Flow Interface for Charge Reduced Electrospray of Nanoparticle Solutions

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Abstract

A charge reduction (CR) interface for electrospray ionization was characterized that permits simultaneous analysis of nanoparticle solutions by multiple detection methods. In the direct infusion configuration, a constant flow of analyte solution undergoes electrospray ionization (ESI). The charged aerosol is sampled directly into the atmospheric pressure inlet of a quadrupole time-of-flight mass spectrometer (QTOF) and to a CR device followed by a differential mobility analyzer (DMA) and condensation particle counter (CPC). In the plug injection configuration, analyte solution is injected into a liquid chromatograph. The effluent is split to an evaporative light scattering detector (ELSD) and the ESI interface. The charged aerosol is then sampled through the CR device directly into the CPC. Performance characteristics of the two configurations were studied with sucrose and protein solutions. When a liquid flow rate in the low μL/min range was used, the reconstructed droplet size distribution from the ESI interface had an average diameter of 184 nm with a geometric standard deviation of 1.4. For the first configuration, the linear working range was wider for ESI-MS than CR-DMA-CPC. For the second configuration, the detection efficiency, defined as the fraction of molecules flowing through the ESI interface that are ultimately detected by the CPC, was on the order of 10^{-6}. Simultaneous measurements with ELSD and CPC were consistent with analyte molecular size and may provide a means of estimating the size of unknown particles.

Introduction

Charge reduction (CR) via ion-ion interaction inside a mass spectrometer was originally introduced by McLuckey et al. [1, 2] as a means to overcome problems associated with multiply charged ions produced by electrospray ionization (ESI). [3] An alternative CR approach was reported by Zarrin and co-workers[4] where a radioactive source neutralized highly charged ions at atmospheric pressure. Although atmospheric CR can be used as an ion source for mass spectrometry,[5] singly charged ions of macromolecules and other nanoparticles fall outside the m/z range of most mass spectrometers. For this reason, atmospheric CR is usually combined with a differential mobility analyzer (DMA) and condensation particle counter (CPC) to separate and detect singly charged ions in the kilodalton to gigadalton mass range. [6, 7] A commercial implementation of this approach is the gas-phase electrophoretic mobility molecular analyzer (GEMMA),[8, 9] which incorporates a removable sample holder, a nanospray source and a neutralization chamber equipped with a ^{210}Po radioactive charge neutralization source. Kaufman and co-workers used this technique to analyze globular proteins[8] ranging from 5.7 to 669 kDa in molecular mass. Other applications of GEMMA include DNA,[10] intact virons, [6, 7, 11, 12] protein complexes,[7, 11] water-soluble and water-insoluble polymers,[13, 14] dendrimers,[15] and the production of protein nanoparticles for instrument calibration.[16] A limitation of GEMMA is that samples must be analyzed by direct infusion, so applications

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requiring plug injection, for example chromatography and other on-line separation methods, are not possible.

Plug injection with subsequent particle analysis was studied by Allen and Koropchack[17, 18] who used a nebulizer to generate particles that were detected with a home-built CPC referred to as condensation nucleation light scattering detector (CNLSD). Particle detection by CNLSD was shown to be more sensitive than a commercial evaporative light scattering detector (ELSD). This approach was extended to a variety of separation methods, [19−22] Plug injection with CR electrospray was studied by Lewis et al. who used size exclusion chromatography (SEC) and reverse phase liquid chromatography (RPLC) to separate proteins with subsequent detection by CPC. [23, 24] For RPLC, the CPC response was found to be linear only over a narrow range of concentrations. The limited working range was attributed to aggregate formation in the liquid phase and a nonlinear dependence of the monomer to multimer ratio with concentration.

In the work reported here, a new ESI interface for plug injection was investigated. The CR region was moved further downstream from the electrospray tip than in previous work to allow Rayleigh breakup of droplets/particles to assist the dissociation of aggregates. The design also permitted multiple detection methods to be used simultaneously so that the results could be combined to give more information about the analyte.

Experimental Section

Materials

Insulin from bovine pancreas, lysozyme from chicken egg white, bovine serum albumin, carbonic anhydrase I (human erythrocytes), and thyroglobulin from bovine thyroid were obtained from Sigma-Aldrich (Saint Louis, MO). Sucrose, ammonium acetate, trifluoroacetic acid, 1-butanol, and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Protein stock solutions were prepared in 20 mM ammonium acetate pH 8, with the exception of insulin which was prepared in 0.1% TFA. Prior to use, all mobile phases and sample solutions were filtered through a 0.22 µm membrane filter (Millipore Corporation, Billerica, MA) and sonicated under vacuum for at least 10 minutes.

ESI Interface

A virgin electrical grade PTFE bar 6 in × 4 in × 2 in (McMaster-Carr, New Brunswick, NJ) was machined to firmly house a Micromass QTOF Ultima API-US nanoflow source (Waters, Milford, MA) and subsequently fastened to an XY positioning slide (McMaster-Carr). For micro flow operation, the source was equipped with a 2 in stainless steel megaflow capillary (125 µm ID × 320 µm OD, Waters, Milford, MA). For nano flow operation, the source was equipped with a fused-silica nano spray tip (50 µm ID, 15 µm tip ID, 360 µm OD, New Objective, Woburn, MA). Because of excessive clogging of the nano spray tip, most experiments were performed with micro flow. High voltage was applied to the source via a small piece of stainless steel bolted into the PTFE bar that was also connected to a power supply (Stanford Research Systems, Inc., Sunnyvale, CA, model PS350/5000 V-25 W). The block (PTFE bar, XY stage, and ESI source) was housed within a clear acrylic tube (7.5 in ID, 8 in OD, 12 in length, McMaster-Carr) designed to allow orthogonal aerosol sampling from the spray tip. A grounded counter electrode was bolted into the tube, approximately 30 mm from the spray tip. An adjustable 45x dual purpose microscope (Edmund Optics, Barrington, NJ) illuminated by a 300-watt flexible fiber optic illuminator system (Edmund Optics) was placed on top of the acrylic chamber to visually monitor the spray. A stainless steel sampling cone (2-in long, 3 mm ID) connected to an in-line ^{210}Po source (10 mCi, NRD, Grand Island, NY) for

charge reduction (CR) was positioned approximately 5 mm from the axis of the spray tip and 20 mm orthogonally to the aerosol plume.

