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Lysine-Based Zwitterionic Molecular Micelle for Simultaneous Separation of Acidic and Basic Proteins using Open Tubular Capillary Electrochromatography

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Abstract

In this work, a zwitterionic molecular micelle, poly-ε-sodium-undecanoyl lysinate (poly-ε-SUK), was synthesized and employed as a coating in open tubular capillary electrochromatography (OT-CEC) for protein separation. The zwitterionic poly-ε-SUK containing both carboxylic acid and amine groups can be either protonated or deprotonated depending on the pH of the background electrolyte; therefore, either an overall positively or negatively charged coating can be achieved. This zwitterionic coating allows protein separations in either normal or reverse polarity mode depending on the pH of the background electrolyte. The protein mixtures contained 4 basic proteins (lysozyme, cytochrome *c*, α-chymotrypsinogen A, and ribonuclease A) and 6 acidic proteins (myoglobin, β-lactoglobulin A, β-lactoglobulin B, α-lactalbumin, and albumin). Protein separations were optimized specifically for acidic (reverse mode) and basic (normal mode) pH values. Varying the polymer thickness by changing the polymer and salt concentration had a great influence on protein resolution, while all peaks were also baseline resolved in both modes using the optimized poly-ε-SUK coating concentration of 0.4%. Proteins in human sera were separated under optimized acidic and basic conditions in order to demonstrate the general utility of this coating. Nanoscale characterizations of the poly-ε-SUK micellar coatings on silicon surfaces were accomplished using atomic force microscopy (AFM), to gain insight into the morphology and thickness of the zwitterionic coating. The thickness of the polymer coating ranged from 0.9–2.9 nm based on local measurements using nanoshaving, an AFM-based method of nanolithography.

Keywords

protein separation; zwitterionic; molecular micelle; open tubular capillary electrochromatography; human serum; atomic force microscopy; nanoshaving; lysine coating

Introduction

Chromatographic separation and quantification of proteins in complex biological mixtures remains a challenge for many bioanalytical applications including bioprocessing,[1] sensory devices,[2] tags in drug delivery,[3] and medical diagnostics.[4] Many proteins have been identified as biomarkers for detection of diseases such as lysozyme for Sjögren' s syndrome,[5] ribonuclease A for ovarian cancer,[6] and myoglobin for cardiac injury due to anthrax lethal

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toxin in mice.[7] However, separation of an individual protein from a pool of proteins in a biological fluid or tissue is a daunting task.

High performance liquid chromatography (HPLC) can be used for protein separation; however, HPLC analyses often require large sample volumes and relatively high protein concentrations. In addition, HPLC techniques are typically limited to separation of proteins based on either their size and/or charge, i.e. size-exclusion and ion-exchange. Recently, polymer beads grafted with a zwitterionic stationary phase have been applied to HPLC analyses for separating two acidic and three basic proteins simultaneously.[8] In contrast, electrophoretic techniques, such as capillary electrophoresis (CE), can achieve separation of proteins based on both the size and charge of the protein.

Apart from its use in protein separations, CE is routinely used for separation of a variety of small organic molecules including drugs,[9] amino acids,[10] and DNA.[11] Some advantages of CE over HPLC are higher resolution, higher efficiencies, smaller sample consumption, and shorter analysis time. However, the use of CE for the separation of biomolecules such as proteins can be difficult due to analyte-wall adsorption resulting in peak tailing, unstable baseline, and poor reproducibility.[12, 13] One strategy for minimizing protein-wall adsorption is to use packed-column capillary electrochromatography (PC-CEC). PC-CEC is a technique in which the capillary is filled with micron-sized particles modified with chemical moieties such as octadecyl silica beads.[14] Separations are based on the electrophoretic mobilities of the analytes as well as interactions with the stationary phase. This technique typically yields highly efficient separations. However, it also requires frits at the ends of the capillary, which inevitably produce bubbles and affect the performance of the separation.[15]

Open-tubular capillary electrochromatography (OT-CEC), a hybrid of CE and HPLC, is another mode of chromatography in which a capillary is coated with a stationary phase (e.g. charged polymers) to facilitate interactions with analytes.[16, 17] Chen *et al.* have applied positively charged amine based polymers as coatings to capillaries in separating peptides and basic proteins.[18] Advantages of this approach include low polymer consumption, ease of preparation, and possible coupling to mass spectrometry.[19] Coupling to mass spectrometers can be accomplished with OT-CEC because the stationary phase is electrostatically bound to the capillary wall, rather than present in the background electrolyte (BGE) as in micellar electrokinetic chromatography (MEKC).[20] Capillaries modified with either adsorbed or covalently attached polyelectrolyte coatings can be used for OT-CEC, which can minimize electrostatic interactions between the protein and capillary wall.[17, 21–25]

Questions yet to be addressed when using molecular micelles as coatings for OT-CEC are the nature and resilience of individual micellar structures and the thickness of the coatings that are formed on columns. It is not known if micelles undergo significant structural rearrangements when interacting with charged surfaces of the silica capillaries, or if the spherical geometries of the micelles persist. In this manuscript, atomic force microscopy (AFM) is used to image zwitterionic micelles in order to evaluate the structural persistence, shape, and film thickness under conditions that simulate buffered conditions within the capillary column. Polished, flat silicon substrates were used for AFM studies of micelle attachment under conditions that mimic the buffered media and immersion intervals that were used for OT-CEC with zwitterionic micelles. Studies using AFM are emerging as a practical strategy for probing mechanisms of surface adsorption and the integrity of micellar structures. For example, pioneering studies have been achieved by Manne and Gaub, who first resolved the structure of micelles of quaternary ammonium surfactants in 1995 in liquid media.[26] For their cationic surfactant system, an adsorbate layer of roughly spherical micelles was observed to form on silicon substrates; however, surfaces of mica, MoS_2, and graphite produced different surface

morphologies, ranging from a flat bilayer to stripes of cylindrical or even hemispherical aggregated structures.

Scanning probe characterizations have also been accomplished for micelles adsorbed at the solid-liquid interface comprised of cationic surfactants,[27–29] polymers,[30, 31] and block copolymers.[32–38] Primary types of surfaces which have been used for such studies include mica, glass, gold, or graphite. It has been demonstrated that the morphology observed for micelles on various surfaces is often different from that observed in bulk solution. Thus, the substrate plays a critical role in determining the micelle surface structure. Surface immobilization of the micellar structures results from strong electrostatic interactions between the surface and surfactants. Depending on the surfactant concentration, interactions with highly charged surfaces have been shown to change micellar structures to form self-assembled layer-like structures in which surfactant molecules organize and interact with other surfactant molecules and with the underlying surface. Thus, to achieve molecularly-resolved images of micelle layers, *in situ* AFM protocols were accomplished in liquid solutions to prevent micelles from floating in solution or from being destroyed by the AFM tip during contact-mode scanning.

