Surface Modified Nano-Electrospray Needles Improve Sensitivity for Native Mass Spectrometry

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ABSTRACT: Native mass spectrometry (MS) and charge detection-mass spectrometry (CD-MS) have become versatile tools for characterizing a wide range of proteins and macromolecular complexes. Both commonly use nano-electrospray ionization (nESI) from pulled borosilicate needles, but some analytes are known to nonspecifically adsorb to the glass, which may lower sensitivity and limit the quality of the data. To improve the sensitivity of native MS and CD-MS, we modified the surface of nESI needles with inert surface modifiers, including polyethylene-glycol. We found that the surface modification improved the signal intensity for native MS of proteins and for CD-MS of adeno-associated viral capsids. Based on mechanistic comparisons, we hypothesize that the improvement is more likely due to an increased flowrate with coated ESI needles rather than less nonspecific adsorption. In any case, these surface modified needles provide a simple and inexpensive method for improving the sensitivity of challenging analytes.

INTRODUCTION

Native mass spectrometry (MS), which uses non-denaturing ionization prior to mass analysis, has become a useful tool for characterizing protein and macromolecular complexes.[1-3] For applications like intact adeno-associated viral (AAV) capsids that yield unresolvable charge states, native MS is coupled with charge detection-mass spectrometry (CD-MS) to simultaneous measure the charge and *m/z*, enabling mass analysis for highly complex systems.[1-2,4] AAVs are used for gene therapy, vaccines, and drug delivery,[5-8] and CD-MS provides direct ratios of empty versus filled capsids.[9-10] However, CD-MS analysis of AAVs often requires relatively high concentrations of sample in the range of $5–100\times10^{12}$ capsids per ml, which is roughly 10–300 nM.[11-13] More broadly, native MS is also limited in sensitivity and usually requires approximately micromolar concentrations of analyte.[14] A comparable method, mass photometry, has lower nanomolar levels of detection for protein complexes but with lower resolution than native MS.[15-16]

Both native MS and CD-MS commonly use static nano-electrospray ionization (nESI) from pulled capillary needles. One possible reason for the limited sensitivity is nonspecific adsorption or other unfavorable interactions with the surface of the borosilicate nESI needles.[17-19] A range of important targets are prone to nonspecific adsorption to glass, including plasma proteins, antibodies, lipoproteins, and neuronal peptides.[20-23] Due often to their positive charge at neutral pH, these proteins may adsorb and/or unfold on the negatively charged glass surface of nESI needles.[17,19-24] Similarly, because AAVs can adsorb to glass, plastic, and metal surfaces,[25-27] we hypothesized that the nonspecific adsorption of AAVs with the borosilicate glass needles used for nESI could be limiting the sensitivity of CD-MS.

Williams and coworkers previously showed that surface interactions with the glass needles can denature positively charged proteins. This surface-induced unfolding is especially prevalent with submicron nESI emitters, which have higher surface area to volume ratio and lower diffusion length from the bulk solution to the glass surface.[17,19] They proposed that passivating the surface of the glass nESI needles would nullify the adsorption and unfolding of analytes to the glass surface.

To improve nESI, different needle geometries have been tried and optimized for native MS.[19,28-29] Microfluidic devices modified for nESI have used polyfluorinated coatings to lower the wettability of the glass surface, which allowed Taylor cones to be formed at lower spray voltages.[30] Coatings on the outside of the nESI emitter have been used to improve electrospray,[31-32] but fewer methods have attempted modifications at the sample/emitter interface on the inside of the nESI needle to lower nonspecific adsorption.[33]

To increase the sensitivity and improve robustness of nESI of proteins and AAVs, we modified the surface of borosilicate capillaries with polyethylene glycol (PEG) 6-9-dimethylchlorosilane or (tridecafluoro-1,1,2,2-