Direct infusion

Figure 1a shows the configuration used for direct infusion experiments. Analyte solution was continuously fed into the ESI interface with a syringe pump (Harvard Apparatus, Cambridge MA). The electosprayed aerosol was sampled orthogonally by a QTOF mass spectrometer (Ultima API-US, Waters, Milford, MA) equipped with MaxEnt1 software for data acquisition and processing, and a scanning mobility particle sizer consisting of a differential mobility analyzer (DMA) and condensation particle counter (CPC) (Models 3080 and 3025A, TSI, Inc., Shoreview, MN). The QTOF sampling cone was held at a potential of 60 V in all cases and the ESI source at 3.5 and 4.7 kV, for analysis in nano and microspray, respectively. While the same ESI plume was simultaneously sampled by both instruments, the aerosol flow rate into the SMPS was approximately 1.5 L/min, much higher than the estimated flow rate into the MS (about 0.1L/min). Single-component protein solutions were prepared in 30% methanol-water and infused at the rate of 4 µL/min unless otherwise noted. Sucrose solutions of 0.25 and 0.02 percent in 30% methanol were infused at micro (4µL/min) and nano (250 nL/min) flow rates, respectively. ESI sources are usually operated with desolvation/nebulizing gas at a high flow rate for efficient spray and CO_2 to prevent electrical discharge. To assess the effect of nebulizing gas on spray efficiency and signal detection, a solution of lysozyme was analyzed with and without nitrogen at 60 psi. Use of the nebulizing gas was found to severely reduce the CPC response. Corona discharge [7, 8] was assessed by applying potential to the ESI source and analyzing the size distribution of the aerosol around it. At both 3.5 and 4.7 kV (nano and microspary modes, respectively), a "clean" particle size spectrum characteristic of background air was observed, indicating the absence of corona discharge. Based on these preliminary results, the ESI interface was operated at room temperature, without pneumatic assistance. The DMA was operated in the high-flow mode, with a sheath and an aerosol flows of 20 and 1.9 L/min, respectively, to mimic the conditions used by Kaufman and co-workers.[8] The total scanning time was 135 s, over a range of 2 to 30 nm. QTOF mass spectra were acquired from 200 to 4000 *m/z*.

Plug injection

Figure 1b shows the configuration used for plug injection. Samples were injected onto a liquid chromatograph system (Models LC-20AD, CTO-20A, CBM-20A; Shimadzu, Columbia, MD). The HPLC effluent was split between an evaporative light scattering detector (ELSD-LTII, Shimadzu, Columbia, MD) and the micro flow ESI interface. The electrosprayed aerosol was sampled orthogonally through the radioactive neutralizer into the CPC. Some experiments were performed with a 4.6×300 mm, 3 µm size exclusion chromatography (SEC) column (Sepax Technologies, Inc, Newark DE) to separate proteins prior to detection by ELSD and CR-CPC. SEC was conducted using a mobile phase of 100 mM ammonium acetate pH 7 to mimic the column manufacturer's recommendation of 150 mM phosphate buffer at pH 7 and the HPLC flow rate was set to 300 µL/min. An adjustable splitter was used to direct approximately 50 µL/min of the effluent to the ESI interface, delivering the remaining 250 µL/min to the ELSD. The ELSD was operated at a gain of 9 (near maximum sensitivity) and a temperature of 60 ° C to efficiently evaporate the relatively concentrated buffer solution. The ESI voltage was set at 4.9 kV and the CPC was operated in the high flow mode.

Results and discussion

Direct Infusion

The aerosol produced by the ESI interface used in this work was characterized in both nano and micro flow modes by direct infusion of a sucrose solution in 30% methanol/water. The dry

particle size distribution measured with the DMA-CPC was used to reconstruct the original droplet size distribution based on the sucrose concentration. The dry and wet particle diameters are related by the equation: [25]

$$\frac{d_{\text{dry}}}{d_{\text{wet}}} = \left[(f_{\text{solute}}) \frac{\rho_{\text{dry}}}{\rho_{\text{wet}}} \right]^{1/3}$$

where d_{dry} and d_{wet} are the dry and wet particle diameters, f_{solute} is the weight fraction of the solute (sucrose), and ρ_{dry} and ρ_{wet} are the dry and wet particle densities. Figure 2 shows the results. As expected, the dry particle number distribution for micro flow operation (Fig. 2a) is shifted to larger particle diameter than the distribution for the nano flow operation (Fig. 2b) and the micro flow distribution is wider. The reconstructed size distributions for the original wet (droplet) aerosols are shown in Figures 2c and 2d. The nano flow distributions are similar to those reported in the literature. [7, 8] The micro flow distributions are somewhat different from those reported in the literature in that our measurements show little evidence for droplets larger than about 300 nm dia. [26] The apparent lack of large droplets with the apparatus in Figure 1 may be due to evaporation and Raleigh disintegration prior to neutralization in the CR device. Alternatively, it is possible that large droplets are not efficiently sampled into the CR device.