Previously, we have demonstrated the effectiveness of several amino acid-based molecular micelles in MEKC and as coatings in OT-CEC for separation of achiral[39, 40] and chiral molecules,[41–44] as well as proteins[16]. It has been demonstrated that molecular micelle coated capillaries provide enhanced separation performance over capillaries coated with conventional micelles.[41–44] The principal advantage of molecular micelles is that they can be used at concentrations well below the monomer's critical micelle concentration (CMC) due to covalent bonds formed between individual monomers. Therefore, this eliminates the dynamic equilibrium of conventional micelles. Recently, Luces *et al*. have demonstrated amino acid based molecular micelles in polyelectrolyte coatings (PEMs) for the separation of four basic proteins.[16] These amino acid based anionic molecular micelles (e.g. sodium poly(N-undecanoyl-L-leucyl-alaninate), sodium poly(N-undecanoyl-L-leucyl-valinate)) were used in combination with cationic polymers to form the multilayers. A PEM coating consists of alternating layers of positively and negatively charged polymers. We hypothesized that a zwitterionic coating would eliminate the need for multiple layers due to its dual charge property. At acidic pH values the polymer has a more overall positive charge, while at basic pH values a net negative charge is achieved. This property allows separation of acidic and basic proteins simultaneously.

The use of conventional zwitterionic micellar coatings for OT-CEC has been reported.[23, 24] Of the many different types of OT-CEC capillary coatings, zwitterionic systems are of particular interest because they can provide a means of controlling the ionic characteristics of the capillary surface and the electroosmotic flow (EOF) as a function of pH.[45–48] Herein, we report the synthesis of a novel lysine-based zwitterionic molecular micelle and its first application to protein separations in OT-CEC.

Experimental

Materials and Reagents

N-hydroxysuccidimide, N,N′-dicyclohexylcarbodiimde (DCC), 10-undecylenic acid, sodium bicarbonate, tetrahydrofuran, acetone, and ethyl acetate were purchased from Sigma (Milwaukee, WI). N_{ε}–Boc-L-lysine, dichloromethane (DCM), trifluoroacetic acid (TFA), ethyl acetate (EtOAc), Tetrahydrofuran (THF), hydrochloric acid, lysozyme (chicken egg white), cytochrome *c* (bovine heart), α-chymotrypsinogen A (bovine pancreas), ribonuclease A (bovine pancreas), deoxyribonuclease I (bovine pancreas), α-lactalbumin (bovine milk), β-lactoglobulin A (bovine milk), β-lactoglobulin B (bovine milk), myoglobin (equine heart),

albumin (bovine serum), human sera, and individual human sera proteins (IgG, transferrin, and albumin) were purchased from Sigma-Aldrich (St. Louis, MO). All proteins were ≥ 85% pure. Sodium phosphate dibasic, sodium hydroxide, methanol, and acetone were purchased from Fisher Scientific (Fair Lawn, NJ). All materials were used as received without further treatment or purification. The pH of the BGE, 20 mM phosphate buffer, was adjusted using 1 M HCl. The buffer was filtered using a 0.45 μm polypropylene filter (Nalgene, Rochester, NY) and sonicated for 15 minutes prior to use. Protein stock solutions were prepared at 4 mg/mL in the BGE and diluted to a working concentration of 0.4 mg/mL.

Synthesis of Lysine-based Zwitterionic Molecular Micelle

The poly-ε-sodium-undecanoyl lysinate (poly-ε-SUK) shown in Figure 1 was prepared by use of a modified method reported earlier.[20] The undecylenic acid N-hydroxysuccinimide ester and ε-Boc-lysine were stirred for 48 hrs to form the lysine based surfactant monomer. Sodium bicarbonate was used to neutralize the carboxylic acid to obtain the sodium salt of the surfactant monomer. The mass spectrum as well as ^{1}H and ^{13}C NMR characterization (in D_2O) are shown in the supporting material (Figures S-1, S-2, and S-3, respectively). The polymerization of e-Boc-sodium-undecenoyl lysinate (e-Boc-SUK) was achieved by γ-irradiation of a 0.1 M surfactant solution using a ^{60}Co source. The ^{1}H and ^{13}C NMR characterization (in D_2O) are shown in the supporting material (Figures S-5 and S-6, respectively). Deprotection of the t-Boc group from the resulting polymer was accomplished with a 50% TFA/DCM solution.[49]

Tensiometric Characterization of Micellar Solutions

Surface tension measurements for all micellar solutions were acquired in triplicate at room temperature using a KSV Sigma 703 Digital Tensiometer. Special care was taken to thoroughly clean the platinum-iridium ring of the tensiometer with deionized water and acetone prior to each measurement. The CMC of ε-Boc-SUK surfactant in water was determined by measuring the surface tension of the surfactant solution over a range of concentrations. The resultant plot of surface tension versus ε-Boc-SUK concentration is shown in the supporting material (Figure S-4). Note that the interception of the two lines indicates the CMC of ε-Boc-SUK. The CMC value obtained for ε-Boc-SUK was 9.05 mM.

Atomic Force Microscopy

Images of the poly-ε-SUK films were acquired at ambient conditions with a model 5500 scanning probe microscope (SPM) equipped with Picoscan v5.3.3 software (Agilent Technologies, Inc., Chandler, AZ). Topographic images were acquired using contact mode in buffer with a scan rate of 3.0 nm/s for 512 lines/frame. The AFM probes used for surface characterizations were oxide-sharpened silicon nitride tips, MSCT-AUHW (Veeco, Santa Barbara, CA) with an average force constant of 0.5 N/m. To minimize tip-sample adhesion, the AFM tips were coated with octadecyltrichlorosilane (OTS) purchased from Gelest, Inc, (Morrisville, PA).[50] To coat the AFM tip, the cantilever was first exposed to UV light (254 nm) for 1 h, then immersed in a solution of 1 mM OTS in a 7:3 v/v mixture of hexadecane and chloroform for 1 h. The tips were then rinsed in chloroform, dried in air and stored until needed. Images were processed using Gwyddion version 2.5, open source software for data visualization and analysis, supported by the Czech Metrology Institute (http://gwyddion.net/).

Substrates used for AFM investigations were pieces of polished silicon wafers (Virginia Semiconductors, Fredericksburg, VA) that were cleaned by immersion in piranha solution for 1 h. Pirahna solution was freshly prepared by mixing sulfuric acid and 30% hydrogen peroxide (3:1 v/v). Caution should be taken since piranha is highly reactive and corrosive. The surfaces were then rinsed with copious amounts of deionized water. To simulate the conditioning of a fused-silica capillary column, the cleaned substrates were sonicated in 1 M NaOH for 30 min

and deionized water for 15 min. To prepare films of poly-ε-SUK micelles, clean substrates were immersed in 0.4% (w/v) solution of poly-ε-SUK in 20 mM phosphate buffer pH 3.0 for 20, 90, and 420 min, respectively. The samples were then analyzed immediately using contact-mode AFM in 20 mM phosphate buffer, pH 3.

OT-CEC Column Fabrication and Separation Methods

Separations were performed using a Beckman P/ACE MDQ capillary electrophoresis system equipped with a UV photodiode array detector (Fullerton, CA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). Each capillary was 50 cm total length (40 cm effective length) and had an internal diameter of 50 μm for all separations. Capillaries were conditioned by rinsing with 1 M NaOH for 30 minutes followed by deionized water for 15 min. The conditioned capillaries were then coated by rinsing with poly-ε-SUK solutions. Coated capillaries were flushed with the corresponding 20 mM sodium phosphate dibasic buffer. All rinses were performed using a pressure of 20 psi. Separations were performed under reverse or normal polarity at temperature of 15 to 25°C with an applied voltage of 15 to 25 kV. Electrokinetic injections were performed by applying 5 kV for 5 s. Electropherograms were collected at 200 nm.