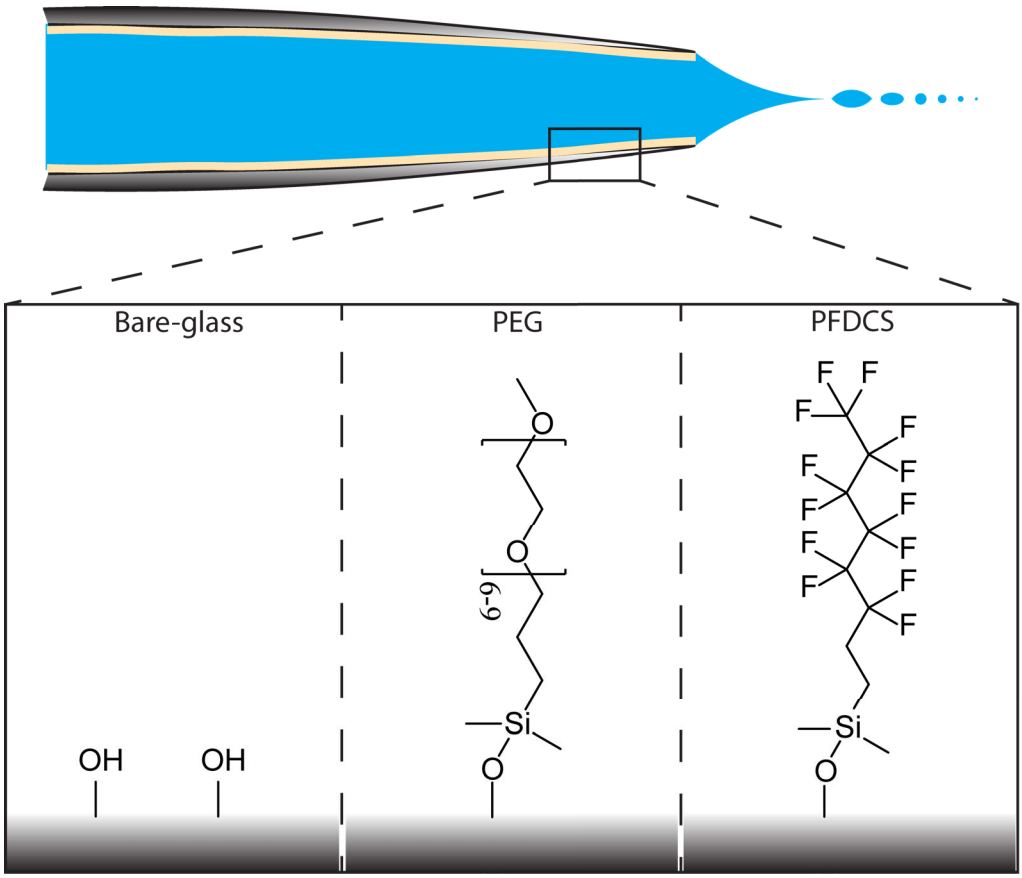

Figure 1: Schematic for surface modified needles. Unmodified bare silanol groups are depicted to the *left* with the PEG modification in the *middle* and the PFDCS modification to the *right*.

tetrahydrooctyl) dimethyl-chlorosilane (PFDCS). These coatings were chosen because they are commercially available, and similar coatings have previously been shown to form inert monolayers that reduce nonspecific adsorption on surfaces.[34-35] We then pulled them the coated capillaries into nESI needles (Figure 1). Recently a similar approach used a hydrophilic coating to reduce nonspecific adsorption of AAVs to pipette tubes and quantitative polymerase chain reaction plates.[36] Also, glass surfaces have previously been passivated for other applications using adsorbed bovine serum albumin (BSA),[32,37] PEG,[23,38] gold nanoparticles,[21,39] and phospholipids.[21] We chose covalent attachment of PEG or PFDCS to the silanol surface to ensure there is no contamination of PEG or PFDCS with the sample. We tested the improvements in sensitivity for several standard proteins and an AAV sample on needles with different geometries. We also explored several potential mechanisms, which revealed that our initial hypothesis is likely either incorrect or incomplete. Although we set out to improve signal by reducing nonspecific adsorption, our data suggests that improvements in flow rate likely contribute to the improvements.

METHODS

Materials. Ammonium acetate, ubiquitin, BSA, and lysozyme were purchased from Sigma-Aldrich. Glass Capillaries were purchased from World Precision Instruments. AAV2 capsids were purchased from Virovek. Vivaspin 100 kDa molecular weight cutoff filters were purchased from Sartorius. Bio-spin 6 columns were purchased from Bio-Rad. 2-[methoxypoly(ethylenoxy)6-9propyl] dimethyl chloro-silane (PEG6-9-dimethyl chlorosilane) and (tridecafluoro-1,1,2,2-tetrahydrooctyl) dimethyl chlorosilane (PFDCS) were purchased from Gelest Inc. Anhydrous acetonitrile (ACN) was purchased from Supelco Inc. Acetone was purchased from EMD Chemical Inc. Ethanol was purchased from Decon Laboratories.

Protein and AAV Capsid Preparation. BSA and lysozyme were buffer exchanged using two consecutive Bio-spin 6 columns (BioRad) into 0.2 M ammonium acetate. AAV capsids were buffer exchanged as previously described.[40] Briefly, AAV capsids were buffer exchanged by diluting the stock capsids with 0.2 M ammonium acetate and then concentrated using a 100 kDa molecular weight cutoff filter at least two consecutive times. Concentrations of AAV2 were calculated by first denaturing the viral capsids by adding 0.1% SDS and heating to 75 ^{0}C for 10 minutes then taking the average of three A280 measurements with a Denovix Model DS-11+ spectrophotometer.[41] The extinction coefficient for denatured AAV2 at 280 nm has been reported as 6.61×10^{6} M^{-1} cm^{-1}.[41] Avogadro's number was then used to convert to capsids per ml based off of the measured molarity. This assumes that all the viral proteins form capsids and have a correct stoichiometry, so this is a rough estimate for viral capsid concentration.