When nanoparticle solutions are electrosprayed, the larger droplet volumes for micro flow operation make it more likely that multiple particles exist within a single droplet. This phenomenon is illustrated in Figure 3 where the dry particle size distributions for nano and micro flow operation are compared for electrosprayed solutions containing bovine serum albumin. Both distributions are dominated by a peak at 6.9 nm corresponding to an individual bovine serum albumin molecule. The measured mobility diameter is within experimental error of literature values. [7, 8, 27] A second peak at ca. 8.3 nm corresponds to the dimer. The similar relative intensities of the monomer and dimer peaks in the nano and micro flow distributions suggest that the dimer species arises mostly from aggregation in solution rather than the random probability that two molecules co-exist within a single droplet [24]. The multimer region in the 10 to 18 nm range is significantly enhanced in the micro flow distribution and most likely arises from large droplets where several BSA molecules randomly co-exist [23]. It should be noted that a 16 nm dry particle would contain approximately 17 BSA molecules. For the solution concentration electrosprayed, a particle containing this number of molecules would have a diameter of about 700 nm, which is beyond the upper end of the droplet distribution in Figure 2c suggesting that relative to the sucrose solutions either additional aggregation has occurred or the droplet size distribution has changed with solution conductivity. While multimer formation is greater with the micro flow mode, monomer and dimer peaks still dominate the size distribution and the measured mobility diameters are accurate.

The direct infusion configuration also provides the opportunity to directly compare ESI mass spectra and CRES size distributions from the same electrosprayed aerosol. Figure 4a shows the mass spectrum of lysozome obtained in the micro flow mode. The spectrum is similar in character to that obtained with a conventional ESI interface. The highest charge state in this spectrum is +11, which is typical of electrosprayed proteins where on average approximately one positive charge is imparted for every 1000 Da. Figures 4b and 4c show plots of particle number concentration (SMPS-CPC detection) and ion signal intensity (MS detection) vs. solution concentration obtained for micro flow operation. While the detection limit is a bit lower for DMA-CPC detection (0.02 µM) than MS detection (0.07 µM), the upper concentration limit of linearity is about an order of magnitude in concentration greater for MS detection. The limit of linearity, defined as the concentration where the correlation coefficient for the calibration line falls below 0.99, is about 1 µM for DMA-CPC detection and 10 µM for MS detection.

Several possibilities exist for the wider linear range with MS detection. First, particle coagulation may occur inside the DMA-CPC apparatus since the transit time through it is longer than for MS. However, the coagulation rate for lysozyme under the conditions of Fig. 4b, estimated by the procedure described by Hinds,[28] is less than 10 particles for every 120 s and cannot explain the magnitude of nonlinearity observed. Another possibility is that dimer and multimer species in the liquid phase become more plentiful as the solution concentration increases. Formation of these species would decrease the particle number count in SMPS-CPC detection, but would not affect the ion signal intensity in MS detection if the multimers are dissociated by high energy collisions in the atmospheric pressure inlet. To test this possibility, the data in Fig. 4b were re-plotted as volume/cm^3 vs. concentration. If increasing multimer formation with increasing solution concentration was the reason for nonlinearity, then one would expect the volume concentration of the aerosol to be linear with solution concentration. However, this plot (not shown) becomes nonlinear in the same solution concentration range as Fig. 4b, so multimer formation appears not to be the source of nonlinearity. A third possibility is an aerosol concentration dependent change in charge neutralization during DMA-CPC analysis. If the number of charged protein particles at high concentration exceeds the neutralization capacity of the polonium source, then fewer particles will acquire a single charge and pass through the DMA at the proper voltage for detection by the CPC. The net result would be nonlinearity as observed in Fig. 4b. This explanation is consistent with plug injection results (see below) showing a wider range of linearity. In the plug injection mode, all particles are detected by the CPC regardless of charge. No matter what the mechanism, it is clear that the loss of linearity for CR analysis is not a function of the ESI interface itself, since a broad linear range is obtained with direct sampling into the QTOF mass spectrometer.

Plug Injection

System performance was further investigated by introducing analyte through a HPLC equipped a 20 µL-injection loop. In this configuration (Figure 1b), the HPLC effluent was split between the ESI interface and an ELSD and the electrospray aerosol was sampled by CR-CPC. The DMA was not used in this configuration because the sample elution time window was much shorter than the DMA scan time. Initially, injections were performed without a column to assess signal linearity. Stock solutions of insulin, carbonic anhydrase I, and bovine serum albumin were prepared and immediately serial-diluted to yield working solutions of 0.086 to 22.1 µM in 30% methanol/water. For each protein, 20 µL of the dilute solution was injected and split between the ELSD and CRES source under the conditions described in the experimental section. The time-dependent CPC raw data were processed by 1) correcting the CPC unit count (particles/cc/s) for background noise and converting each negative value to zero, 2) multiplying the particle concentration rate by the measured aerosol flow rate (1.5 L/min) and 3) integrating the count rate (particles/s) over the elution peak to obtain the total number of particles (molecules) detected. Figure 5a is a plot of particle count rate (particles/s) vs. time for an injection of bovine serum albumin. A single peak is observed whose area is related to the amount injected. Similar plots were obtained for all three proteins over a wide range of concentrations. These data are shown in Figure 5b, plotted in units of total particles (molecules) detected integrated over the elution band vs. molecules flowing through the ESI interface (total molecules injected multiplied by the fraction split to the ESI interface). In ESI, larger particles (proteins) typically reach higher charge states than smaller particles. Therefore, more charge must be removed per particle by the polonium source and it becomes easier to exceed the neutralization capacity as the particle concentration increases. This phenomenon provides a reasonable explanation why nonlinearity in Figure 5b becomes more pronounced as the particle size increases.