Results and Discussion

OT-CEC protein separations are influenced by at least three factors: electrophoretic mobility, stationary phase interactions, and the EOF. Proteins are separated in CE due to differences in electrophoretic mobility based on their size to charge ratio. Protein interactions with the stationary phase are also an important factor to consider in order to provide greater peak resolution. The EOF is the bulk flow of the solution and a major component of an OT-CEC method. The EOF is established by the charge of and/or on the capillary wall, an applied electric field, and attraction of ions of the BGE to the capillary surface charge.[51, 52] Due to the isoelectric point of the zwitterionic coating, the overall charge can be more positive if the pH of the BGE is below the pI of the coating or more negative if above. Thus, μ_{EOF} can be manipulated through controlled changes in pH.

Evaluation of Zwitterionic Character of the Micellar Stationary Phase as a function of pH

Tunablity of the EOF is an important factor for a zwitterionic coating capable of separation at both acidic and basic pH values. Control of the EOF is due to protonation of both the amine and carboxyl groups of the zwitterionic coating, resulting in an overall positive charge in acidic media. Conversely, deprotonation of the amine and carboxyl groups results in an overall negative charge. Increased dominance of one of the charged species relates to increased electroosmotic mobility of the EOF marker, i.e. μ_{EOF}.

In Figure 2, the influence of the μ_{EOF} is demonstrated for pH values ranging from 3.0 to 9.0. To acquire these data, each new coating was applied to a bare capillary at the corresponding separation pH. The capillary was then flushed for 2 minutes with the same buffer. The absence of the EOF marker in reverse or normal mode at pH values near the isoelectric point of the zwitterionic molecular micelle was due to severely diminished EOF. At pH values approaching the isoelectric point, i.e. approximately pH 5.5, the zwitterionic molecular micelle had a net charge of zero, and thus no attraction to the cationic and anionic components in the solution, which caused stagnation of the bulk flow. Deviations from the isoelectric point, yielded an increased μ_{EOF} due to either higher net positive or net negative charge. Coated capillaries reflected an anodic EOF at low pH and conversely a cathodic EOF at high pH. Such changes cannot be observed in a bare capillary which can only exhibit a cathodic EOF as has been previously reported by Wang et al.[53]

Characterizations of Zwitterionic Micellar Coating on Silicon using AFM

It is expected that increasing the thickness of a stationary phase in OT-CEC will yield more interactions with analytes. Nanofabrication with AFM was used to evaluate the changes in thickness for coatings formed on the silicon surface. Changes in the thickness of micellar coatings were evaluated at acidic pH, using the conditions optimized for protein separation. To prevent the surface from super saturation at higher concentrations of polymer, a parallel study using AFM with varying polymer coating times was investigated while holding concentration constant. As a baseline, AFM topography views of the clean, uncoated silicon surface are presented side-by-side with the coated substrate in Figure 3. Considerable changes are observed for the AFM topography images of the clean surface of silicon (Figure 3A) versus the film of poly-ε-SUK formed after 420 min immersion (Figure 3B). The silicon surface has a local surface roughness measuring 0.75 nm for the $4 \times 4\ \mu m^2$ area displayed in Figure 3A. The brighter colors represent taller features, whereas shallow features are darker. The images displayed are representative of views for many different areas of the sample. The cleaned surface of silicon exhibits a somewhat irregular and uneven morphology at the nanoscale, with depressions or pits ranging from 0.3 to 2.9 nm in depth. The silicon surface does not contain visible contaminants or residues, indicating success of the cleaning procedure. Complete coverage of the surface with a film of micelles was apparent after 420 min of immersion in poly-ε-SUK solution (Figure 3B). The surface is coated with densely packed micelles, which range in height from 0.8 to 6 nm, corresponding to single and multiple layers. The shapes of the molecular micelles are nearly spherical, and are consistent with observations gleaned from previous reports for different types of micelles formed on mica[54] and silicon.[33] The slightly non-spherical shape of the micelles may be attributed to the shape of the probe, caused by the well-known tip-surface convolution effects of AFM tip geometry. The holes and roughness of the underlying substrate are still apparent after forming a coating of poly-ε-SUK. The surface roughness measured for the topography frame in Figure 3B measures 1.3 nm. The size of the micelles is quite uniform and monodisperse, as evident in the topograph and corresponding histogram of Figure 3C. Most of the micelles (52%) measure from 1.6 to 2.4 nm in height, with an overall average size of 2.3 ± 1.6 nm. However, the roughness of the bare silicon substrate (0.75 nm) complicates the precise measurement of the micelle diameter, and the peaks and valleys of the substrate contribute to the overall dimensions plotted in the histogram.

Nanoshaving was used to evaluate the local thickness of poly-ε-SUK coatings deposited onto silicon substrates. Tip induced displacement of surface films of multilayers of polymeric micelles was previously demonstrated by Emoto, et al.[55] Nanoshaving is accomplished by applying a sufficiently high force to the AFM tip to remove or “shave” away a small region of the film in order to expose a bare area of the silicon substrate. The uncovered area then provides a baseline for measuring the film thickness.[56] For the film of adsorbed zwitterionic micelles, the nanoshaved rectangular area of the silicon surface measures $600 \times 900\ nm^2$ and is clearly visible in both the topography and lateral force images of Figures 4A and 4B, respectively. Note that the area was swept seven times with an applied set point of 10 nN to ensure complete removal of poly-ε-SUK. Micelle nanostructures are visible throughout the scan area except within the nanoshaved pattern. The lateral force image (Figure 4B) shows that micelles have been cleanly removed from the substrate, since the exposed polished silicon wafer has a smooth, homogeneous contrast as compared to the unshaved region. A representative cursor line profile across the pattern is presented in Figure 4C. The local thickness of the film measured 2.4 ± 0.7 nm, in agreement with the height values measured for individual micelles from Figure 3.

To better evaluate the film thickness of poly-ε-SUK formed under different immersional intervals, additional nanoshaving experiments were conducted (AFM images not shown). These results are summarized in Table 1 for thickness values obtained from cursor measurements of nanoshaved patches from several nanoshaving experiments. After only 20

min of immersion, incomplete surface coverage was observed. As one would expect, as the length of immersion was increased, a thicker layer of micelles was observed to form.

In situ AFM characterizations of films of lysine-based zwitterionic molecular micelles demonstrate that uniform and structurally precise micelle structures have been produced, with regular spherical shapes that persist after adsorption on silicon. The supramolecular assembly of zwitterionic micelles into multilayered coatings can be controlled by immersion intervals, within time frames of a few hours. Although micelles have been previously shown to disassemble and form bilayer coatings on certain substrates,[57] our results with *in situ* AFM clearly demonstrate that controlling pH and solvent parameters ensure conditions for robust, persistent molecular micellar structures.

Simultaneous Acidic and Basic Protein Separation by OT-CEC under Acidic Conditions

The use of poly-ε-SUK as a coating in OT-CEC allowed minimization of protein-wall adsorption and provided a stationary phase for analyte interaction. Figure 5 is a display of the effect of poly-ε-SUK concentration on the simultaneous separation of 10 acidic and basic proteins (Table 2) under cathodic EOF. The polymer concentration ranged from 0.3 to 0.5% (w/v). At lower concentrations (0.3% w/v), long migration times were observed, as well as low separation reproducibility which is likely the result of partial coverage of the polymer on the capillary wall. Having non-uniform coating allowed protein-wall adsorption which may have resulted in low peak efficiencies. Correspondingly, not all individual proteins could be identified. R_{s1} denotes the resolution between the first and second peaks, R_{s2} for the second and third peaks, R_{s3} for the third and fourth peaks, and so on.