Capillary Coating. The procedure for capillary coating was adapted from previously described methods.[34] Capillaries were coated prior to pulling into nESI needles. First, borosilicate glass capillaries were cleaned, and the surface was activated by immersion in a 1 M HNO_3 solution for 30

min. Note, this is a strong acid solution and should be handled with care. Next, the capillaries were rinsed consecutively with nanopure water and 100% ethanol before being dried in a vacuum overnight. Once dried, the glass capillaries were quickly submerged in 2% PEG6-9-dimethylchlorosilane in dry acetonitrile (v/v). The reaction was allowed to proceed for 12 h at room temperature. For the PFDCS modification, the glass capillaries were submerged into 2% PFDCS in dry toluene (v/v). The reaction with PFDCS was allowed to proceed for 6 hours at room temperature.

Following the surface modification, excess silane was removed with successively rinsing with acetonitrile/toluene, acetone, water, and ethanol, and the capillaries were dried and stored in vacuum before being pulled into nESI needles with a P-97 or P-1000 pipette puller (Sutter Instruments). Control needles were pulled with the same program but used capillaries that lacked the coating. All coated capillaries were acid washed, but the control capillaries were not. Unless otherwise noted, capillaries were pulled using the standard pulling program (see Table S1) and were manually clipped under a microscope. Needles with fixed geometries of either 2 µm or 0.1 µm were pulled using different programs (Table S2 & S3) and did not need manual clipping.

It was important to keep the modified capillaries in a vacuum or a desiccator for long term storage. Even in a dry climate, needles left on the bench for a week reacted with water vapor in the air and released PEG that contaminated the sample to some degree. To help readers reproduce the method, a step-by-step protocol for preparing surface modified needles and 0.1 µm needles are provided in the Supplemental Methods.

Native MS of BSA, Lysozyme, and Ubiquitin. Mass spectra were collected with a Q-Exactive HF quadrupole-Orbitrap mass spectrometer equipped with ultra-high mass range (UHMR) modifications (Thermo Fisher Scientific, Bremen). BSA and lysozyme were diluted to 500 nM prior to MS analysis. Five replicate measurements were collected with the control and chemically modified needles, alternating between each during MS analysis. Mass spectra were collected for 3 minutes. MS parameters of note include a trapping gas setting of 3 and a capillary temperature of 200 ^{0}C. The high-collisional dissociation (HCD) cell and in-source trapping (IST) voltages were set to 0 V. The source fragmentation was set to 30 V for BSA and 0 V for lysozyme and ubiquitin. Detector optimization and transfer optics were set to low *m/z*, and the spray voltage was set to 1.1 kV. The resolution was set to 15,000 for BSA and 60,000 for lysozyme and ubiquitin. Positive ionization mode was used for all MS experiments.

For BSA, lysozyme, and ubiquitin the signal intensity was calculated by summing the 3-minute dataset in Thermo QualBrowser and measuring the intensity of the most abundant charge state, which was +8 for lysozyme, +5 charge state for ubiquitin, and +17 or +16 for BSA. Error bars indicate the standard deviation for control and surface modified needles (n=5 for each).

CD-MS of AAV Capsids. Single ion CD-MS was performed on the same UHMR Orbitrap mass spectrometer used for native MS analysis. Specifically, we directly measured the signal intensity of single ions to determine the