The linear working range is wider for plug injection than direct infusion. There are two contributions to the wider linear working range. First, the detection limit decreases for plug

injection because removing the DMA eliminates the dilution factor associated with sheath vs. sample flows, and both charged and uncharged particles are detected. Second, the high solution concentration limit of linearity is greater for plug injection than direct infusion – the highest concentration data points for carbonic anhydrase and insulin in Fig. 5b correspond to peak solution concentrations in the elution band of about 20 µM, well beyond the limit of linearity for lysozyme in Fig. 4b.

The detection efficiency, defined as total particles (molecules) detected by the CPC divided by the total number of molecules injected post-split, was determined from the slope of the initial linear portion of each plot. The detection efficiency was found to increase with increasing molecular size: 5.0×10^{-7} for insulin, 7.6×10^{-7} for carbonic anhydrase and 3.2×10^{-6} for bovine serum albumin. This increase is consistent with the work of Lewis et al [23] showing that smaller and lighter molecules diffuse more rapidly and are more likely to experience wall loss in the transfer line. In the current setup, the detection efficiency is influenced by several size dependent processes: 1) sampling from the electrospray aerosol into the transfer line, 2) transmission through the transfer line and CPC, and 3) particle growth and detection within the CPC. Of these, the first process is most likely the greatest contributor to particle loss, since the majority of the electrospray current flows to the counter electrode. For the size range of particles studied, the second and third processes are likely to cause less than one order of magnitude loss.[9]

Size exclusion chromatography was performed to separate a protein mixture comprising carbonic anhydrase I and thyroglobulin. Stock solutions were prepared as described previously, but serial-diluted in 20 mM pH 7 ammonium acetate buffer to produce working solutions of 0.041 to 5.20 µM. SEC analysis required a mobile phase of 100 mM ammonium acetate pH 7 flowing at a rate of 50 µL/min into the electrospray interface. Under these conditions, the observed spray was very different from that produced with 30% methanol/water flowing at a rate of 4 µL/min. The shape of the liquid at the tip was neither cone-jet (i.e. a stable Taylor cone) nor silver bullet (i.e. a stable liquid meniscus in the shape of a bullet) as observed by Chen.[25] Spraying in the cone-jet mode (at much lower flow rate) was first attempted; but this option was abandoned due to excessive band broadening. Nonetheless, particles were efficiently detected as illustrated below.

A CPC chromatogram of a mixture of the two proteins (0.650 µM each within the injected solution) is shown in Figure 6a. The nonzero baseline is caused by particle formation via electrospray of the solvent. The ELSD chromatogram of the same mixture is shown in Figure 6b. Calibration plots of CPC response to amount injected were linear for both proteins up to the highest solution concentration studied (5.2 µM). The linear working range is comparable to that of Figure 5 and suggests that despite the non-ideal spray conditions, individual protein molecules were generated by the spray source. Absolute detection limits for CPC detection were in the sub picomole range after the ESI-ELSD split, 280 fmol for carbonic anhydrase and 140 fmol for thyroglobulin.

As expected, the thyroglobulin and carbonic anhydrase peaks in Figure 6a are of similar magnitude since similar moles (molecules) were injected and the CPC detector responds to number of particles (molecules). Also as expected, the thyroglobulin peak is larger by about a factor of 2–3 since its molecular size and corresponding detection efficiency are greater. While in principle the CPC signal in Figure 6a includes both monomers and multimers, multimer formation is not expected to represent a significant fraction of the peak area since the analysis was performed within the linear working range of the experiment. Also, the lack of additional peaks in the chromatogram indicate that multimers, if they exist in solution, do not migrate in distinct bands from the respective monomers. The peaks in Figure 6b are quite different in signal intensity with the thyroglobulin peak about 24 times larger than the carbonic anhydrase

peak. Again, this result is expected since the ELSD generates "large" particles (micron and submicron dimensions) and the scatter signal depends on the total mass concentration of nonvolatile analyte in solution. The relative magnitudes of the peaks in Figure 6b match the difference in molecular weight: the molecular weight of thyroglobulin is about 23 times larger than that of carbonic anhydrase.

Conclusion

The experimental configurations studied in this work can be applied to variety of separation methods and a wide range of nanoparticles in solution such as individual proteins, biomolecular assemblies, virons, industrial polymers, and natural and engineered nanoparticles. In principle, simultaneous detection by ELSD and CPC in the plug injection configuration can give an estimate of the mass of a single particle (and its size if the density is known or inferred) provided that 1) injections are made at concentrations in the linear working range of the detectors and 2) and the detection efficiency vs. size is accurately known.