Using 0.4% (w/v), 9 proteins peaks were resolved (R_{s1} = 3.42 R_{s2} = 1.02 R_{s3} = 2.07 R_{s4} = 0 R_{s5} = 1.83 R_{s6} = 5.13 R_{s7} = 0.59 Rs_8 = 1.32 R_{s9} = 4.58). Partial separation occurred between β-lactoglobulin A, and β-lactoglobulin B while α-lactalbumin and ribonuclease A coeluted. All other peaks were baseline resolved owing to thicker coating which in turn promoted interactions with the analytes. As the polymer concentration was increased to 0.5% (w/v) protein migration times increased and efficiencies decreased, while protein peak migration order was the same as 0.4%(w/v), with 9 protein peaks being resolved (R_{s1} = 3.29 R_{s2} = 0.51 R_{s3} = 0.95 R_{s4} = 0 R_{s5} = 1.90 R_{s6} = 3.16 R_{s7} = 0.41 R_{s8} = 1.54 R_{s9} = 4.70). Due to increased thickness of the coating, further increases in elution times produced longer analysis times. Therefore, 0.4% (w/v) was chosen as the optimal polymer concentration value as a result of this study.

Influence of NaCl concentration

Previous studies have demonstrated that the presence of NaCl in capillary coatings increases the thickness of the coating[58] and in turn increases protein separation resolution.[16, 40, 59] Figure 6 shows the effect of NaCl concentration in poly-ε-SUK coatings on the above protein system in a selected concentration range of 15–25 mM (Table 3). When 25 mM NaCl was used, 8 peaks were observed. Protein peaks 4, 5 (α-lactalbumin and ribonuclease A) and 7, 8 (β-lactoglobulin A and β-lactoglobulin B) were not resolved. Using 20 mM NaCl, all 10 protein peaks were distinctively resolved. All peaks with the exception of peaks 4, 5 (α-lactalbumin and ribonuclease A) and 7, 8 (β-lactoglobulin A and β-lactoglobulin B) were baseline resolved. At 15 mM, 9 protein peaks eluted. Protein peaks 2, 3 (α-chymotrypsinogen A and albumin) were not resolved while 4, 5 (α-lactalbumin and ribonuclease A) and 7, 8 (β-lactoglobulin A and β-lactoglobulin B) were partially resolved. Therefore, 20 mM NaCl was selected as the optimal concentration and adopted for all subsequent separations. Column robustness was also evaluated using polymer concentration, polymer coating time, and NaCl concentration in the supplemental material (Figures S-7, S-8, and S-9, respectively). Minor changes were observed in experiments involving evaluation of the coating times and NaCl concentrations. However,

increased polymer concentration by 10% produces deviations from optimum for the coating conditions, causing only 8 of the 10 proteins to be resolved. Protein peaks 2, 3 (α-chymotrypsinogen A and albumin) showed no separation along with peaks 6, 7 (myoglobin and β-lactoglobulin A). Partial separation of peak 8 (β-lactoglobulin B) with coeluting peaks 6, 7 was also observed. The coelution of some proteins observed with such small variance in polymer concentration indicates chromatographic behavior.

Simultaneous Acidic and Basic Protein Separation by OT-CEC under Basic Conditions

Due to the zwitterionic properties of the coating, separations performed under cathodic EOF can be achieved as quickly as under anodic EOF. To examine the performance of this coating under anodic EOF conditions, the separation was performed at pH 11.5. Figure 7 shows the optimized separation of acidic and basic proteins under basic experimental conditions. The voltage was lowered to 10 kV and the poly-ε-SUK coating time was lowered to 15 min in contrast to the optimized separation at acidic pH. All other variables were held constant (0.4% (w/v) poly-ε-SUK, 20mM sodium phosphate, 15°C). The EOF was observed at 9.8 minutes and 9 protein peaks were resolved. All resolved protein peaks had at least baseline resolutions, i.e. greater than 1.5. However, peaks 7 and 8 (β-lactoglobulin A and β-lactoglobulin B) co-eluted and were not resolved under any variations of voltage, salt concentration, temperature, or other experimental conditions.

Reproducibility of a Lysine-Based Zwitterionic Molecular Micelle-Coated Capillary

To determine the stability of the poly-ε-SUK coating, run-to-run, day-to-day, and week-to-week studies were investigated. The reproducibility of the EOF was evaluated by monitoring the average retention time of the EOF marker and % relative standard deviation (%RSD). The results of this study are summarized in Table 4. Each capillary was prepared according to the coating procedure previously described for the optimized separation at acidic pH. The run-to-run reproducibility for the EOF was calculated based on 50 consecutive runs using the same capillary. The day-to-day reproducibility for the EOF was evaluated based on the use of one capillary for 5 days with replicate analyses; the week-to-week reproducibility was evaluated based on 5 different capillaries with 3 consecutive runs for each. All measured %RSD were below 4%, indicative of highly acceptable reproducibilities.

Separation of Proteins from a Human Serum Sample using a Lysine-Based Zwitterionic Molecular Micelle Coating

A sample of human serum, used as received from Sigma Aldrich, was evaluated using the optimized conditions for both acidic and basic pH values. The results of these studies are provided in Figure 8. For the acidic pH separation, the human serum was diluted with buffer by a factor of 8. However, under basic conditions a more concentrated mixture with a dilution factor of 2 was used. Peak areas were observed to correlate with the amount of protein found from normal human serum samples reported in the literature (IgG 8–18 mg/mL, transferrin 2–3 mg/mL, and albumin 35–50 mg/mL).[60] The separations achieved at acidic and basic pH values were obtained under the same optimized conditions used in Figures 6B and 7, respectively. An injection-to-injection study, shown in the supporting material (Table S-1), was investigated to evaluate the reproducibility of the migration times of major protein peaks. Less than 1% RSD was observed for all protein peak migration times at both acidic and basic pH. Contributions from sample impurities along with degradation products of the three major proteins may lead to peaks of low efficiencies since protein standards did not exhibit such behavior. Both acidic and basic optimized conditions resulted in the same elution order of proteins. However, separation under basic conditions provided better resolution than under acidic conditions. Thus, this separation shows the versatility of using lysine-based molecular micelles for the separation of proteins from human serum with no purification of the sample.

