plotted and the linear region was determined to be for dilutions ≥1000× (Figure S-1c,d in the Supporting Information). The total concentration of IgG was then determined using the slope and intercept of the linear regression fit applied in the linear region and the measured droplet volume. The procedure above in the linear region of the concentration dilutions was repeated six times over 5 days, with four different capillaries and the results are summarized in Figure 5b. Based on the six experiments and the theoretical molecular weight of this IgG 1κ, 148 kDa, our measured mass concentration of the IgG is 113.7 ± 1.6 g/L, and the intrinsic dimer to monomer ratio is about 0.1%. Both results are consistent with the reported estimated value of 100 ± 10 g/L based on UV−vis absorbance at 280 nm, and the dimer to monomer ratio <1% based on size-exclusion chromatography (SEC) measurements.

Application to a Formulated Rituximab. Using the protocol described above, we applied the quantification method to a formulated Rituximab (Rmab) (145 kDa) (Figure S-2 in the Supporting Information) with the experimental data in our previous study.[19] The original formulated Rmab was purified and buffer exchanged. The concentration of Rmab in the working buffer for ES-DMA-CPC measurement was adjusted to 1 mg/mL as verified by measuring the maximum absorbance at 280 nm and using a molar absorptivity of 236 020 $(\text{mol/L})^{-1}$ cm^{-1}. The 1 mg/mL Rmab solution was diluted to m = 10×, 20×, 40×, 100×, and 200× and measured by ES-DMA-CPC. Because at the low concentration of 100×, no trimers were observed (Figure S-2b in the Supporting Information), we only consider monomers and dimers for concentration calculations. The observed dimer to monomer ratios versus $1/2m$ was plotted and the linear region was used for all dilutions (Figure S-2c in the Supporting Information). The total concentration of Rmab was then determined using the slope and intercept of the linear regression fit applied in the linear region and the measured droplet volume. The procedure above was repeated twice over 2 days, and the results are summarized in Figure 5c. Our measured mass concentration of the Rmab is 1.14 ± 0.03 g/L, which is consistent with the measurement value of 1 g/L based on UV−vis absorbance at 280 nm.

DISCUSSION

Droplet entrapped aggregation based on the charge residue mechanism for relatively large proteins (literature reported, 6.5 kDa and up, for multiply charged proteins[14]) is usually not significant in mass spectrometry (MS) where the multiple charges make the final droplets small following serial fissions. In our ES setup (Figure 1), in order to have a significant droplet entrapped effect, a charge neutralizer is used to rapidly decrease the charge state of the droplets, prevent fission, and thus make the final droplets large enough to observe oligomer formation. The charge distribution after a neutralizer follows a known modified Boltzmann equilibrium charge distribution.[20] As a result, the majority of charged protein particles possess a single charge. This charge reduction process should make the charge residue mechanism valid for even smaller proteins than literature reported 6.5 kDa and up for multiply charged proteins[14] because the singly charged droplets would not encounter electric fields as high as those multiply charged present at the Rayleigh limit.

One of the merits of our approach is its accuracy. Our method as applied to NIST SRM 927e, BSA, shows excellent agreement with the NIST certified BSA concentration determined by ID-LC/MS/MS and a commercial amino acid analyzer (Figure 5). The concentration of a high-purity IgG obtained by our approach is consistent with the providers estimate to about 10% uncertainty, based on their reported UV−vis absorbance approach. Our measured dimer to monomer ratio of the IgG is 0.1%, consistent with the estimate of the provider, <1%, based on size-exclusion chromatography (SEC) (Figure 5). The concentration of a formulated Rituximab obtained by our approach is consistent with the UV−vis absorbance result to about 10% uncertainty (Figure 5).

The second merit of this approach is its speed, where analysis time is minutes to an hour. The third merit of our approach is it eliminates the need for specific protein calibration, which is required for other techniques. For example, UV−vis absorption requires knowledge of the extinction coefficient for each protein. Since only ratios are used in our approach, determination of the absolute number concentration of proteins can be obtained without the need for protein specific loss calibration which may depend in an unknown way on the nature of the ES efficiency,[26,27] particle transportation to the DMA or MS, and the efficiency of particle counting instruments.

A fourth merit is that in addition to the total concentration, the concentrations for each oligomer can be quantitatively determined. This offers the opportunity to directly measure protein aggregation and protein colloidal stability.

To take advantage of the above-described properties requires a stable and monodisperse electrospray generated droplet distribution, which places constraints on the quality requirements for manufactured electrospray capillaries. The salt in protein solution is required to be low enough in order to distinguish protein monomer from higher order aggregates because salt would coat the protein after ES droplet evaporation. Implicit in the method is that we rely on the charge residue mechanism for the droplet formation. Thus, there will be a limit to how small a protein that can be characterized by our approach, before the ion emission mechanism[28] becomes important. This limit value could be ∼6.5 kDa for multiply charged proteins based on literature report.[14] Because we measured singly charged proteins with our approach, this limiting value for molecular weight should be even smaller. Despite this limitation, further refinement can be envisioned in which charge neutralization is performed more rapidly to prevent fission.

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CONCLUSIONS

Traditional approaches for measuring the absolute concentration of proteins require calibration and can be time-consuming. Above, we describe the development of a rapid and accurate method for the absolute quantification of proteins in solution that exploits the droplet entrapped aggregation effect[6] from electrospray processes. The essential feature of this approach is that the observed oligomer/monomer ratio is a unique function of the droplet size and the initial (lower and equal order) oligomer concentrations. Once the droplet size is measured, the initial protein concentration can be extracted from the observed oligomer/monomer ratios. This method is validated using NIST standard reference material (SRM) 927e, (bovine serum albumin (BSA)), a high-purity immunoglobulin G 1κ (IgG), and a formulated Rituximab (Rmab). We anticipate that our approach can be readily applied to obtain the absolute number concentrations of nearly any protein solution, if the charge residue mechanism for electrospray droplet formation holds. The real power of the method is that it requires no specific calibration standards for the specific protein.

ASSOCIATED CONTENT

Supporting Information

Application to quantify the concentration of a high-purity Immunoglobulin G 1κ (IgG), application to quantify the concentration of a formulated Rituximab (Rmab), droplet entrapped aggregation of identical particles (monomers) from droplets of identical size, droplet entrapped aggregation from monodispersed droplet size distribution by a serial dilution, droplet entrapped aggregation from multi-modal droplet size distribution, and detailed data analyses (Excel file). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.L. developed the method. M.L. and J.T. performed the ES-DMA-CPC experiments. J.T., M.L, and M.R.Z analyzed data. M.L., M.J.T, and M.R.Z prepared the paper. All the authors discussed the results and commented on the paper.

Notes

The authors declare no competing financial interest.

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