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Varying nanoparticle pseudostationary phase plug length during capillary electrophoresis†

Varuni Subramaniam, Lindsay Griffith, and Amanda J. Haes*

University of Iowa, Department of Chemistry, 204 IATL, Iowa City, Iowa, 52242, USA

Abstract

Capillary electrophoresis based separations of the hypothesized Parkinson's disease biomarkers dopamine, epinephrine, pyrocatechol, L-3,4-dihydroxyphenylalanine (L-DOPA), glutathione, and uric acid are performed in the presence of a 1 nM 11-mercaptoundecanoic acid functionalized gold (Au@MUA) nanoparticle pseudostationary phase plug. Au@MUA nanoparticles are monitored in the capillary and remain stable in the presence of electrically-driven flow. Migration times, peak areas, and relative velocity changes (vs. no pseudostationary) are monitored upon varying (1) the Au@MUA nanoparticle pseudostationary phase plug length at a fixed separation voltage and (2) the separation voltage for a fixed Au@MUA nanoparticle pseudostationary phase plug length. For instance, the migration times of positively charged dopamine and epinephrine increase slightly as the nanoparticle pseudostationary phase plug length increases with concomitant decreases in peak areas and relative velocities as a result of attractive forces between the positively charged analytes and the negatively charged nanoparticles. Migration times for neutral pyrocatechol and slightly negative L-DOPA did not exhibit significant changes with increasing nanoparticle pseudostationary plug length; however, reduction in peak areas for these two molecules were evident and attributed to non-specific interactions (*i.e.* hydrogen bonding and van der Waals interactions) between the biomarkers and nanoparticles. Moreover, negatively charged uric acid and glutathione displayed progressively decreasing migration times and peak areas and as a result, increased relative velocities with increasing nanoparticle pseudostationary phase plug length. These trends are attributed to partitioning and exchanging with 11-mercaptoundecanoic acid on nanoparticle surfaces for uric acid and glutathione, respectively. Similar trends are observed when the separation voltage decreased thereby suggesting that nanoparticle-biomarker interaction time dictates these trends. Understanding these analyte migration time, peak area, and velocity trends will expand our insight for incorporating nanoparticles in separations.

Introduction

Capillary electrophoresis is an efficient, sensitive, and high resolution liquid-phase separation technique that permits fast analysis of molecules in small sample volumes.^{1–4} Capillary electrophoresis, as a result, is often used to separate simple mixtures of small molecules,⁵ proteins,^{6,7} and DNA^{7–10} as well as to evaluate the fast association and dissociation of complex transient species.^{11–13} Capillary electrophoresis based separations

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amanda-haes@uiowa.edu; Fax: +1 319-335-1270; Tel: +1 319-384-3695.

of analytes are achieved *via* differences in electrophoretic mobilities.¹⁴ During the past 20 years, separation selectivity of these species was improved using stationary and pseudostationary phases.¹⁵ Pseudostationary phases, for instance, can migrate with or against electroosmotic flow,¹⁶ and improvements in analyte separation is dictated by analyte partition coefficients between the pseudostationary phase and buffer/background electrolyte.^{15,17}

In 1989, Wallingford and Ewing were the first to incorporate nanoparticles as a pseudostationary phase in capillary electrophoresis to separate five catechol amines.⁵ Subsequently, nanomaterials were utilized as pseudostationary phases in electrically-driven separations^{17–19} because of their inherently large surface area to volume ratios and small sizes. Furthermore, successful separations of acidic and basic proteins,^{6,20} neurotransmitters,²¹ structural isomers of aromatic acids and bases, and DNA^{9,22} were previously achieved using nanomaterial pseudostationary phases in capillary electrophoresis. Gold nanoparticles are commonly used capillary electrophoresis pseudostationary phases because of their size-dependent optical,^{23,24} electrical,²⁵ and magnetic properties;²⁶ long shelf lives;²⁷ and easily modulated surface chemistries.^{21,23–25,28,29} Nanoparticle surface chemistry modification (1) prevents uncontrolled nanoparticle aggregation^{21,30} and (2) facilitates nanoparticle stability and function during separations.^{31,32}

