Electrophoretic Separation of Proteins in Capillaries Filled with Poly(ethylene oxide)-stabilized Silver Nanoparticles

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Poly(ethylene oxide)-stabilized silver nanoparticles (PEO-SSNPs) were synthesized and investigated for their potential use in capillary electrophoretic separation of proteins. It was found that the PEO-SSNPs were relatively stable against changes of the buffer ionic strength, while they began to aggregate with increase of buffer pH. Adding 0.024 g/L PEO-SSNPs to 30 mmol/L phosphate buffer (pH 2.66) led to obvious increase in the peak heights of albumin bovine and albumin egg. Also, the separation efficiency for all the standards enhanced due to the suppressed wall-adsorption of proteins in the presence of the nanoparticles. The above experiments suggest that the PEO-SSNPs are effective pseudostationary phase for CE separation of proteins.

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Introduction

Nanoparticles (NPs) have been intensively studied in the fields of catalysis,1 biocatalysis,2 bioimaging,3 labeling,4 sensing,5 photonics,6 and opto-electronics,5 due to their serviceability, novelty and variety. In recent years, efforts have been made to apply NPs in separation science. Dodecanethiol-protected NPs have been demonstrated as efficient stationary phase for gas chromatography in separating organic compounds.7,8 NPs were also used in electrophoresis techniques, either as modifiers to the wall of capillary or microchip,9-12 or as pseudostationary phases (PSPs) in which they were added into the running buffers.13-15 NPs used as PSPs can be replaced from run to run, and the stationary carry-over effects are thus avoided, favoring sample analysis in complex matrices.6 Since the first report by Wallingford and Ewing,17 various NPs to date have been explored. Silica nanoparticles and organically modified silica nanoparticles were employed in capillary electrochromatography (CEC) separation of polycyclic aromatic hydrocarbons (PAHs),18 aromatic acids19 and catecholamines.20 Colloidal gold nanoparticles were synthesized and added to CE buffers for separation of toluidines.21 Molecularly imprinted polymer (MIP) nanoparticles were synthesized and employed to separate enantiomers of propranolol.22 Viberg et al. reported that the NPs in the buffer did not enter the mass spectrometer connected to the capillary via orthogonal electrospray interface, and the analytes were detected with high sensitivity.13 The same research group employed NPs as PSPs in separation of neutral compounds, and they reported plate numbers as high as 700000/m.14 Chang’s group employed gold nanoparticles (GNPs) as additives in CE separation of DNA fragments.23-26 Due to the interaction of DNA fragments with the GNP, the apparent mobilities of DNA fragments changed, and the plate numbers doubled.

Protein separation is important but very challenging in proteomic studies. CE separation of proteins has been actively studied and now is an attractive alternative to the classical polyacrylamide gel electrophoresis (PAGE) method. The NP-protein conjugation improves the selectivity of NPs toward proteins,27 which might be of help in improving protein separation. However, there are only a few reports on this topic. Yu et al.28 demonstrated the separation of proteins in the capillary filled with surfactant-protected gold NPs. Our group reported CE separation of proteins using silica NPs as PSPs, showing that the analysis time was shortened and resolution was improved in the presence of NPs.29 Silver nanoparticles (SNPs) have superior properties relative to other metallic NPs, such as electrical conductivities,30 antimicrobial effects31 and optical properties.32 Poly(ethylene oxide) (PEO) is an often-used stabilizer for NP suspension.32,33 PEO molecules attach to the surfaces of the NPs through their hydrophobic regions, while their hydrophilic groups will interact with the polar suspension media,32 thus providing another improvement in selectivity for protein separation. However, to the best of our knowledge, there is no report on investigating PEO-stabilized silver nanoparticles (PEO-SSNPs) in CE separation of proteins.

The aim of this work is to study the potential usefulness of PEO-SSNPs in CE separation of proteins. The UV-visible spectra of PEO-SSNPs were investigated and the influences of buffer pH, concentration of stabilizer and concentrations of PEO-SSNPs were studied. By careful optimization of the separation conditions, the detection sensitivity and separation efficiency of the standard proteins from 14 to 66 kDa in molecular weight were improved. The results presented in this paper suggest that PEO-SSNPs are effective PSPs for protein separation.