Acknowledgments

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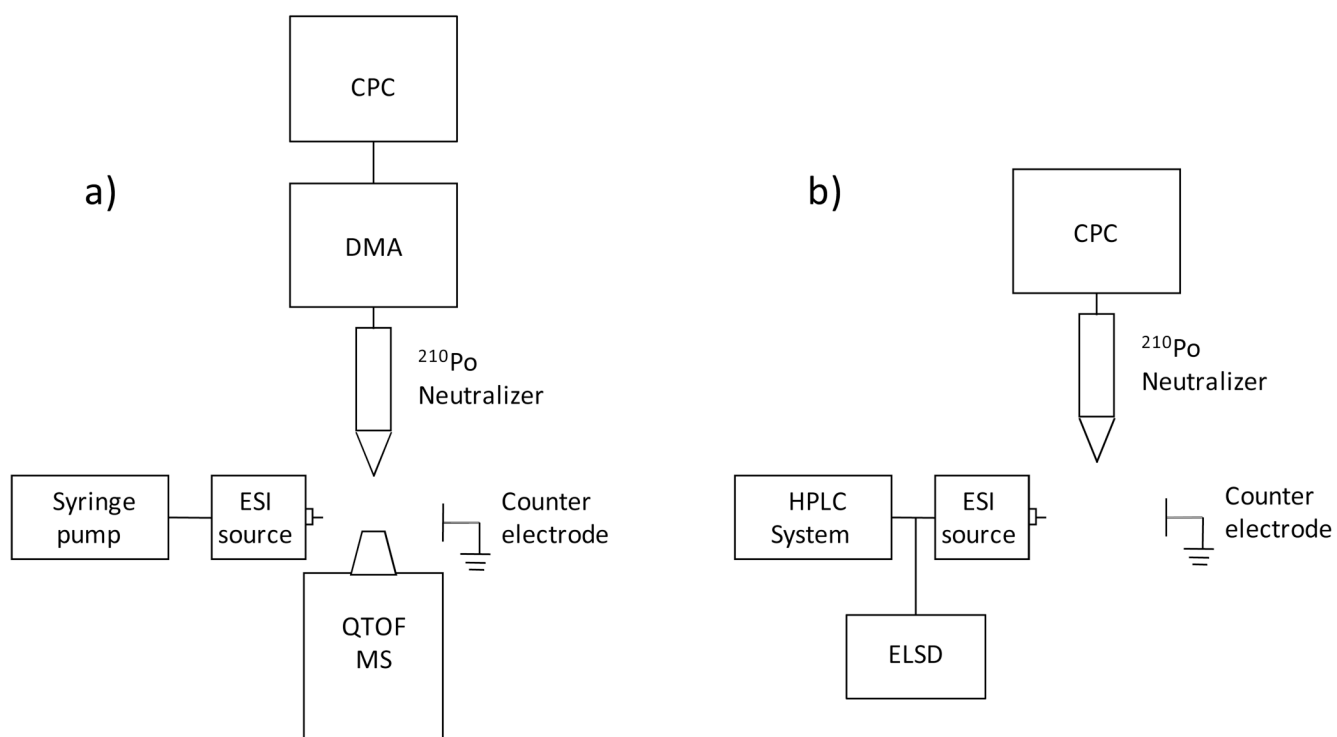
Figure 1.
Two configurations of the ESI interface used in this study: a) direct infusion and b) plug injection.
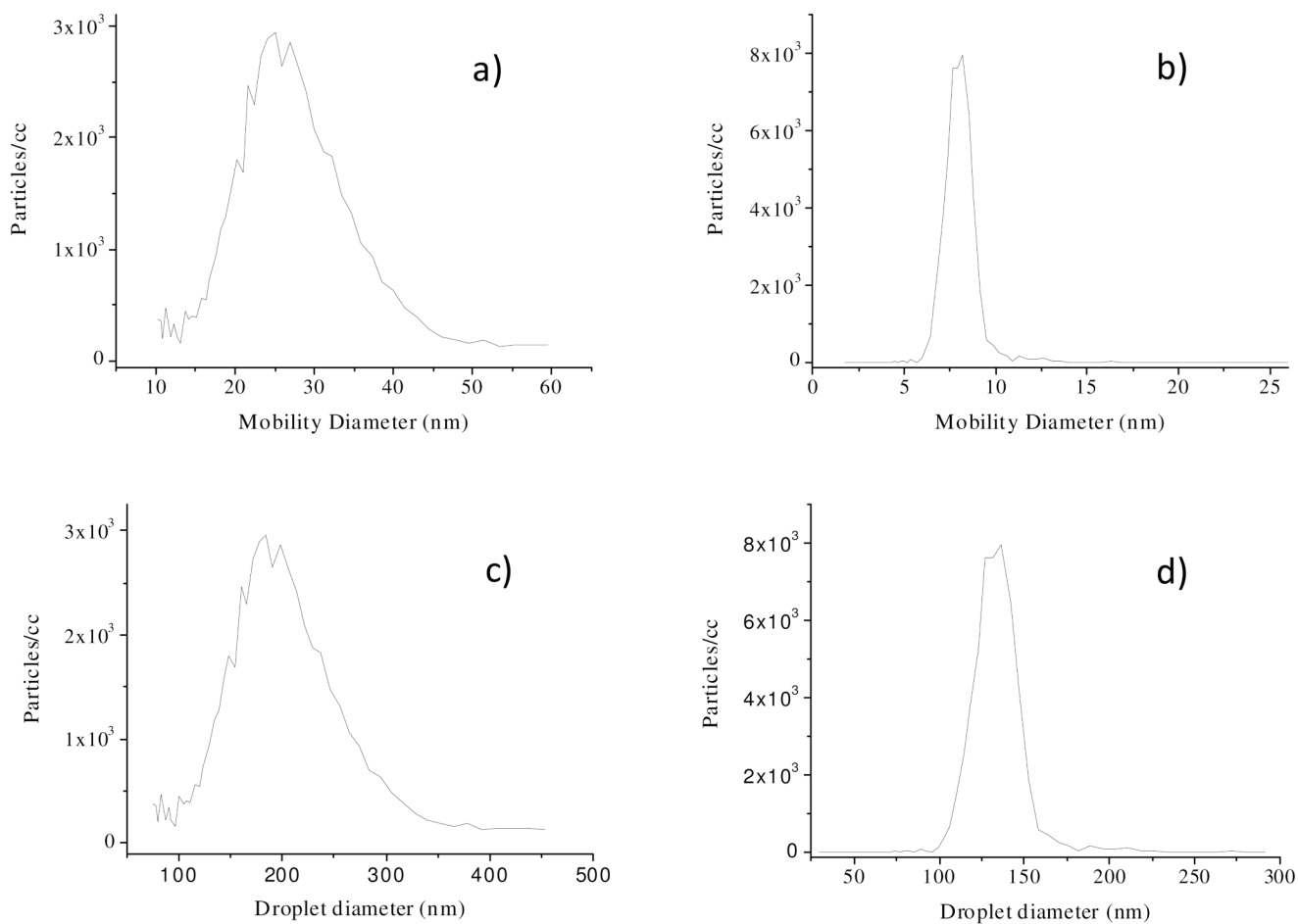
Figure 2.