In this study, pseudostationary phase plug lengths containing 11-mercaptoundecanoic acid functionalized gold (Au@MUA) nanoparticles will be systematically introduced during the capillary electrophoresis based separation of the hypothesized Parkinson's disease biomarkers^{33–38} dopamine, epinephrine, pyrocatechol, L-DOPA, glutathione, and uric acid. To eliminate concentration-dependent aggregation effects, nanoparticle concentration will be maintained at 1 nM. Increasing the Au@MUA nanoparticle pseudostationary phase length at a fixed separation voltage will be shown to increase the migration times of positively charged analytes whereas opposite effects prevail with negatively charged molecules. Similar trends will be presented as the separation voltage decreases for a fixed nanoparticle pseudostationary phase plug length. These migration time and relative velocity trends are attributed to electrostatic interactions between the slow moving and negatively charged Au@MUA nanoparticles *vs.* positively charged analytes (retarding analyte velocity) or negatively charged analytes (enhancing analyte velocity). Finally, interactions between analytes and nanoparticles will be shown to decrease peak areas systematically for all the molecules. In all cases, increasing the probability of nanoparticle-biomarker interaction either from increasing the nanoparticle pseudostationary phase plug length or by decreasing the separation voltage magnifies these results. These findings suggest that relative charges between analytes and nanoparticle surfaces as well as nanoparticle-biomarker interaction time are important parameters to manipulate when optimizing capillary electrophoresis separations carried out in the presence of a nanoparticle containing pseudostationary phase. Recognizing these influences is expected to improve our overall understanding for employing nanoparticles as pseudostationary phases in electrically driven separations.

Experimental methods

Materials

Gold(III) chloride trihydrate (HAuCl_4), trisodium citrate dihydrate (citrate), 11-mercaptoundecanoic acid (11-MUA), dopamine hydrochloride, epinephrine, 3–4-dihydroxy-L-phenylalanine (L-DOPA), pyrocatechol, and L-glutathione (reduced) were purchased from Sigma Aldrich (St. Louis, MO). Sodium dihydrogen phosphate dihydrate, sodium phosphate dibasic hepta-hydrate, sodium hydroxide (NaOH), ethanol, hydrochloric acid (HCl), and nitric acid (HNO_3) were purchased from Fisher Scientific (Pittsburg, PA). Nanopure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) was obtained from a Barnstead Nanopure System (Dubuque, IA) and used for all experiments. Fused silica capillary (internal diameter = $75 \mu\text{m}$, outer diameter = $360 \mu\text{m}$) with an external polyimide coating was purchased from Polymicro (Phoenix, AZ).

Gold nanoparticle synthesis

All glassware was cleaned with aqua regia (3 : 1 $\text{HCl}:\text{HNO}_3$), rinsed with Nanopure water, and oven dried. Au nanoparticles were prepared using previously reported citrate reduction procedures.³⁹ Briefly, 20 mL of citrate (38.8 mM) was rapidly added to a refluxing and stirring aqueous solution of 1 mM HAuCl_4 (200 mL). After 10 min, the characteristic burgundy colored nanoparticle solution was allowed to cool to room temperature. As previously reported,⁴⁰ these nanostructures were centrifuged (Eppendorf – Centrifuge 5424, Germany) at 11,500 RPM ($8797 \times g$) for 40 min to remove excess citrate. The resulting loose nanoparticle pellet was diluted to 10 nM (nanoparticle concentration) using pH adjusted water (adjusted to pH = 11 with 1 M NaOH prior to nanoparticle resuspension). Nanoparticle concentration was estimated using a standard estimation model described by Haiss.⁴¹

Au@MUA nanoparticles were prepared using a four step procedure. First, a 10 mM 11-MUA solution (ethanol) was added to the gold nanoparticle solution in a 1 : 10 (v/v) ratio. Next, the solution was stirred in the dark for 16 h at 20°C to ensure equilibrated 11-MUA monolayer formation. Third, the nanoparticle solution was centrifuged thrice at $8797 \times g$ for 30 min and resuspended in 35 mM phosphate buffer (pH 7.2) to remove excess 11-MUA. Finally, the Au@MUA nanoparticles were diluted to 1 nM in 35 mM phosphate buffer prior to their use in capillary electrophoresis.

Transmission electron microscopy (TEM)

Au@MUA nanoparticle shape and size homogeneity were characterized using TEM (JEOL JEM-1230). Samples were prepared by applying 30 μL of the diluted nanoparticle solution (50% in ethanol) to a 400 mesh carbon and formvar coated copper grid (Electron Microscopy Sciences, Hatfield, PA). Excess solution was removed with filter paper, and the sample was allowed to air dry prior to imaging. The resulting images were analyzed using Image Pro Analyzer, and at least 100 nanoparticles were evaluated per sample.

Extinction spectroscopy

Extinction spectra of Au@MUA nanoparticles were acquired using a UV-visible spectrometer (Ocean Optics HR 4000, Dunedin, FL) configured in transmission geometry to

evaluate the optical properties and overall stability of the nanoparticles in 35 mM phosphate buffer (pH = 7.2). All spectra were collected in disposable, methacrylate cuvettes (path length = 0.5 cm) using the following parameters: integration time = 20 msec, average = 20 scans, and boxcar = 10.