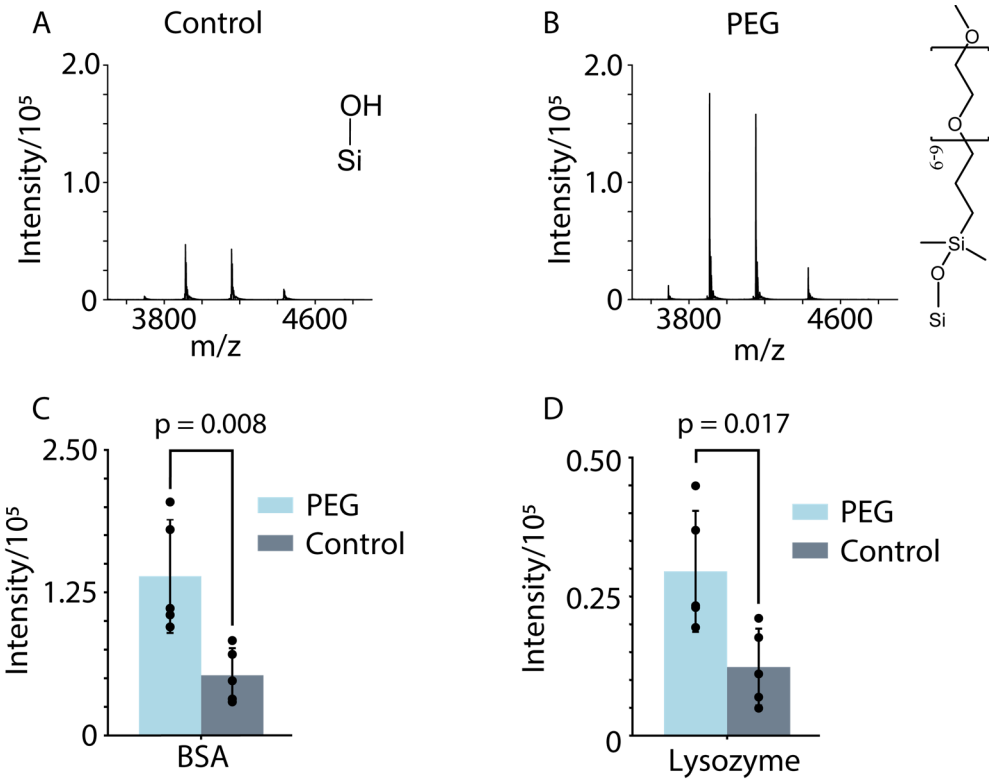

Figure 2: Raw mass spectra of BSA for A) uncoated control needles and B) PEG-coated needles showing an improvement in signal intensity. Signal intensity for the most abundant charge state for C) lysozyme and D) BSA with the PEG coated (*light blue*) and control (*grey*) nESI needles. The PEG coating increases the signal intensity and improves sensitivity for these standard proteins. Dots indicate the individual data points for each needle type with error bars indicating ±1 standard deviation (n=5).

charge.[10] As previously described, we calibrated the S/N ratio against known charges and used this calibration to determine the mass and charge of single ions.[40] AAV capsids were analyzed similarly to what has been previously described,[40] but the spray voltage was set to 1.1 kV for better resolution of AAV capsids and less background noise. We found that increasing the capillary temperature to 350 ^{0}C and lowering the HCD voltage to 100 V reduced adduction and gave more accurate masses for empty AAV2 capsids. CD-MS spectra was acquired for 5 minutes, which was used to calculate the total number of single ions acquired for control and surface modified needles. To reduce experimental variation, control and surface modified needles were prepared on the same day and analyzed alternating between the two.

UniDecCD was used to process and count the number of single ions for AAV2. Parameters for processing and deconvolving AAV capsid spectra have been previously described.[40] Ions were counted and binned within a *m/z* window of 20,000–35,000 and a charge window of 100–200.

Flow Rate Determination. Flow rates of PFDCS coated, PEG coated, and control nano ESI needles were determined by weighing the buffer solution consumed during the spraying process. Needles were backfilled to approximate 95% capacity with 0.2 M ammonium acetate buffer. The weights of the filled needles with an inserted silver wire electrode were recorded on a semi-microbalance (Quintix 125D-1S, Sartorius AG, Göttingen, Germany). The needles were then connected to an ESI power supply (HP020RZZ616B, Applied Kilovolts Ltd, West Sussex, UK) and mounted 5-6 mm away from a grounded metal plate serving as a counter electrode. A spray voltage of 1.2kV was applied, and the sprays were allowed to proceed for one hour with no external pumping or application of back pressure. The weights of the needles were measured again after the one hour spraying period. The density of the buffer was used to determine the actual flow rate of each type of needle.

RESULTS

Modified Needles Improve Sensitivity for Proteins. To determine if the surface modification improved sensitivity, we first used BSA and lysozyme as simple standards because they are known to nonspecifically adsorb to glass surfaces.[37-38,42-43] With the PEG modified needles, we saw a higher signal intensity for BSA and lysozyme compared with uncoated controls (Figure 2). Under proper storage conditions (see above), we found no residual free PEG or PEG associated with BSA or lysozyme, which showed that there was no contamination from the coating. The improvements in signal intensity were roughly two-fold for both. We observed minor adduction for lysozyme after the buffer exchange, but the amounts were similar between the PEG coated and control nESI needles. The similar adduction profile suggests that the droplets sizes may be similar between the two nESI needle types. Overall, the PEG coating significantly improved the native MS sensitivity for standard proteins with manually clipped nESI needles.

