Effectiveness of Charged Noncovalent Polymer Coatings against Protein Adsorption to Silica Surfaces Studied by Evanescent-Wave Cavity Ring-Down Spectroscopy and Capillary Electrophoresis

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Protein adsorption to silica surfaces is a notorious problem in analytical separations. Evanescent-wave cavity ringdown spectroscopy (EW-CRDS) and capillary electrophoresis (CE) were employed to investigate the capability of positively charged polymer coatings to minimize the adsorption of basic proteins. Adsorption of cytochrome *c* (cyt *c*) to silica coated with a single layer of polybrene (PB), or a triple layer of PB, dextran sulfate (DS), and PB, was studied and compared to bare silica. Direct analysis of silica surfaces by EW-CRDS revealed that both coatings effectively reduce irreversible protein adsorption. Significant adsorption was observed only for protein concentrations above 400 μM, whereas the PB−DS−PB coating was shown to be most effective and stable. CE analyses of cyt *c* were performed with and without the respective coatings applied to the fused-silica capillary wall. Monitoring of the electroosmotic flow and protein peak areas indicated a strong reduction of irreversible protein adsorption by the positively charged coatings. Determination of the electrophoretic mobility and peak width of cyt *c* revealed reversible protein adsorption to the PB coating. It is concluded that the combination of results from EW-CRDS and CE provides highly useful information on the adsorptive characteristics of bare and coated silica surfaces toward basic proteins.

Developments in the fields of protein chemistry, biopharmaceuticals, and biotechnology have led to an increasing demand for sensitive and selective analytical tools for the determination of intact proteins. Especially, separation techniques that allow analysis of proteins under mild conditions without the need for, for example, organic solvents or very high salt concentrations gain growing interest. However, when separating proteins by, for example, capillary electrophoresis (CE) or microfluidic systems,

the tendency of protein molecules to adsorb to the internal walls of the glass or (fused-)silica flow channels is a major problem. This is particularly true for basic (i.e., positively charged) proteins. As a result, separation efficiencies deteriorate, unless the surfaces are extensively reconditioned after each run. To prevent these protein−wall interactions, coating of the internal surfaces with agents that minimize protein adsorption is a common strategy.[1−3]

To establish the effectiveness of such coatings, methods are required that allow determination of reversible and irreversible adsorption. In CE, parameters such as protein electrophoretic mobility, peak asymmetry, and plate number can be used to assess reversible adsorption. In addition, changes in peak height or area between runs and in electroosmotic flow (EOF) may indicate irreversible protein adsorption.[2] Regnier et al.[4] proposed a method to probe protein adsorption in CE. They devised a special instrument, which allowed protein detection at several positions along the capillary. A decrement of peak area as measured by the successive detectors quantitatively indicated protein adsorption. Righetti and co-workers[5] developed a capillary equilibration procedure to probe the amount of adsorbed protein. The capillary was first completely filled and conditioned with a background electrolyte (BGE) containing fluorescently labeled protein. After equilibration, the capillary was flushed with buffer to remove any unbound protein. The protein retained on the capillary was then eluted with surfactant-containing buffer and subsequently quantified by laser-induced fluorescence detection. The method was used to evaluate the ability of several additives to prevent protein adsorption.

As discussed above, in a CE setting, protein adsorption is normally studied in an indirect fashion, because probing of the actually adsorbed protein molecules at the capillary wall obviously would be very difficult, if not impossible. In situ monitoring of adsorption and desorption processes at surfaces can in principle be achieved using surface-specific spectroscopic methodologies,

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such as surface plasmon resonance (SPR) and attenuated total reflection (ATR). SPR is based on the measurement of the shift in the plasmon resonance of a thin metal (usually gold) film after adsorption of species to the metal or a layer (e.g., a self-assembled monolayer) deposited on the metal. To use SPR for protein adsorption studies on silica, a synthetic silica layer should be deposited on the gold SPR surface. Such a system would not be an appropriate model for studying the effectiveness of coatings. Kraning et al.[6] recorded polarization-dependent absorption spectra on a single-pass silica ATR prism, which was sufficiently sensitive for the detection of a full monolayer of cytochrome *c*. However, for detection of lower surface coverage, the absorbance signal needs to be enhanced. In ATR spectroscopy, this can be achieved by increasing the optical path-length using a multipass geometry. Still, to get a sufficient number of reflections at the surface, waveguides with dimensions on the order of several centimeters have to be used, necessitating large sample volumes.

Recently, evanescent-wave cavity ring-down spectroscopy (EW-CRDS) was shown to be an effective tool for the probing of surface-specific processes, and its potential for bioanalytical applications was demonstrated.[7−9] CRDS is a very sensitive mode of absorbance spectroscopy based on the detection of the exponential decay of light behind an optically stable cavity after abrupt termination of excitation. Adding an absorber (analyte) in the cavity constitutes an extra loss of light resulting in a shortened $1/e$ or ring-down time of the exponential decay.[10,11] Although CRDS was first developed for measuring very low gas-phase absorbances, more recently, CRDS applications in the liquid phase have also been reported, as reviewed by van der Sneppen and co-workers.[11] In EW-CRDS, one of the reflections in the cavity is a total internal reflection (TIR) event. Only the evanescent wave associated with the TIR is being used to probe the sample. EW-CRDS combines the excellent sensitivity of CRDS with the surface specificity of EW techniques and, therefore, in principle is suited for studying protein adsorption to silica surfaces.[7−9] An adsorbed protein of which the UV−vis absorption spectrum overlaps with the applied laser wavelength can be detected through a decrease in the ring-down time, whereas unbound protein in the bulk solution will hardly contribute to the EW-CRDS signal.