Zeta potential

Au@MUA nanoparticle surface potential was determined from electrophoretic mobility measurements which were carried out in 35 mM phosphate buffer (pH 7.2, conductivity 5 mS cm⁻¹) at 25 °C using a Malvern Zetasizer (Worcestershire, UK). Gold nanoparticles were diluted to 1 nM, and measurements were performed in triplicate. Reported zeta potential data were obtained from monomodal acquisitions and fits according to the Smoluchowski theory.

Buffer and sample preparation

A 150 mM sodium phosphate buffer (stock) was prepared using sodium dihydrogen phosphate dihydrate and sodium phosphate dibasic heptahydrate. The pH was adjusted to 7.2 using 1 M NaOH. Buffers were prepared by diluting this stock solution to 35 mM and filtered using 0.45 µm PTFE membrane filters (VWR International, West Chester, PA).

5 mM stock solutions of dopamine, epinephrine, pyrocatechol, L-DOPA, glutathione, and uric acid (in 10 mM NaOH) were prepared individually in 10 mM HCl unless noted. The sample matrices used in capillary electrophoresis were prepared in 10 mM HCl and diluted to 5 µM final concentrations for dopamine, epinephrine, pyrocatechol, L-DOPA, and glutathione and 2.5 µM for uric acid.

Capillary electrophoresis

All separations were performed in a Beckman Coulter P/ACE-MDQ capillary electrophoresis system equipped with a photodiode array detector. Detection wavelengths (λ_{det} = 193, 520, and 600 nm) were selected to monitor the analytes, stable nanoparticles, and aggregated nanoparticles, respectively. A fused silica capillary (total length = 60.2 cm, length to detector = 50 cm) was thermostated at 25 °C.

The capillary was conditioned with 0.1 M HNO₃ (20 psi for 5 min), water (20 psi for 2.25 min), 1 M NaOH (20 psi for 2.25 min), water (20 psi for 2.25 min), 150 mM sodium phosphate buffer (20 psi for 2.25 min), and 35 mM sodium phosphate buffer (20 psi for 5 min). Next, a Au@MUA nanoparticle pseudostationary phase plug was injected into the capillary. Plug lengths of 0, 1.2₆ (2.5₂%), 2.5₃ (5.0₆%), 5.0₆ (10.1₂%), and 7.5₉ (15.1₈%) cm were achieved by hydrodynamically (1 psi) injecting the nanoparticle solution for 0.5, 10, 20, or 30 s, respectively. A 1 s (1 kV) 35 mM buffer plug was injected after the nanoparticle plug to reduce vial contamination. Next, the biomarker sample matrix was injected into the capillary (1 psi for 5 s). A 20 kV separation voltage was applied across the capillary (normal polarity). Alternatively, a 2.5₃ cm Au@MUA nanoparticle pseudostationary phase plug was injected into the capillary prior to the sample matrix, and the separation voltage was varied from 10–25 kV.

Data were analyzed using OriginPro 7.5, and electropherograms from each run were normalized to the migration time of pyrocatechol (nanoparticle plug length = 0 cm). For Fig. 2, a 40 s window that included the electroosmotic band (*i.e.* the velocity of neutral buffer species which resulted in a large negative drop in baseline) was removed for clarity. Peak areas were determined by fitting each biomarker peak with OriginPro 7.5 equipped with a peak fitting module. Specifically, a two point linear baseline was manually assigned for peaks in the electropherogram. After baseline subtraction, peaks were fit with a Gaussian or EMGaussian peak function for symmetric and asymmetric peaks, respectively. Reported peak areas are for the best fits (*i.e.* lowest chi-squared values). Average analyte peak areas were normalized to the average peak area collected with a 0 cm nanoparticle pseudostationary phase plug length. Averages and standard deviations for normalized migration times and peak areas were evaluated from six replicate separations and propagated errors, respectively.