Experimental

Reagents and solutions

Albumin egg (AE, Mw = 45000, pI = 4.7), hemoglobin (HG, bovine, Mw = 64000, pI = 6.8) and lysozyme (LZ, Mw = 14300, pI = 11) were from Sigma (St. Louis, MO); albumin bovine fraction V (AB, Mw = 66000, pI = 4.7) was purchased from

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Amresco (Solon, OH). Poly(ethylene oxide) (PEO, $M_w$ 1000000) was supplied by Alfa Aesar (Ward Hill, MA). Silver nitrate (AgNO₃), sodium borohydride (NaBH₄) and sodium citrate, purchased from Beijing Chemical Plant (Beijing, China), were of analytical grade and were used without further purification. Double-distilled water was passed through a 0.22-$\mu$m filter (Jiuding High Tech., Beijing, China) before being used to prepare all buffers and solutions in the experiment. Individual protein stock solutions of ca. 1000 mg/L each were prepared by dissolving the corresponding standards in water and were stored at –20°C. More dilute solutions were prepared as required.

**Preparation of PEO-SSNPs**

The procedures of Zhang et al. were slightly modified and adopted to prepare the stock solution of PEO-SSNPs. Briefly, 60 mL of 1.5 mmol/L AgNO₃ aqueous solution was added with 20 g/L PEO solution to a desired concentration (3, 2, 1, 0.5, 0.3 and 0.05 g/L, respectively) under vigorous stirring. Then, 200 mL of 400 mmol/L freshly prepared NaBH₄ was added dropwise into the above stirred mixture. The as-formed brown-yellowish colloidal solutions were stored below 4°C. For preparation of running buffer, appropriate volumes of the stock solutions of NP colloidal, phosphoric acid and sodium dihydrogen phosphate were mixed and diluted with double-distilled water to desired concentrations. The background electrolytes containing NPs were not filtered in order to prevent aggregation of NPs on the filter (as observed in our preliminary experiments). However, buffers void of NPs were filtered.

**Instruments and methods**

A GBC UV-visible spectrophotometer (Model Cintra-10, Victoria, Australia) was used to measure the absorbance of the PEO-SSNP suspensions. CE was performed on a DW-P303-1AC capillary electrophoresis system (Sanhuang High Tech, China) conjugated with a CE-10UV detector (Johnsson Separation Science, Liaoning, China) that was operated at 214 nm. A polyimide-coated fused-silica capillary of 75-μm i.d. and 375-μm o.d. (Yongnian Photoconduction Fibre, Hebei, China) was employed in the study. The total length of the capillary was 50.0 cm and the effective length was 40.0 cm. The fresh capillary was rinsed for 30 min with 1 M NaOH, 10 min with double-distilled water and 5 min with running buffer, consecutively. Samples were hydrodynamically injected into the capillary by dipping the anodic end into the sample vial and lifting the vial 15.0 cm for 20 s. Electrophoretic separations were carried out at a voltage of 20 kV for all the experiments in this report. When the pH of the running buffer was changed, the capillary was rinsed with the new buffer for 20 min. Between runs, the capillary was rinsed for 2 min. Data corresponding to absorbance values were transferred from the UV detector to a personal computer via an A/D interface data acquisition unit (Qianpu, Shanghai, China) and processed by an HW-2000 Chromatography Workstation (Qianpu). The CE experiments were conducted at a temperature of 22 ± 1°C.

**Results and Discussion**

**UV-visible spectra of PEO-SSNP suspension**

Figure 1A shows that the absorbance peak of PEO-SSNPs is located at 388 nm, indicating the formation of SNPs. The resonance wavelength of the surface plasmon in metallic NPs depends on the surrounding medium. The absorbance at 388 nm increases with the concentration of phosphate, suggesting that a high concentration of phosphate solution helps to stabilize the NP suspension. This finding coincided with the observation by Huang et al., however, increasing the buffer concentration from 5 to 40 mmol/L only results in an increase of about 0.02 (AU) in UV absorbance, indicating the limited stabilizing ability of high concentration electrolyte towards the PEO-SSNP suspension.

Variation in solution pH caused significant changes in UV-visible spectra of the NPs (Fig. 1B). The absorbance at 590 nm increases with pH, while the absorbance at 388 nm decreases simultaneously. The absorbance at 590 nm corresponds to the aggregation of PEO-SSNPs, which leads to the longitudinal plasmon resonance. It was reported that the extinction ratio was more accurate to assess the degree of aggregation. The curve of extinction ratio ($A_{388}/A_{590}$ in this study) versus pH in the up-right inset of Fig. 1B suggests that the aggregation degree of PEO-SSNPs increases obviously with buffer pH.