Conclusions

The synthesis, characterization, and application of a lysine-based zwitterionic molecular micelle (poly-ε-SUK) for capillary coatings in OT-CEC are presented in this study. The versatility of poly-ε-SUK is demonstrated through simultaneous separation of acidic and basic proteins at both low and high pH values. Most of the proteins studied were baseline resolved. This novel zwitterionic coating also contributed to variations in the μ_{EOF} as a function of systematic changes in pH. It was found that μ_{EOF} approached zero as the pH approached the estimated pI of the zwitterionic molecular micelle (~pH 5.5). High stability of the coating was demonstrated with up to 50 runs on the same capillary, resulting in an RSD of 1.24%. Views acquired with AFM demonstrate that regular, robust spherical geometries of micelles are formed as multilayers on silicon, which retain their structural integrity after surface adsorption. Proteins from an untreated human serum sample were separated and identified under both acidic and basic pH conditions to demonstrate the general utility for separation of complex samples. As a result of these findings, poly-ε-SUK is considered a unique coating with great potential for simultaneous separation of acidic and basic protein systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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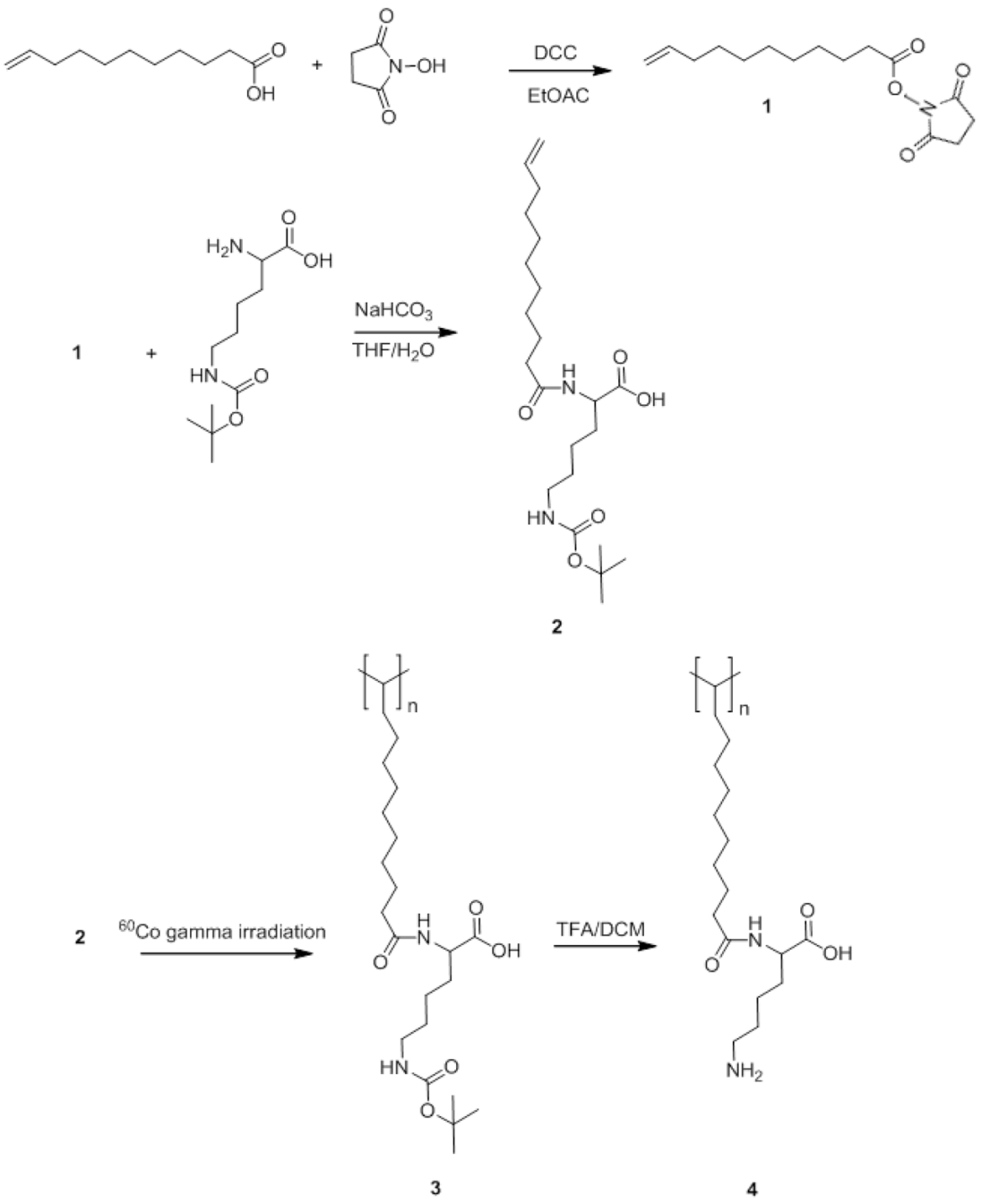

Figure 1.
Synthesis of poly-ε-SUK

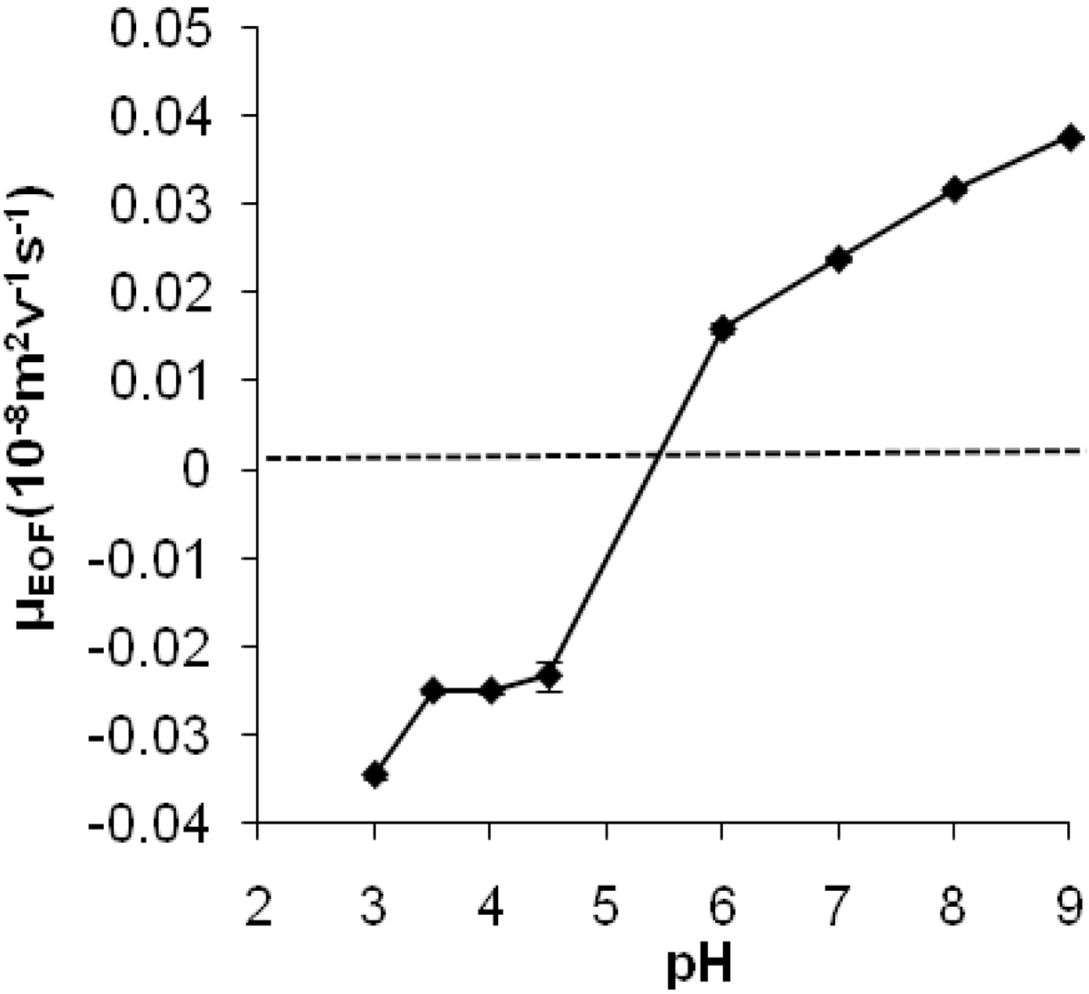

Figure 2.
Effect of BGE pH on μ_{EOF} using poly-ε-SUK coating. **Conditions: Coating time:** 20 min at pH 3; **NaCl concentration:** 20mM; **Background electrolyte:** 20mM sodium phosphate dibasic; **Applied voltage:** 25 kV; **Temperature:** 15 °C; **Capillary:** 50 cm (total length), 40 cm (effective length), 50 μm i.d.; **Analyte concentration:** 0.4 mg/mL; **Injection:** 5 kV for 5 s; **Detection:** 254 nm.