Results and discussion

Structure and surface characterization of Au@MUA nanoparticles

Following synthesis, surface modification, and purification; Au@MUA nanoparticles were systematically evaluated using TEM, zeta potential, and extinction spectroscopy to assess structural morphology, effective surface charge, and optical stability, respectively. TEM characterization reveals that the Au@MUA nanoparticles are homogeneous and exhibit a ~spherical morphology with a mean diameter of $12.3_6 \pm 0.6_8$ nm (Fig. 1A inset). While not an absolute property of a material, zeta potential measurements in the separation buffer indicate the effective surface charge and stability of the functionalized nanostructures at a certain pH. Upon three centrifugation and rinsing cycles in phosphate buffer (pH = 7.2), the Au@MUA nanostructures were equilibrated for several hours and evaluated. Upon equilibration, centrifugation, and resuspension in buffer, the Au@MUA nanoparticles exhibit an average zeta potential of -30.5 ± 0.3 mV at pH 7.2 (*vs.* -16.7 ± 3.1 mV for citrate stabilized nanoparticles). A negative zeta potential value indicates that the carboxylic acid terminated self assembled monolayers (SAMs) are deprotonated. This negative surface charge facilitates the formation of a repulsive layer on nanoparticle surfaces thereby stabilizing the nanomaterials for further applications.

To further evaluate Au@MUA nanoparticle homogeneity and stability, extinction spectroscopy was used. Noble metal nanoparticles (*i.e.* copper, gold, silver, *etc.*) exhibit strong extinction (sum of absorption and scattering) spectra at visible wavelengths^{42–49} which arise when incident photon frequency is in resonance with the collective oscillation of the conduction electrons in the nanoparticles and is known as the localized surface plasmon resonance (LSPR).⁵⁰

The LSPR is sensitive to changes in nanoparticle shape, size, stability, and the local dielectric constant of the surrounding medium.^{51,52} Previously, the LSPR spectrum of ~13 nm Au nanoparticles was centered at ~520 nm; however, growth of a characteristic low energy (~620 nm) extinction band was observed when coupling between nanoparticles occurred.²¹ In these studies, a 1 nM Au@MUA nanoparticle solution reveals an extinction spectrum with a single LSPR band centered at 524.5 nm and characteristic UV features

when suspended in 35 mM sodium phosphate buffer (Fig. 1A). This extinction maximum wavelength (λ_{max}) is slightly red-shifted from the unfunctionalized (*i.e.* citrate stabilized) value and is indicative of stable, electromagnetically isolated nanostructures.

Despite the high degree of stability in bulk solution, nanoparticles were previously shown to be unstable during electrically-driven capillary separations.^{21,31,53} Nanomaterial instability was attributed to nanoparticle composition, concentration, and/or surface chemistry as well as buffer characteristics. To assess nanoparticle stability during electrically-driven separations, absorbance measurements at 193, 520, and 600 nm were used to generate complementary electropherograms after a 7.5₉ cm plug containing 1 nM Au@MUA nanoparticles was injected into the capillary. Fig. 1B displays representative electropherograms (electric field strength = 333 V cm⁻¹), which reveal a single band centered at ~24.5 min at all detection wavelengths. As previously reported,²¹ the integrated areas of the LSPR-relevant bands (Area₅₂₀/Area₆₀₀) reveal information regarding the stability of gold nanoparticles during electrophoresis. As shown in Fig. 1C, a ratio of 3.5 is obtained for these 1 nM Au@MUA nanoparticle pseudostationary phase plugs which suggests that the functionalized nanoparticles are stable under these separation conditions.

Separations as a function of Au@MUA nanoparticle pseudostationary phase plug length

The separation of the hypothesized Parkinson's disease biomarkers dopamine (d), epinephrine (e), pyrocatechol (p), L-DOPA (l), glutathione (g), and uric acid (u) is evaluated using a nanoparticle pseudostationary phase plug in capillary electrophoresis (Fig. 2). To perform these experiments, a 1 nM Au@MUA nanoparticle plug was hydrodynamically injected into a capillary for 0–30 s before the biomarker mixture. This resulted in a nanoparticle pseudostationary phase plug that varied in initial length from 0–8 cm. A 333 V cm⁻¹ potential was applied across the capillary causing the fast moving analytes to elute through the nanoparticle pseudostationary phase and subsequently detected at 193 nm. Regardless of the nanoparticle pseudostationary phase plug length used, the migration time of the neutral molecule pyrocatechol did not vary and was used to account for slight run to run variations during each separation.