Modified needles improve AAV CD-MS analysis. AAV capsids are currently being used as drug and gene therapy delivery systems,[6-7] and major strides have been made with CD-MS and native MS to characterize empty and filled AAVs.[9-10,44-45] However, even single ion CD-MS methods can require high concentrations of AAV samples and long

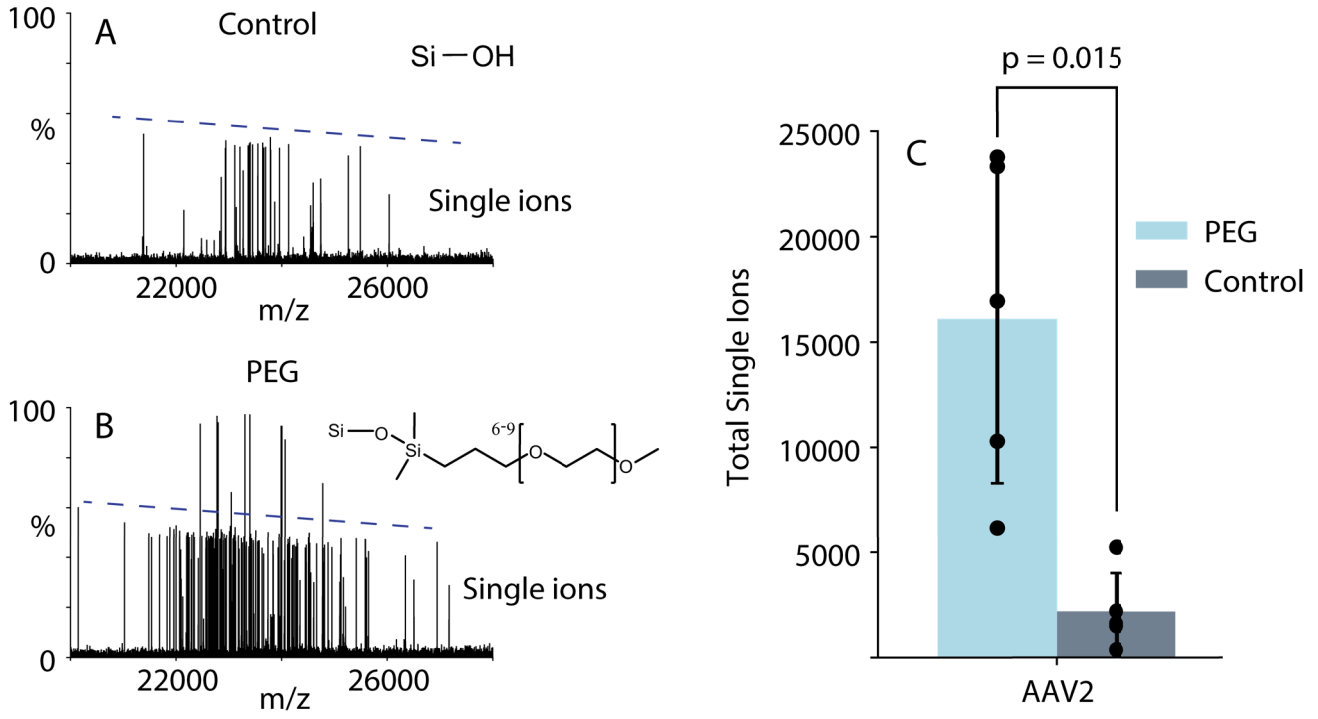

Figure 3: CD-MS analysis of AAV2 capsids. Five averaged single scans for a replicate of A) control and B) PEG coated needles. The blue dashed line indicates the single ion level. C) Total number of single ions collected from a 5-minute CD-MS acquisition for empty AAV2 capsids. PEG coated needles (*light blue)* increase the total amount of single ions compared to the control (*dark grey*). Dots indicate the individual data points for each needle type with error bars indicating ±1 standard deviation (n=5).

acquisition times to acquire the required number of ions.[40,44] Building on the promising results with standard proteins, we next tested CD-MS of AAV capsids to see if the surface coating would increase the signal and lower acquisition times for dilute samples.

We found that PEG modified needles yielded more than eight times higher total number of ions collected compared to the control needle at 2×10^{12} capsids per ml (Figure 3). Clearly, the PEG needles significantly improved the ion current at lower concentrations. At higher concentrations, e.g., 1×10^{13} capsids per ml, there was higher signal for the PEG coated needles, but the statistical significance of the results was weak (p=0.1). This indicates that PEG coated needles are not as beneficial for concentrated AAVs over 1×10^{13} capsids per ml (Figure S1).

Overall, the surface modified needles provide higher ion currents for dilute AAV preparations, which is valuable for the often dilute preparations of filled and partially filled capsids.[8,46] At the lower concentration, PEG modified needles gave an average of $16.1\pm7.8\times10^3$ single ions after a 5-minute acquisition compared to $2.2\pm1.8\times10^3$ ions for the control needles. Also, the relative standard deviation observed for the total number of single of ions was 48% for the modified needles compared to 82% control needles, which was significantly lower according to the F-test, indicating an improve reproducibility with PEG coated needles.