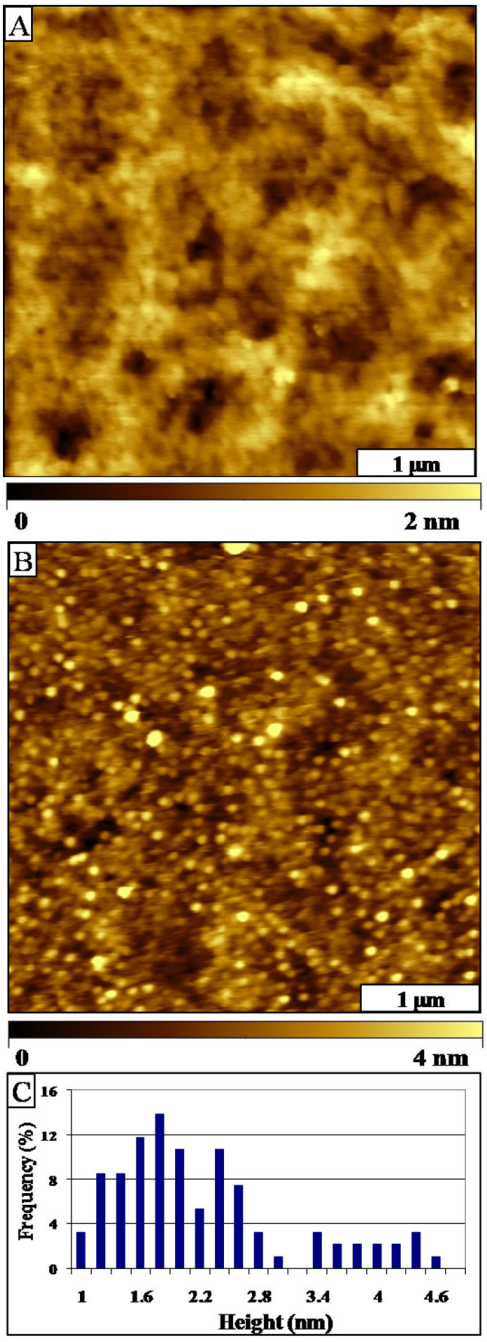

Figure 3.
Changes in surface morphology viewed with contact-mode AFM topographs. [A] Clean surface of polished silicon; [B] after immersion in 0.4% (w/v) poly-ε-SUK; [C] size distribution for the micelles measured from individual cursor height profiles.

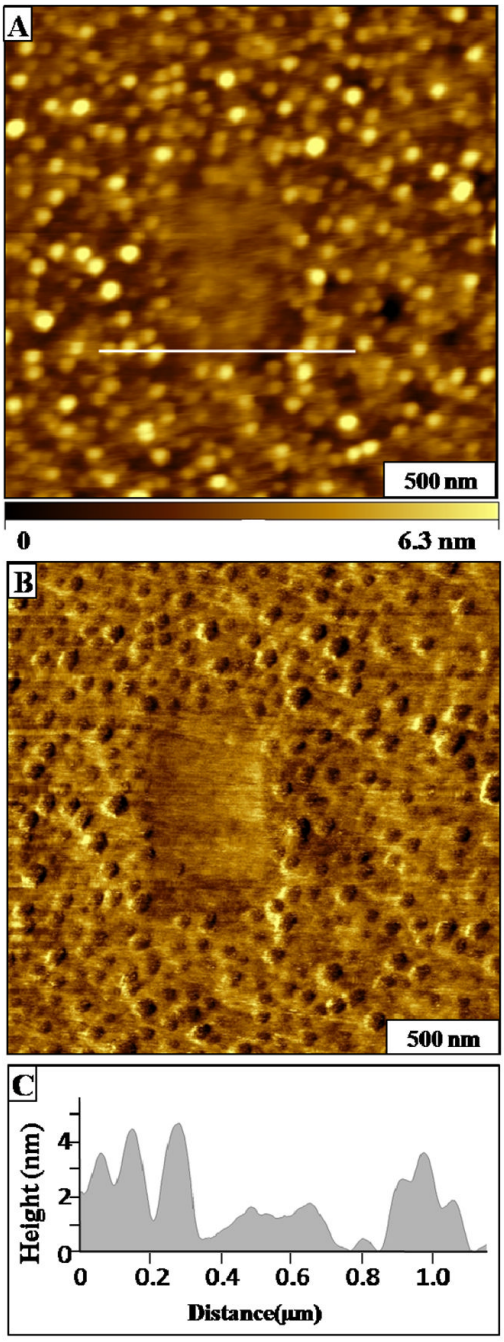

Figure 4.
Nanoshaved area produced by increasing the force applied to an AFM tip. [A] Contact-mode topograph; [B] corresponding friction image; [C] height profile for the line in **A**.

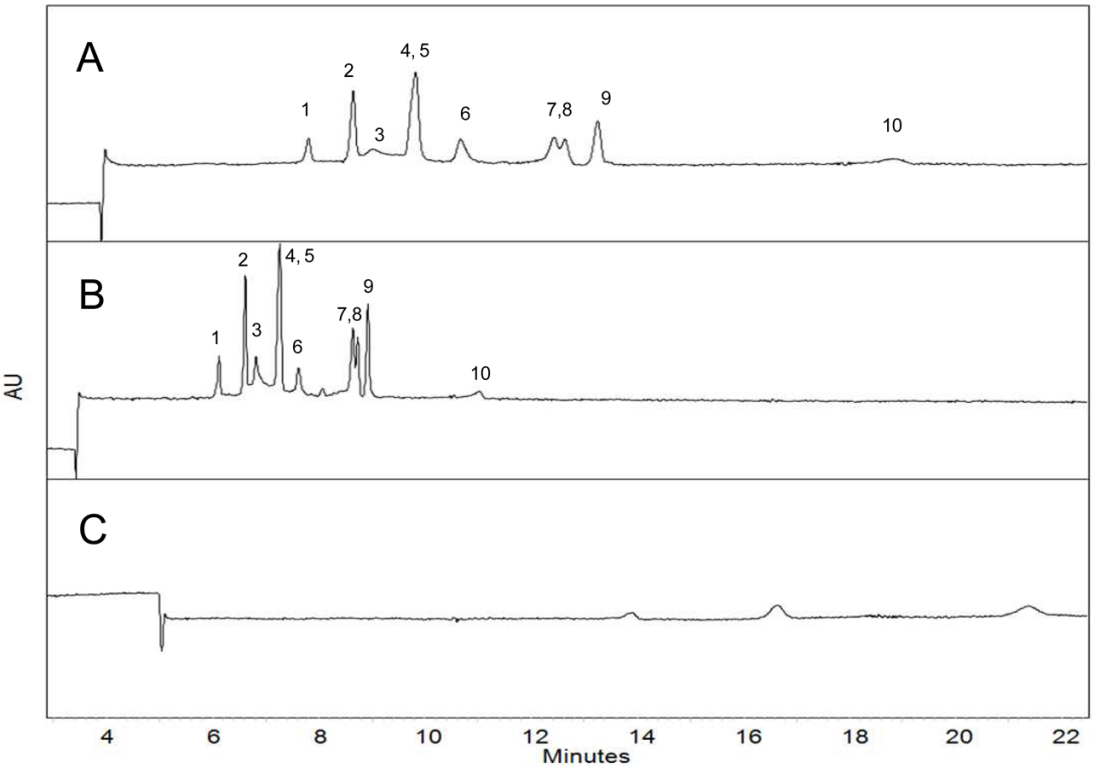

Figure 5.