Particle size distributions: a) dry sucrose particle size distribution in micro flow mode, b) dry sucrose particle size distribution in nano flow mode, c) reconstructed droplet size distribution in micro flow mode, d) reconstructed droplet size distribution in nano flow mode. Sucrose concentrations were 0.25 and 0.02 wt. %, respectively, for the micro and nano flow experiments.
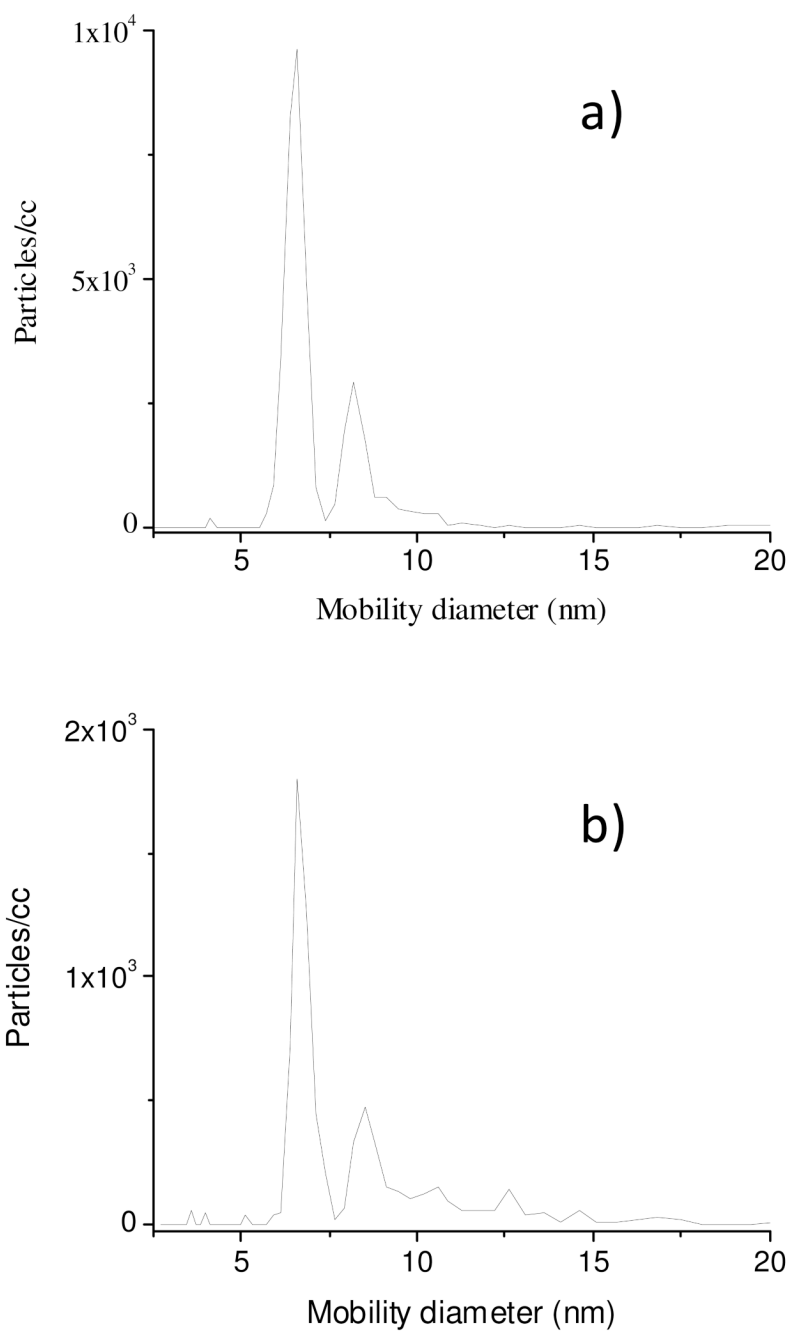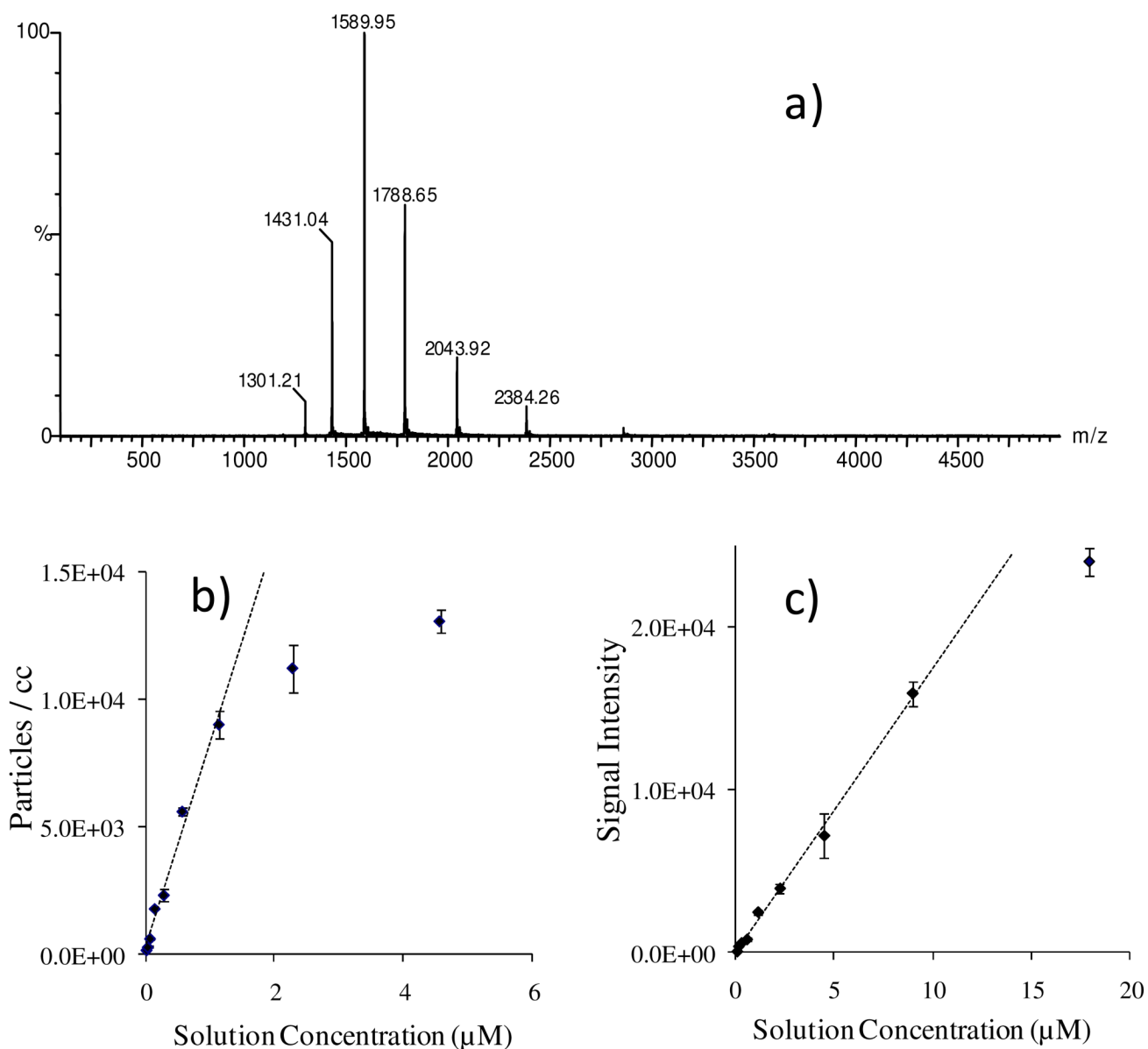
Figure 3.
Size distribution of bovine serum album in a) nano flow mode, 0.52 µM in 30% methanol, and
b) micro flow mode, 0.15 µM in 30% methanol.