Improving Reproduciblity with 2 µm nESI Needles. Although the coated needles significantly improved the signal intensity at lower concentrations, both the control and modified needles had large needle-to-needle variation (Figures 2 and 3). Initially, we hypothesized that the high standard deviations in signal intensity were due to differences in the tip diameter caused by the manual clipping of the electrospray needles. Manual clipping of the needle tips after pulling is common for native MS, and we found it gave a range of 1.5–4 µm tip diameters, as measured using a microscope (Figure S2A).

To minimize the variation of tip sizes and geometry, we developed a 6-line program (Table S2) for the pipette pullers to produce nESI needles that reproducibly had tip diameters of 2 µm and did not have to be manually clipped. The tip diameter was confirmed with a microscope (Figure S2B). It is important to note that we used a tungsten wire in the back of the needle to apply the electrospray voltage for all samples in this study, so we did not coat the needles in metal.

We tested the 2 µm needles with BSA, and we found that the uncoated control single pull needle signal intensities were not statistically significant from control clipped needles (Figure S3). However, the relative standard deviation for the single pulled needles were 32% which was significantly lower than the clipped needles at 67%, confirmed with an F-test (Figure S3). PEG-coated 2 µm needles improved the signal intensity by almost ten-fold compared to uncoated 2 µm needles, and the data was more consistent run-to-run (Figure 4).

The higher signal improvement with the coated 2 µm needles compared with coated manually clipped needles may be due to the shorter taper on the 2 µm needles. The shorter taper likely yields a higher density of the coating close to the tip of needles compared to the manually clipped needles, which have a longer taper and may have a more damaged coating near the tip from the pulling process (Figure S2). However, further investigation will be needed to confirm this hypothesis.

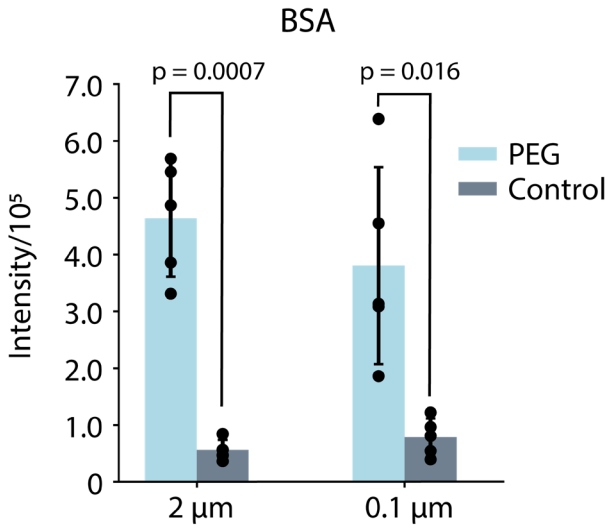

Figure 4: Signal intensity of most abundant charge state of BSA with PEG coated (*light blue*) and uncoated control (*grey*) nESI needles with 2 µm (*left*) and 0.1 µm (*right*) tip diameters.

Together, these results indicate a much higher improvement in the signal intensity compared to clipped nESI needles. They may also suggest that the geometry of the tip affects the signal intensity for these coated tips, but further exploration of this phenomenon is required.

Coated Submicron nESI Needles. We then explored submicron emitters to determine if the tip diameter would affect the improvement from the surface modification. Submicron emitters have previously been shown to improve desolvation via the formation of smaller droplets during the ESI process.[19,29,42] We found that the control 0.1 µm nESI needles had similar signal intensities for BSA compared to 2 µm needles. Like the 2 µm tips, the PEG modification increased the signal intensity for BSA five-fold for the submicron tips (Figure 4). These data confirm that surface coating significantly improves nESI sensitivity for needles of different geometries and can be implemented in needles of different diameters without manual clipping.

One important point to note is that pulling the nESI needles likely either burns the coating off or dilutes the coating through stretching at the tip of the needle. Attempts to prepare needles that were coated after pulling led to consistent problems with the silane coating clogging the needle tip. Future studies will explore coating the capillary after needle pulling in greater depth. However, it may be that the shorter taper on the 2 µm and 0.1 µm tips compared with the clipped needles (Figure S2) means that more of the coating is intact close to the tip surface, which may contribute to the improved signal observed with this different geometry.

Why do modified needles improve sensitivity? We have demonstrated that the PEG modification improves sensitivity for nESI for several systems. Although a full examination of the causes of the improved signal is beyond the scope of this manuscript, we performed a few experiments to shed light on the potential mechanisms involved.