Influence of polymer concentration on separation of 10 acidic and basic proteins. **Conditions: Polymer concentration:** (A) 0.5% (B) 0.4% (C) 0.3% (w/v) poly-ε-SUK; **Coating time:** 20 min; **Background electrolyte:** 20mM sodium phosphate dibasic (pH 3); **Applied voltage:** 25 kV; **Temperature:** 25 °C; **Capillary:** 50 cm (total length), 40 cm (effective length), 50 μm i.d.; **Analyte concentration:** 0.4 mg/mL; **Injection:** 5 kV for 5s; **Detection:** 200 nm; **Analytes: 1.** deoxyribonulease I, 2. α-chymotrypsinogen A, **3.** albumin, **4.** α-lactalbumin, **5.** ribonuclease A, **6.** myoglobin **7.** β-lactoglobulin A, **8.** β-lactoglobulin B, **9.** lysozyme, **10.** cyctochrome *c*.

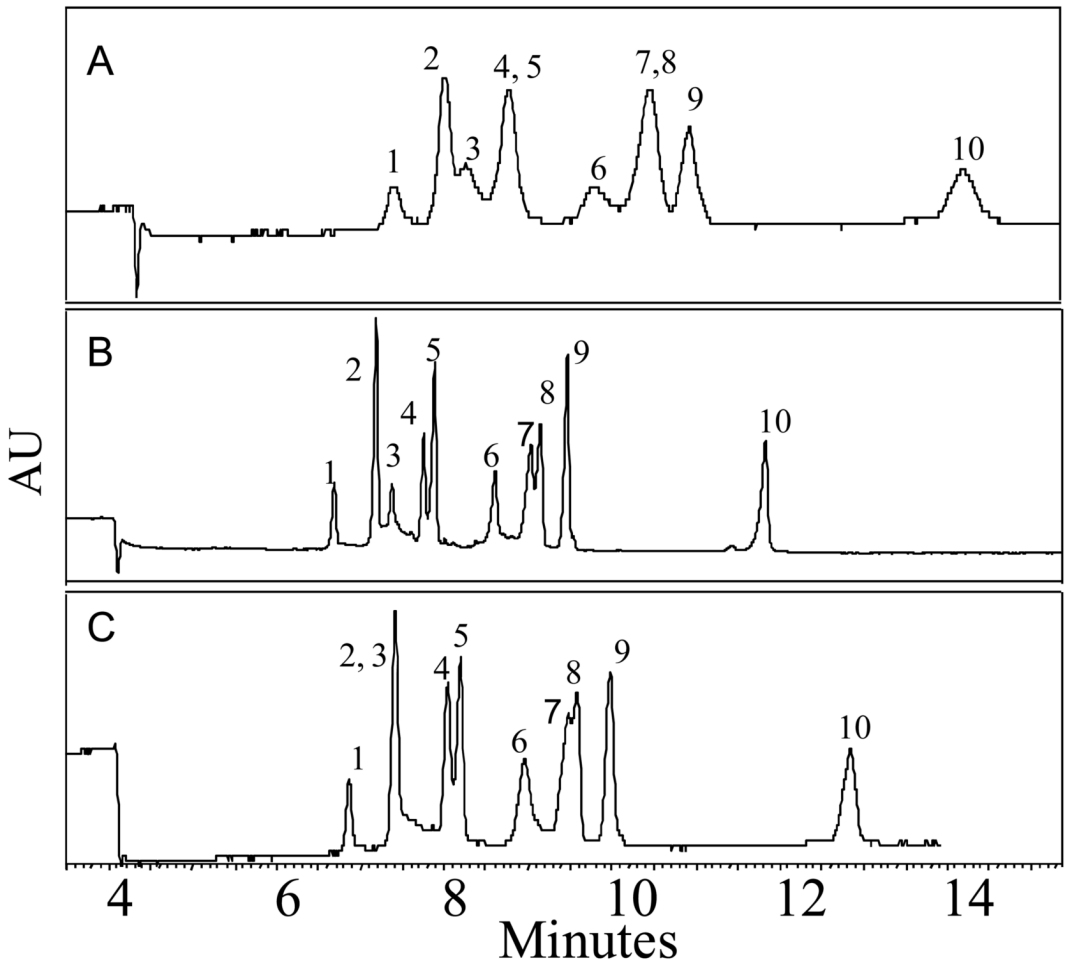

Figure 6.

Effects of NaCl concentration on separation of 10 acidic and basic proteins. **Conditions:** **Polymer concentration:** 0.4%(w/v) poly-ε-SUK; **Coating time:** 20 min; **NaCl concentration:** (A) 25mM (B) 20mM (C) 15mM; **Background electrolyte:** 20mM sodium phosphate dibasic (pH 3); **Applied voltage:** 25 kV; **Temperature:** 15 °C; **Capillary:** 50 cm (total length), 40 cm (effective length), 50 μm i.d.; **Analyte concentration:** 0.4 mg/mL; **Injection:** 5 kV for 5 s; **Detection:** 200 nm; **Analytes:** 1. deoxyribonulease I, 2. α-chymotrypsinogen A, 3. albumin, **4.** α-lactalbumin, **5.** ribonuclease A, **6.** myoglobin **7.** β-lactoglobulin A, **8.** β-lactoglobulin B, **9.** lysozyme, **10.** cyctochrome *c*.

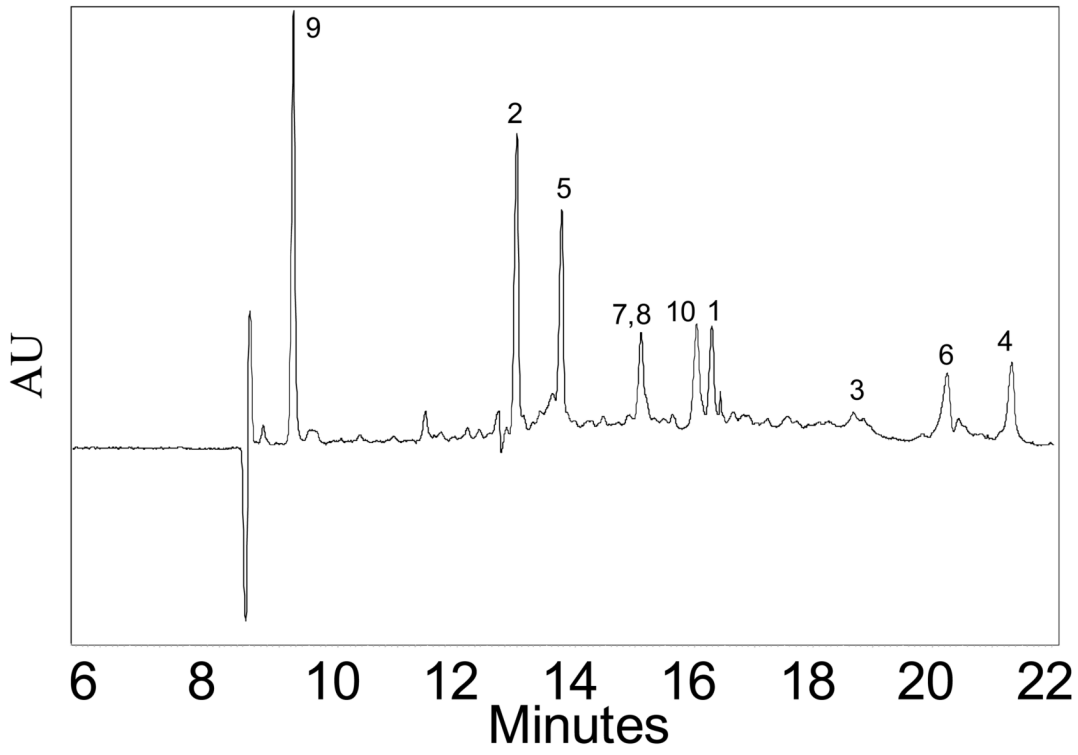

Figure 7.