Five trends are observed in the representative electropherograms shown in Fig. 2. First, electroosmotic flow is constant at 0.029 cm²V⁻¹min⁻¹ (96 mm min⁻¹) and independent of nanoparticle pseudostationary phase plug length. Second, the biomarker elution order does not change upon addition of a nanoparticle pseudostationary phase. In all cases, the molecules elute according to their charge to Stokes radius. It should be noted that the nanoparticle pseudostationary phase plug always eluted at ~24.5 min – well after the biomarkers were detected (and is only shown in Fig. 1B). Third, increasing the Au@MUA nanoparticle pseudostationary phase plug length causes the cationic dopamine and epinephrine biomarkers to exhibit increased migration times whereas the anionic species L-DOPA, glutathione, and uric acid migrated more quickly relative to controls. For instance, the difference in migration time between dopamine (the first analyte detected) and uric acid (the last analyte detected) is 8.14 and 6.02 min for 0 and 7.5₉ cm nanoparticle pseudostationary phase plug lengths, respectively. Fourth, the peak areas of all biomarkers decrease upon the addition of nanoparticles. The peak area for uric acid, for example,

decreases by ~70% when a 7.5 μ m nanoparticle pseudostationary phase plug is used *vs.* when no nanoparticles are included. Finally, resolution between dopamine and epinephrine as well as between pyrocatechol and L-DOPA decreases upon inclusion of a nanoparticle pseudostationary phase plug (Table 1). Resolution was calculated using the migration times and full width at half maxima values of the fitted peaks. Because analyte band widths increase slightly as nanoparticle plug length increases, changes in resolution are attributed to nanoparticle-biomarker interactions and/or an Eddy diffusion-like phenomenon as analytes migrate through the nanoparticle pseudostationary phase plug.

Evaluating trends in biomarker migration times (fixed separation voltage)

As previously reported, nanoparticles can impact separations in a composition,^{54–59} concentration,⁶⁰ shape,^{61,62} size,^{15,61,63} and/or surface chemistry^{21,60,64} dependent manner. Because nanoparticle composition, shape, size, and surface chemistry are held constant in these studies; observed trends in the separations shown in Fig. 2 likely arise because of variations in effective nanoparticle concentration and/or pseudostationary phase plug length.

To evaluate variations in analyte migration time trends more closely, distinct changes in migration time and velocity for each biomarker as a function of nanoparticle pseudostationary phase plug length are shown in Fig. 3A and 3B, respectively. The migration time of each biomarker is evaluated in the absence of nanoparticles and normalized as a “0” response. A nanoparticle pseudostationary phase plug length was then injected into the capillary prior to the sample matrix in lengths varying from 1.2 μ m–7.5 μ m. For all charged biomarkers, relative migration times and velocities vary exponentially with respect to the nanoparticle pseudostationary phase plug length.

As shown in Fig. 3A, the migration times for dopamine and epinephrine increase slightly by 18 and 15 s, respectively when a 7.5 μ m nanoparticle pseudostationary plug is incorporated into the separation *vs.* when no nanoparticles are included. Migration times for pyrocatechol reveal no pseudostationary phase plug length dependence. For anionic L-DOPA, glutathione, and uric acid, relative migration times decrease systematically as the pseudostationary phase plug length increases. Interestingly, the largest effects in migration times are observed for uric acid, the molecule that spends the longest time in the capillary.

To account for various migration time differences, relative variations in biomarker velocity were calculated for these data. To do this, the absolute velocity of each biomarker was calculated by dividing the inlet-to-detector capillary length by the average migration time. Changes in these absolute velocities *vs.* separations in the absence of a nanoparticle pseudostationary phase plug were calculated as relative changes in velocities (D velocity) and then plotted. As shown in Fig. 3B, the slight (~20 s) increase in migration time for dopamine results from a reduction in analyte velocity by ~10 mm/min. Similar trends are observed for epinephrine. L-DOPA which migrated slightly more quickly upon inclusion of a nanoparticle pseudostationary phase plug exhibited a slight increase in relative velocity. Glutathione and uric acid, the anionic species which eluted last from the capillary, experienced the greatest decrease in migration time upon addition of the nanoparticle pseudostationary phase plug and experienced an increase in relative velocity by ~8 and ~9 mm min⁻¹ *vs.* controls, respectively.

If an Eddy diffusion-like phenomenon is dictating the observed variations in analyte migration times, similar trends should be observed for each molecule – that is, biomarker velocities should decrease with increasing nanoparticle pseudostationary phase plug length. This is not observed. Instead, these systematic trends likely arise from electrostatically driven interactions between the biomarkers and nanoparticles. During these separations, the negatively charged Au@MUA nanoparticle pseudostationary phase plug moves slower than the biomarkers. Because the nanoparticle plug is injected before the biomarkers, both specific and/or non-specific interactions between the analytes and nanoparticles will occur.