First, to test whether the improvement in sensitivity was due to reduced nonspecific adsorption to the glass, we compared our needles modified with PEG to needles modified with PFDCS (Figure 1), a polyfluorinated molecule.[35] PFDCS provides an amphiphobic surface that repels both polar and nonpolar molecules, in contrast to the PEG coating that is hydrophilic.[34] Although the surface chemistry is different, both reduce nonspecific adsorption.[34,47] Thus, if the improvement was due only to decreased nonspecific adsorption, then we would expect that both the PFDCS and PEG coating would improve the signal intensity.

Instead, we found that there were no statistically significant differences between the control and PFDCS modified needles (Figure S4). Because PEG coatings improved signal but not PFDCS, we think our initial hypothesis is either incorrect or incomplete, and that the improvement is likely not solely due to less nonspecific adsorption. However, we cannot rule out that the beneficial reduced nonspecific adsorption with PFDCS was cancelled out by other confounding deleterious properties of the coating, such as reduced capillary action as described below or the amphiphobic nature.

To further test whether the improvement was due to less nonspecific adsorption, we compared the signal intensity of ubiquitin, which does not adsorb to glass nESI needles at neutral pH,[19] with 2 µm control and PEG coated needles. If the improvement in signal intensity was due to only reduced nonspecific adsorption, then the signal intensity of ubiquitin should not be different between the control and PEG coated needles. Instead, we found a 3-fold improvement in the signal intensity with the PEG coating (Figure S5). The fact that the signal improvement is still observed for a protein that does not adsorb further suggests that the improvement is not likely due to decreased adsorption to the needle. Some other factor is playing a more important role.

Comparing the improvements from PEG and PFDCS, we can rule out changes in the wettability of the needle surface as a cause for the improvement. The contact angle of water with a given coated surface is an indicator of the wettability of the coating.[48] PEG and bare borosilicate glass have a similar water contact angle of 34°,[47,49] but PFDCS has a very high water contact angle of 108°,[34] indicating a lower wettability. Other attempts at surface modified electrospray needles used similar polyfluorinated compounds to lower the wettability and theorized that this would improve electrospray.[30-31,33] Because the PEG-modified needles performed better than PFDCS-modified and control needles, the improvements are likely due to other factors.

Finally, we investigated the capillary action of surface modified needles, which affects movement of a solution through a capillary. We found that the bare borosilicate capillaries have the highest capillary action, PEG capillaries have an intermediate capillary action, and PFDCS capillaries have little to no capillary action (Figure 5A). Then, we investigated the flow rate of PFDCS modified, PEG modified, and control nESI needles under static conditions with no fluid pumping or applied back pressure (Figure 5B). We found that PEG modified nESI needles had the highest flow rate compared to PFDCS and control nESI needles, and the PFDCS modified and the control nESI needle flow rates were not statistically different.

Because the flow rate trends most closely matched the native MS data, we hypothesize that the improvement in sensitivity is mostly likely driven by the increased flow rate for these needles. We envision that higher capillary forces