Optimized separation of acidic and basic proteins under basic experimental conditions. **Conditions: Polymer concentration:** 0.4%(w/v) poly-ε-SUK; **Coating time:** 15 min; **NaCl concentration:** 20mM; **Background electrolyte:** 20mM sodium phosphate dibasic (pH11.5); **Applied voltage:** 10 kV; **Temperature:** 15 °C; **Capillary:** 50 cm (total length), 40 cm (effective length), 50 μm i.d.; **Analyte concentration:** 0.4 mg/mL; **Injection:** 5 kV for 5 s; **Detection:** 200 nm; **Analytes:** 1. deoxyribonulease I, 2. α-chymotrypsinogen A, 3. albumin, **4.** α-lactalbumin, **5.** ribonuclease A, **6.** myoglobin **7.** β-lactoglobulin A, **8.** β-lactoglobulin B, **9.** lysozyme, **10.** cyctochrome *c*.

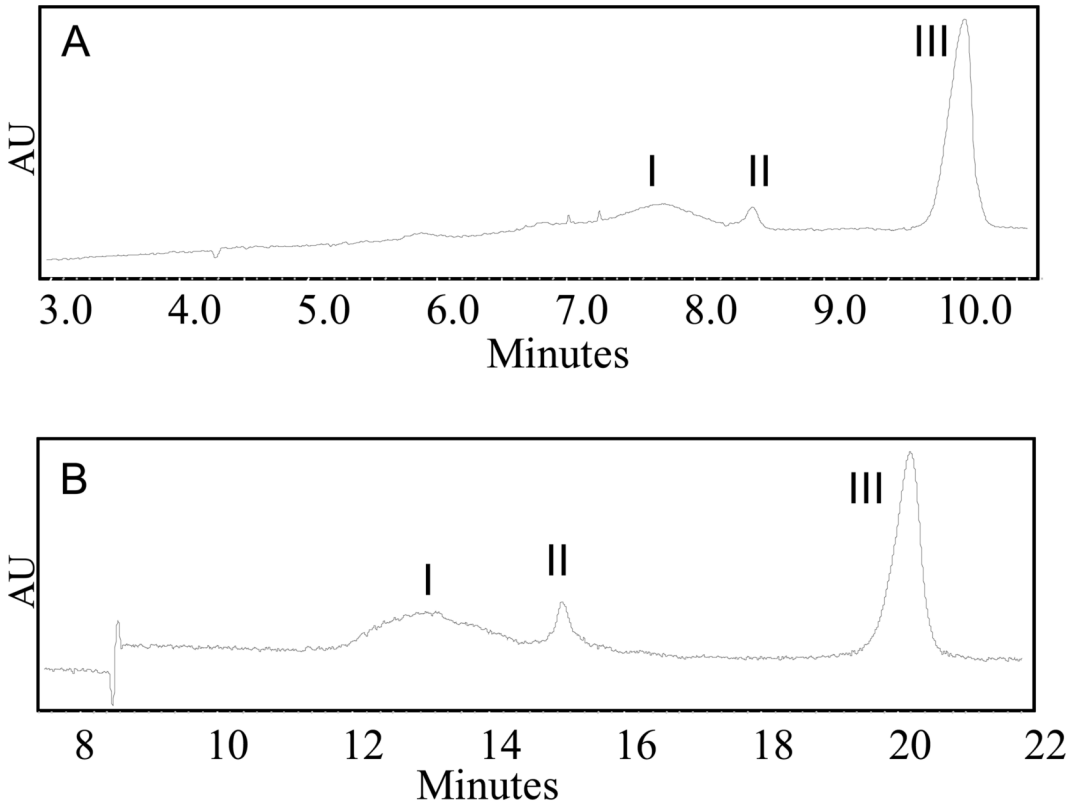

Figure 8.
Separation of Human Serum Proteins using Zwitterionic Coating.
Conditions: (A) same as Figure 6B, pH 3 (B) same as Figure 7, pH 11.5; **Human Serum:** (A) dilution factor of 8, (B) dilution factor of 2; **Peaks:** I. IgG II. Transferrin, III. Albumin.

Table 1

Thickness of micelle layers formed on silicon after different immersion intervals.

Immersion time (min)	Film thickness (nm)
20	0.98 ± 0.12
120	1.9 ± 0.38
420	2.4 ± 0.74

Table 2

Protein system selected for OT-CEC separation.

Proteins	Source	pI	MW
α-lactalbumin	bovine milk	4.8	14.2
β-lactoglobulin A	bovine milk	5.1	36.7
β-lactoglobulin B	bovine milk	5.3	36.6
albumin	bovine serum	4.7	69.0
deoxyribonuclease I	bovine pancreas	6.7	31.0
myoglobin	equine heart	6.8	17.0
α-chymotrypsinogen A	bovine pancreas	9.2	25.0
ribonuclease A	bovine pancreas	9.3	13.7
cytochrome *c*	bovine heart	10.2	12.4
lysozyme	chicken egg white	11.1	14.3

Table 3

Resolutions calculated from protein separations.

		R_{s1}	R_{s2}	R_{s3}	R_{s4}	R_{s5}	R_{s6}	R_{s7}	R_{s8}	R_{s9}
pH 3	25mM NaCl	3.01	2.94	0.76	2.30	0.88	0.21	1.08	7.80	0
	20mM NaCl	2.26	1.50	2.55	1.01	4.52	1.96	0.52	1.96	13.62
	15mM NaCl	1.17	0.34	0.60	0	1.17	0.92	0	0.45	4.05
pH 11.5	20mM NaCl	19.65	4.15	5.53	0	3.10	0.95	3.81	2.25	3.37

*0.4% (w/v) poly-ε-SUK was used for each salt concentration

Table 4

Reproducibility studies of the poly-ε-SUK coating.
Conditions: Polymer concentration: 0.4%(w/v) poly-ε-SUK; **Coating time:** 20 min; **NaCl concentration:** 20mM; **Background electrolyte:** 20mM sodium phosphate dibasic (pH 3); **Applied voltage:** 15 kV; **Temperature:** 25 °C; **Capillary:** 50 cm (total length), 40 cm (effective length), 50 μm i.d.; **EOF marker:** acetone; **Injection:** 5 kV for 5 s; **Detection:** 254 nm.

	Ave. EOF migration time(min)	%RSD of EOF
Run to Run (n=50 runs)	3.95	1.24
Day to Day (n=5 days)	4.08	1.20
Week to Week (n=3 weeks)	4.19	1.85
Capillary to Capillary (n=5 capillaries)	4.09	3.15