Opposing migration time trends for positively and negatively charged analytes are rationalized from differences and/or similarities between the Au@MUA nanoparticles and the biomarkers. For instance, electrostatic attractive forces between the positively charged dopamine and epinephrine molecules and the slow moving, negatively charged nanoparticles decrease analyte velocities than if no nanoparticle pseudostationary phase was included (because of the retarding electrostatic forces). Opposite effects are observed for the negatively charged analytes as interactions with the negatively charged nanoparticles are repulsive. As the nanoparticle plug length increases, analyte velocities systematically increase leading to enhanced effects (*i.e.* decrease) in migration time.

Evaluating trends in biomarker peak areas (fixed separation voltage)

Although pseudostationary phases generally offer larger separation selectivity than if no pseudostationary phase is used, analyte loss by specific and/or non-specific interactions with the nanoparticle surfaces can either limit and/or facilitate separations. For instance, hydrophobic and covalent interactions between proteins and surface modified gold nanoparticles were previously exploited to reduce non-specific binding of proteins to the capillary wall.⁶ In contrast, interactions between analytes and nanoparticles were shown to increase peak tailing and width which consequently reduced plate numbers.^{15,16} Evaluation of analyte peak area changes in the presence of nanoparticles, however, are often not compared but could be useful in understanding the extent of nanoparticle-biomarker interactions.

To investigate how biomarker peak areas vary as a function of nanoparticle pseudostationary phase plug length, average analyte peak areas were calculated from at least four independent experiments. All individual peak areas were normalized to that analytes' peak area when no nanoparticle pseudostationary phase was included in the separation. As shown in Fig. 4, normalized peak areas decrease for all the analytes when a nanoparticle pseudostationary phase of any length is incorporated into the capillary at a fixed separation voltage. These area changes indicate that some amount of each analyte is (1) specifically and/or non-specifically interacting with the nanoparticle surfaces and/or (2) experiencing Eddy diffusion-like band broadening which results in a decrease in detectable peak areas.

To evaluate the trends for each analyte as a function of nanoparticle pseudostationary phase plug length, the analytes were separated into three classes: (1) analytes electrostatically attracted to, (2) analytes electrostatically repulsed by, and (3) analytes which could covalently bind to (*i.e.* replace 11-MUA molecules on) the nanoparticle surface. In the first classification, electrostatically attracted dopamine (d) and epinephrine (e) are evaluated in

Fig. 4A. The normalized peak areas for both species undergo similar decreases in peak area as the pseudostationary phase plug length increases. For instance, peak areas for both analytes decrease by ~30% when a 1.2₆ cm nanoparticle pseudostationary phase plug is injected into the capillary *vs.* no nanoparticle pseudostationary phase. For the longest nanoparticle pseudostationary phase plug studied, the peak areas for dopamine and epinephrine decrease by 58% and 52%, respectively, relative to the control.

Clearly, increasing the nanoparticle pseudostationary phase plug length results in additional peak area loss. These trends are non-linear and suggest that either nanoparticle-biomarker interaction time and/or analyte charge may be important in understanding the extent of interaction. Dopamine, which exhibits the fastest absolute migration time, is impacted by the longest nanoparticle pseudostationary phase to a greater extent than epinephrine. This suggests that both analyte charge and nanoparticle-biomarker interaction time are important in understanding these trends. As with any analyte, both positively charged dopamine and epinephrine interact with the nanoparticle pseudostationary phase non-specifically *via* hydrogen bonding and van der Waals forces. Dopamine ($pK_a = 8.87$)⁶⁵ is slightly more positive than epinephrine ($pK_a = 8.55$)⁶⁶ and is hypothesized to undergo more significant electrostatic interactions with the negatively charged nanoparticle surface. As a result, the peak area for dopamine decreases slightly more than that for epinephrine.

While pyrocatechol (p) and L-DOPA (l) revealed no migration time dependence on the presence or length of the nanoparticle pseudostationary phase, peak area analysis does reveal differences in the presence and absence of nanoparticles (Fig. 4B). For both analytes, peak area decreases by ~55–60% (relative to the absence of nanoparticles) when the longest nanoparticle pseudostationary phase plug length is included in the separation. These trends are attributed to hydrogen bonding and van der Waals forces. Uric acid data are in contrast to the other biomarkers (u) where normalized peak areas gradually decreases then saturates at ~70% loss (*vs.* controls) with increasing nanoparticle pseudostationary phase plug length. Uric acid, however, does reveal a dependence on nanoparticle pseudostationary phase plug length. Previous studies revealed that the solubility of the urate ion (*i.e.* deprotonated form of uric acid) is reduced at physiological pHs⁶⁷ and decreases with increasing sodium ion concentration.^{67,68} Because the separation buffer contains sodium ions, the urate anion is likely precipitating out of solution and/or partitioning into the 11-MUA self assembled monolayer thereby reducing the observed peak area.