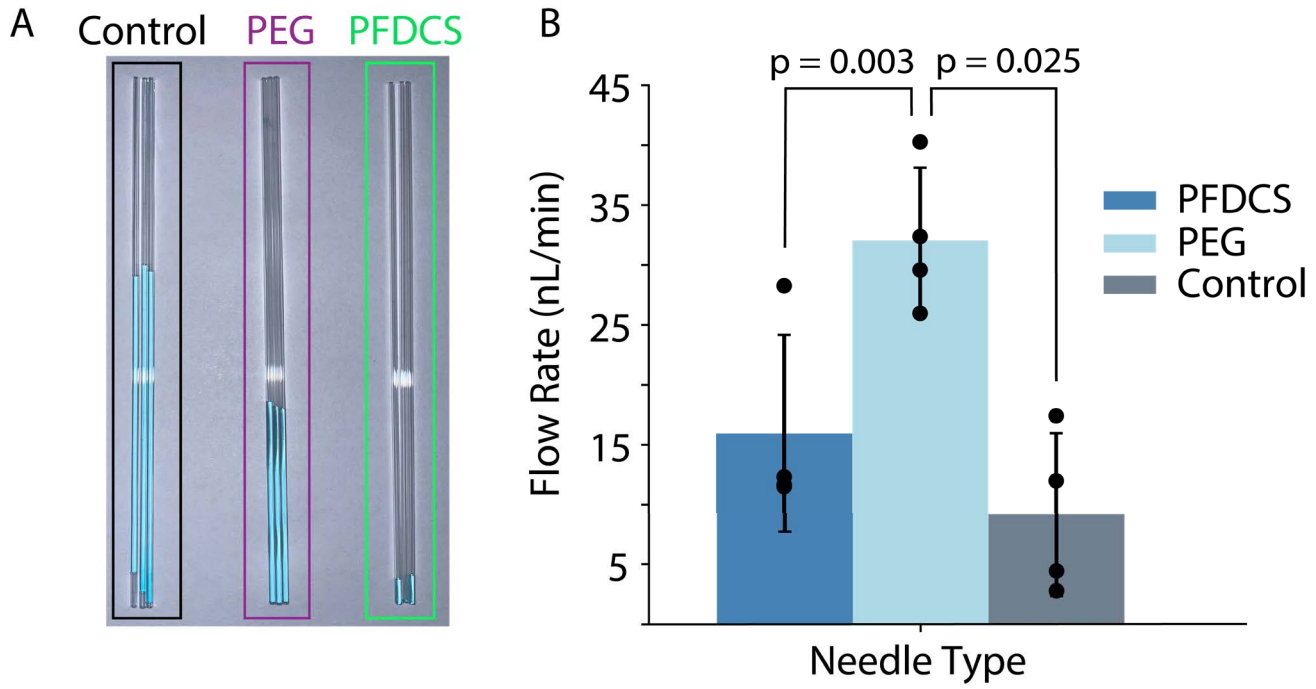

Figure 5: Photograph of the (A) capillary action of control (*black*), PEG modified (*purple*), and PFDCS modified (*green*) nESI needles. Three capillaries of each type were submerged on the bottom side in blue #2 dye and allowed to reach equilibrium. Control needles have the highest capillary action, PEG modified needles are intermediate, and PFDCS needles have little to no capillary action. (B) Flow rate of PFDCS modified (*dark blue*), PEG modified (*light blue*), and unmodified (*grey*) needles. Control and PFDCS modified needles have similar static flowrates, and PEG modified nESI needles have the highest flow rate.

in conventional needles lower the liquid flow at a given ESI voltage because the liquid clings tightly to the glass. Lowering this capillary drag may increase the flow of liquid to the tip, thus increasing the signal. However, the lower capillary action with PFDCS does not correlate with the flow rate, so the differences in surface chemistry between PEG (hydrophilic) and PFDCS (amphiphobic) may be complicating the results. It may be that a certain amount of capillary action is necessary for optimal flow, but future experiments will be needed to explore this hypothesis in more depth.

Interestingly, we observed less sodium adduction of ubiquitin with the 2 μm PEG coated needles compared to the control needles (Figure S6), which may suggest that the PEG needles may produce smaller droplets or reduce nonspecific addition in some other way. Results from lysozyme on manually clipped needles, described above, showed similar levels of salt adduction between control and PEG needles, so the reduction of adduction may be protein or tip geometry dependent. Future research will be necessary to explore this further.

It is important to note that these experiments were all performed under static conditions with no applied fluid pumping or back pressure. Future work will explore whether the same effects are observed with a flowing nESI system where capillary drag is less problematic. Overall, our current hypothesis is that reduced increased flow rate improves the nESI sensitivity of PEG coated needles.

CONCLUSION

We demonstrated that surface modified nESI needles increase the signal intensity and sensitivity of multiple analytes. We discovered that CD-MS analysis of dilute AAVs is significantly improved with PEG modified nESI needles, and non-clipped needles and submicron emitters especially benefit from the coating. Although our initial hypothesis was that PEG coating reduced nonspecific adsorption, we now hypothesize that higher flow rates from the tip drive the improvement in signal. Future studies will explore other surface modifications that will create a hydrophilic surface that reduce some capillary action.[34] Future research will also be required to explore this mechanism in more depth. In any case, this technology demonstrates a relatively quick and inexpensive method for improving the sensitivity of difficult analytes like AAV capsids at lower concentrations that will enable native mass spectrometry of challenging analytes.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

The supporting information contains a step-by-step protocol for performing the surface modification and for making 2 μm and 0.1 μm needles. It also contains supplemental figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

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CONFLICT OF INTEREST

The authors at the University of Arizona have applied for a provisional patent on this technology.

ABBREVIATIONS

PEG, polyethylene glycol; MS, mass spectrometry; PFDCS, (tridecafluoro-1,1,2,2-tetrahydrooctyl) dimethylchlorosilane; CD-MS, charge detection mass spectrometry

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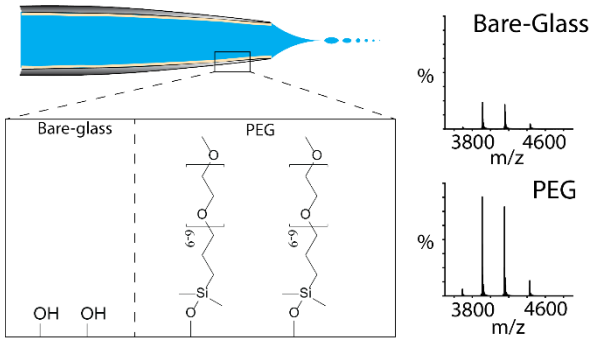
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