Figure 4.
a) Lysozyme QTOF mass spectrum showing the +11 (1301 m/z), +10 (1431 m/z), +9 (1590 m/z), +8 (1789 m/z), and +7 (2044 m/z) charge states. Calibration curves for lysozyme with b) SMPS-CPC and c) QTOF MS detection. Each data point is the average of three replicate measurements. The SMPS and QTOF data were obtained simultaneously for the same solutions.
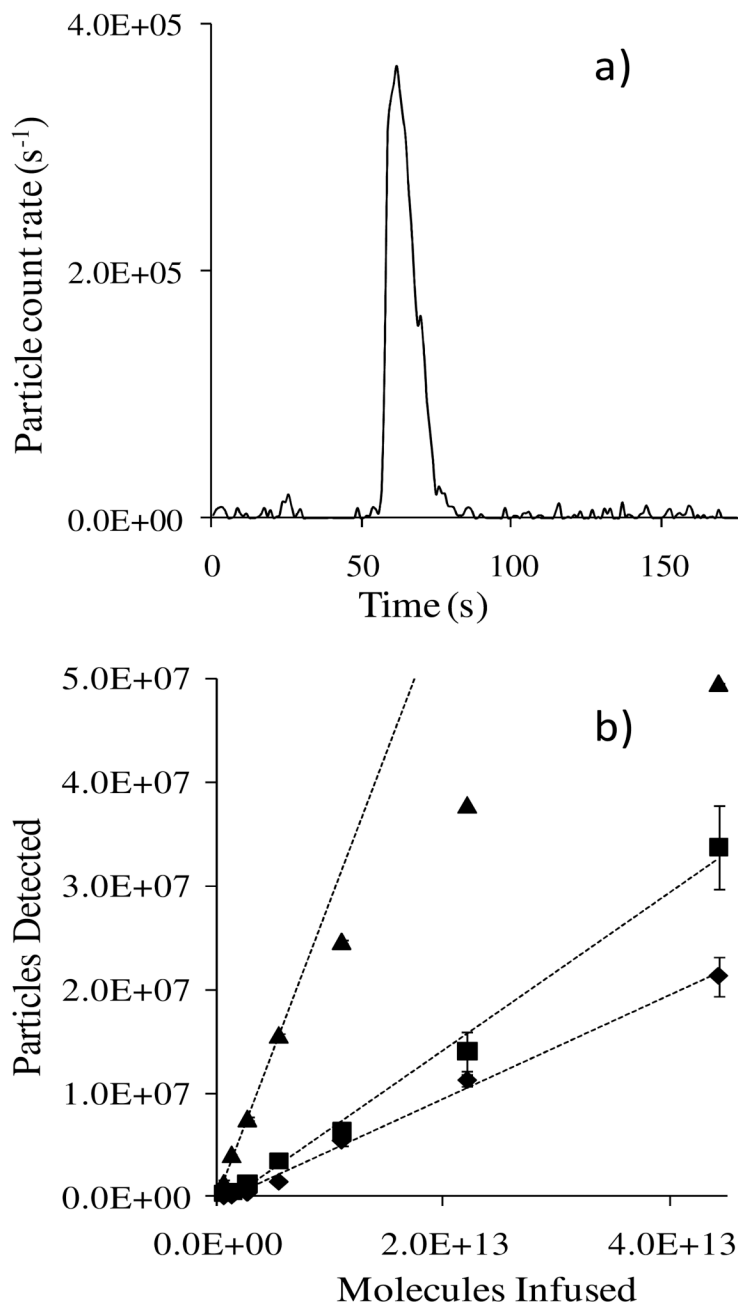
Figure 5.
a) CPC chromatogram of bovine serum albumin, 0.690 μM in 30% methanol. b) CPC response (total particles detected within the elution peak) for insulin (diamonds), carbonic anhydrase I (squares), and bovine serum albumin (triangles) as a function of molecules infused into the ESI source.
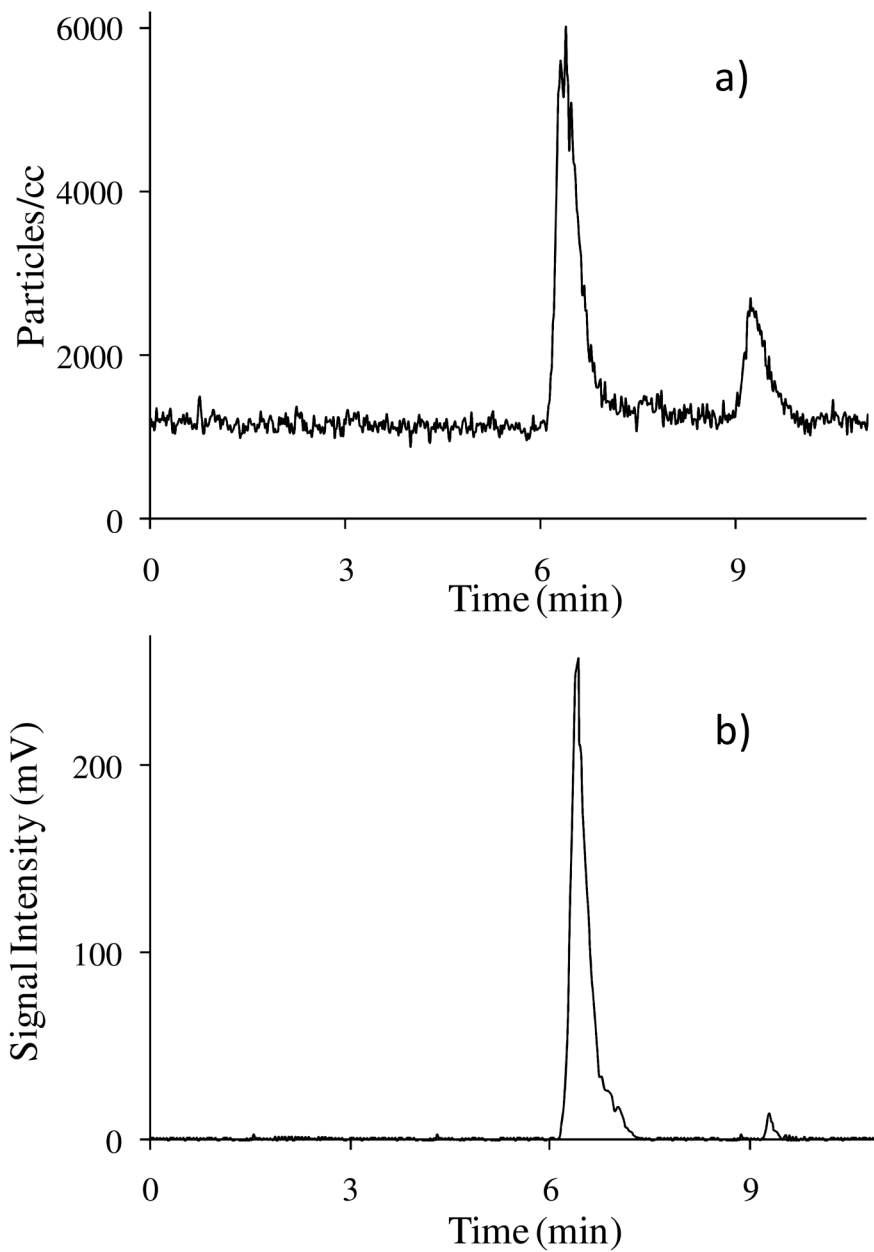
Figure 6.
Size exclusion chromatograms with a) CPC detection and b) ELSD detection of 0.650 µM carbonic anhydrase I (second, smaller peak in both cases) and thyroglobulin in 20 mM ammonium acetate pH 7. Mobile phase: 100 mM ammonium acetate pH 7. Injection volume: 20 µL. ELSD/CPC split ratio: 6:1.