For all analytes studied, glutathione (g) revealed the most significant reduction in peak area with increasing nanoparticle pseudostationary phase plug length (Fig. 4C). Glutathione peak area decreased by 43% and 80% (*vs.* controls) when a 1.2₆ and 7.5₉ cm nanoparticle pseudostationary phase plug length was used, respectively. Because of this large, exponential decrease in glutathione peak area with increasing nanoparticle pseudostationary phase plug length, we hypothesize that glutathione is exchanging with 11-MUA groups on the nanoparticle surfaces. This exchange behavior was previously observed for glutathione with a variety of thiolated molecules in cell based assays involving proteins,^{69,70} drugs,^{69,71} and gene delivery.^{69,72} Because glutathione peak area loss is more significant with increasing nanoparticle pseudostationary phase plug length, the probability of molecular exchange should systematically increase as observed in these studies.

Evaluating trends in biomarker peaks (fixed nanoparticle pseudostationary phase plug length)

If nanoparticle-biomarker interactions dictate the observed relative migration time, velocity, and peak area trends for the biomarkers, then the nanoparticle concentration and the amount of time the analytes can interact with the nanoparticles will influence these parameters. Because nanoparticle stability limits the reproducibility of electrically driven separations,^{21,73} varying nanoparticle concentration leads to irreproducible data (results not shown). Second, varying solution pH is a straight forward method to disrupt/encourage these interactions in bulk solutions; however, both electrophoretic and electroosmotic flow velocities would be simultaneously impacted during a capillary electrophoresis separation. In contrast, the separation voltage (*i.e.* electric field strength) applied during a separation is a straight forward method to vary nanoparticle-biomarker interaction time without impacting the stability and/or charged state of the biomarkers, nanoparticles, or capillary wall.

In these studies, a 1 nM Au@MUA nanoparticle pseudostationary phase plug (length = 2.5₃ cm) was injected into the capillary prior to the biomarker matrix. The samples were then subjected to varying field strengths (166–415 V cm⁻¹) by using discrete separation voltages (10–25 kV). Importantly, the nanoparticle pseudostationary phase remains stable in these electrical environments (Fig. 1C). As observed in Fig. 5, peak area loss becomes more significant as separation voltage decreases thereby suggesting that the interaction time between the biomarkers and nanoparticles is important in understanding both limitations and advantages of using nanoparticle pseudostationary phases.

Trends in relative migration times and velocities further support this hypothesis. As shown in Fig. 6A, as the separation voltage decreases, the relative migration times for all analytes vary systematically, and the largest migration time differences occur at the lowest (10 kV) separation voltage for all biomarkers. At this separation voltage, electroosmotic flow is the slowest (46 mm min⁻¹), and the probability of nanoparticle-biomarker interactions should be greatest.

If this is the case, then the relative velocities should be most impacted at the lowest separation voltage *vs.* the other voltages. This is observed in Fig. 6B. Relative percent changes in biomarker velocity (velocity change/velocity without nanoparticles) are reported as a function of separation voltage. Linear changes are observed for all biomarkers, and the slope of these lines reveal interesting trends regarding the degree of velocity change. Uric acid and dopamine exhibited the largest negative and positive slopes, respectively; thereby suggesting that biomarker-nanoparticle interaction times are crucial in understanding and reproducing these separations.

Conclusions

In summary, the capillary electrophoresis based separation of the hypothesized Parkinson's disease biomarkers dopamine, epinephrine, pyrocatechol, L-DOPA, glutathione, and uric acid were evaluated in the presence of a 1 nM Au@MUA nanoparticle pseudostationary phase. As the nanoparticle pseudostationary phase plug length was varied at a fixed separation voltage, all biomarkers exhibited changes in their migration times and/or peak

areas. The positively charged dopamine and epinephrine species migrated more slowly and revealed a slight decrease in peak area as the nanoparticle pseudostationary phase plug length increased. These effects were attributed to electrostatic attractive forces between the positively charged analytes and negatively charged nanoparticles.

The neutral marker pyrocatechol and the slightly negative analyte L-DOPA revealed the smallest separation changes. In both cases, variations in nanoparticle pseudostationary phase plug did not significantly influence migration times. For all pseudostationary phase plug lengths studied, peak areas were reduced relative to the assays performed in the absence of nanoparticles. This suggests that non-specific interactions which are governed by hydrogen bonding and van der Waals attractive forces likely induce the observed peak area reduction.