**Influence of buffer pH**

Buffer pH is an important factor influencing not only the properties of the NPs but also the surface charge properties of proteins and consequently the NP-protein interactions. Figure 2 shows that the peak heights of AB and AE increase more in the
presence of 0.01618 g/L silver NPs at pH 2.66 (trace A2). Under pH 2.91, the peak height of AB is also higher with the buffer containing NPs (trace B2). However, separation deteriorated in the presence of NPs at pH 4.68 (traces C1 and C2). In the buffer of pH less than 4.7, all the protein standards are positively charged, so their adsorption onto the capillary wall is highly influential on the peak heights. The results presented in Fig. 2 suggest that addition of NPs into the buffer suppresses wall-adsorption of analytes, which was also confirmed by others.\textsuperscript{25} Compared with the working concentration of 0.3 g/L with silica NPs in our previous report,\textsuperscript{29} the silver NPs synthesized in this experiment are more effective in suppressing adsorption of proteins. More increase in the peak heights of AB and AE compared with the other two proteins suggests the selectivity of PEO-SSNPs towards proteins. The peak height of protein is influenced by protein-wall and protein-NP interactions. The protein-wall interactions can be divided into electrostatic and hydrophobic interactions. In acidic buffer of pH < 3, the surface charge density of silica wall is very low, hence the hydrophobic interaction is predominant. The hydrophobic parameters of LZ, AB and AE are 890, 1000 and 980, respectively,\textsuperscript{42} suggesting LZ is more water-soluble. The hydrophobic parameter of bovine hemoglobin could not be found, but it is soluble in water (1 part HG with 7 of water).\textsuperscript{43} So, peaks of LZ and HG could be observed in most of the separation conditions. Furthermore, the LZ molecule is rigid\textsuperscript{44} and the basic/acidic groups on the molecule surface are homogeneously distributed;\textsuperscript{45} both factors further suppress the adsorption.\textsuperscript{45} Therefore, the peak width of LZ is narrow in comparison with those of other model analytes. The protein-silver NP interaction is usually initialized by conjugation between the cysteine residues of the protein and the NPs \textit{via} thiol groups.\textsuperscript{46} The number of cysteine residues in LZ, AB, AE and HG are 8, 35, 6, and 6, respectively.\textsuperscript{47} For the hydrophobic AB and AE, addition of silver NPs suppresses the wall-adsorption by the protein-NP interaction (traces A1 and A2). At pH 4.68, the peak of HG was missing. Although the mechanism is not clear, we think the enhanced aggregation degree of PEO-SSNPs (as illustrated in Fig. 1B) is responsible. It was found that the migration time difference between the first and the last peaks (LZ and HG) increases with buffer acidity, from 1.77 min at pH 2.91 to 2.12 min at pH 2.66, creating the higher peak capacity. So the buffer pH was chosen to be 2.66 in this study.

\textbf{Influence of the stabilizer concentration}

PEO was present during colloid preparation; therefore, it could directly influence particle nucleation and the NP properties.\textsuperscript{46} Increases in the concentration of stabilizer will make the NP suspension more stable, favoring the strong NP-protein interactions. The analysis time shows a trend of decrease with addition of PEO till 0.05 g/L, and then it increases with further addition (Fig. 3). The peak intensity of AB increases with addition of PEO, but the peak height of AE reaches a zenith at PEO concentration of 0.1 g/L. Considering the parameters of analysis time and the peak height of AE, we can deduce from Fig. 3 that the optimal concentration of PEO in the buffer is located between 0.05 and 0.1 g/L. Excess PEO will increase the buffer viscosity and some might adsorb onto the inner wall of the capillary, leading to prolonged analysis time. At the optimal PEO concentration, the four proteins were baseline resolved with high detection sensitivity; the analysis time was shortened from \textit{ca}. 9.5 to 8 min. The experiments suggest that PEO also has some ability to elute AB; the peak height of AB increases dramatically in traces “e” and “f” of Fig. 3.
Conclusions

The PEO-SSNPs were prepared and applied for the separation of proteins. High ionic strength favors dispersion and stabilization of PEO-SSNPs; however, alkali solutions will cause aggregation. SSNPs added in the running buffer suppress the wall-adsorption of proteins, leading to the enhanced detection sensitivity and separation efficiency. Secondly, NPs showed selectivities to the proteins studied, rendering them more able to improve the separation. Further, the analysis time shortened in the presence of NPs due to the increased EOF intensity stemming from the adsorbed negative silver nanoparticles. Our experiments suggest that the PEO-SSNPs are suitable PSPs for protein separation.

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