The negatively charged species uric acid and glutathione exhibited the largest changes in migration times and peak areas. Systematic decreases in the migration times of both species as the nanoparticle pseudostationary phase plug length increased suggest that electrostatic repulsive forces increased the velocities of the species. Peak areas for both analytes surprisingly decreased with increasing pseudostationary phase plug length. These systematic changes were attributed to partitioning and ligand exchange with 11-MUA SAMs for uric acid and glutathione, respectively. Trends for all biomarkers were further supported by reducing the separation voltage (*i.e.* increasing the amount of time the biomarkers could interact with the nanoparticle pseudostationary phase plug).

Clearly, relative charges between analytes and nanoparticle surfaces are important parameters to control and understand in the development of any nanoparticle based assay. As separation conditions are varied, these interactions could be maximized or eliminated to better understand the role of nanoparticles during capillary electrophoresis. Understanding how nanoparticles (*i.e.* composition, concentration, surface chemistry, and nanoparticle-biomarker interaction time) influence separations will provide further insight for effectively employing nanoparticles as pseudostationary phases in electrically driven separations.

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Fig. 1. Characterization of Au@MUA nanoparticles. (A) Extinction spectrum and TEM micrograph (inset) of 1 nM Au@MUA nanoparticles. Au@MUA ($d = 12.36 \pm 0.68$ nm) exhibit an extinction maximum, $\lambda_{\text{max}} = 524.5$ nm in 35 mM phosphate buffer (pH = 7.2). (B) Representative electropherograms from multi-wavelength detection of a 1 nM Au@MUA nanoparticle plug (length = 7.59 cm) in a capillary collected at $\lambda_{\text{det}} =$ (1) 193, (2) 520, and (3) 600 nm, separation voltage = 20 kV. (C) Relative peak areas for a Au@MUA nanoparticle plug length = 7.59 cm and evaluated at varying separation voltages.

Fig. 2.

Representative electropherograms as a function of Au@MUA nanoparticle pseudostationary plug length: (1) 0, (2) 1.2₆, (3) 2.5₃, (4) 5.0₆, and (5) 7.5₉ cm. The analyte peaks are labeled as follows: dopamine (d), epinephrine (e), pyrocatechol (p), L-DOPA (l), glutathione (g) and uric acid (u). In each case, 35 mM phosphate buffer (pH = 7.2) is used as the separation buffer and the “sample” injection order is 1 nM Au@MUA nanoparticles (1 psi), buffer (1 kV for 1 s), and analytes (1 psi for 5 s). Separation voltage = 20 kV (field strength = 333 V cm⁻¹), $\lambda_{\text{det}} = 193$ nm.

Fig. 3.

Trends in biomarker (A) migration time variation and (B) relative velocities vs. Au@MUA nanoparticle pseudostationary phase plug length. The dotted line represents data collected in the absence of nanoparticles. Identical separation conditions described in Fig. 2 are used.

Fig. 4.

Trends in normalized analyte peak area vs. Au@MUA nanoparticle pseudostationary phase plug length for (A) dopamine and epinephrine; (B) pyrocatechol, L-DOPA (l), and uric acid (u); and (C) glutathione. Identical separation conditions described in Fig. 2 are used.

Fig. 5.

Trends in normalized biomarker peak area vs. separation voltage for a 2.53 cm Au@MUA nanoparticle pseudostationary phase plug length for (A) dopamine and epinephrine; (B) pyrocatechol, L-DOPA (l), and uric acid (u); and (C) glutathione. In each case, 35 mM phosphate buffer (pH = 7.2) is used as the separation buffer and the “sample” injection order is 1 nM Au@MUA nanoparticles (1 psi for 10 s), buffer (1 kV for 1 s), and analytes (1 psi for 5 s). Separation voltage = varied from 10–25 kV, $\lambda_{\text{det}} = 193$ nm.

Fig. 6. Trends in biomarker (A) migration time variation and (B) relative velocities vs. separation voltage. The dotted line represents data collected in the absence of nanoparticles. Identical separation conditions described in Fig. 5 are used.

Table 1

Peak resolutions for dopamine/epinephrine and pyrocatechol/L-DOPA as a function of nanoparticle pseudostationary phase plug lengths

Au@MUA nanoparticle plug length (cm)	Resolution	
	Dopamine/ Epinephrine	Pyrocatechol/ L-DOPA
0	2.32 ± 0.11	1.41 ± 0.01
1.2 ₆	2.06 ± 0.03	1.36 ± 0.01
2.5 ₃	1.97 ± 0.04	1.35 ± 0.02
5.0 ₆	1.91 ± 0.03	1.33 ± 0.02
7.5 ₉	1.89 ± 0.02	1.32 ± 0.03