EW-CRDS studies of compounds on coated prisms have been reported.[7,12−16] The applied surface coatings mainly served to bind the compounds of interest[7,12−15] and not to prevent adsorption.

The coatings were either covalently[7,13,16] or noncovalently[12,14,15] attached to the silica surface. Covalent coatings can be very effective; however, their production can be tedious and time-consuming, and may not be reproducible. Noncovalently attached coatings offer a more practical solution, as they can be made simply by flushing the solution of the proper coating agent along the silica surface. This type of coating is generally based on the physical adsorption due to electrostatic interaction between the coating agent and the silica surface. Such a coating procedure is relatively fast and can be easily repeated. An interesting and highly flexible approach, as introduced by Katayama et al. for CE,[17] is the use of successive multiple charged-polymer layers to construct stable coatings. Powell et al.[12] applied EW-CRDS to study the pH-dependent adsorption of a ruthenium-based dye to a noncovalent bilayer coating of poly-L-lysine and poly-L-glutamic acid.

In the present study, EW-CRDS is used to evaluate the effectiveness of charged noncovalent polymer coatings in minimizing adsorption of basic proteins. The coatings were applied to the surface of a fused-silica EW-CRDS prism. The studied coatings were a single layer of polybrene (PB) and a triple layer composed of PB, dextran sulfate (DS), and PB (PB−DS−PB).[18] These positively charged coatings were prepared by flushing the silica surface with PB, or successively with PB and DS solutions. Subsequently, the respective coatings were exposed to solutions of cytochrome *c* (cyt *c*) in sodium phosphate buffer (pH 7.4). Cyt *c* has a pI of 10.5 and is consequently positively charged at medium pH. It is chosen as a test compound as it tends to adsorb strongly to bare silica[4,6,16] and features a strong absorption in the visible range. Cyt *c* adsorption to bare and coated silica prisms was measured directly with EW-CRDS at 538 nm, which overlaps with the strongest Q-band transition of cyt *c* and for which wavelength appropriate mirrors were available in our laboratory. Cyt *c* was also analyzed by CE using a fused-silica capillary without and with the respective coatings in combination with a BGE of sodium phosphate (pH 7.4). The CE results in terms of magnitude of the EOF, peak area, electrophoretic mobility, and peak width are interpreted with respect to protein adsorption and discussed in conjunction with the EW-CRDS measurements.

EXPERIMENTAL SECTION

Chemicals. Potassium hydroxide, polybrene (hexadimethrine bromide, PB), and dextran sulfate (DS) sodium salt were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfuric acid, acetonitrile, and potassium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Formamide was obtained from Fluka (Steinheim, Germany) and used as an EOF marker. A 10 mM phosphate buffer (pH 7.4) was prepared by dissolving 0.136 g of potassium dihydrogen phosphate in 100 mL of deionized water and adjusting the pH with potassium hydroxide. Solutions of 10% (w/v) PB and 3% (w/v) DS were prepared in deionized water. The solutions were filtered over a 0.45 μm filter type HA (Millipore, Molsheim, France) prior to use.

For the EW-CRDS measurements, a stock solution of 1 mM (12.5 mg/mL) bovine heart cytochrome *c* (Sigma-Aldrich) was

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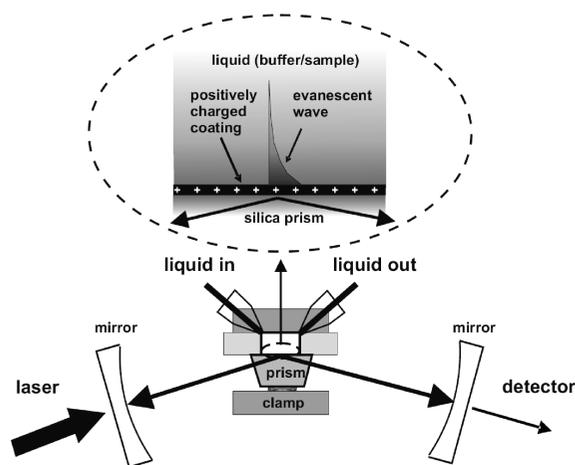(18) Haselberg, R.; De Jong, G. J.; Somsen, G. W. *J. Sep. Sci.* **2009**, *32*, 2408–2415.

Figure 1. Schematic representation of the EW-CRDS setup, the flow cell attached to the TIR surface, and the evanescent wave probing the sample near the interface.

made in 10 mM potassium phosphate buffer and was further diluted to the required concentration with the phosphate buffer. For the CE experiments, a stock solution of 80 μM (1 mg/mL) cyt c was prepared in deionized water, and diluted in deionized water containing 0.25% (v/v) formamide (EOF marker) to a final cyt c concentration of 16 μM.

EW-CRDS System. The setup was similar to the one used in a previous study[16] and is shown in Figure 1. A crucial difference was that a 10 Hz Nd:YAG-pumped pulsed dye laser system was used; as a consequence, in this study the light was s-polarized, as opposed to p-polarized. An optical cavity was constructed using mirrors with a reflectivity $R \geq 99.996\%$ at 532 nm, 50 mm radius of curvature from REO Inc. (Boulder, CO), in combination with a 70° Dove prism at normal incidence. This way, reflections at the intracavity surfaces do not lead to losses, but are maintained within the cavity. Entrance and exit faces of the prism were both polished to a flatness of $\lambda/10$ at 632.8 nm to minimize losses at these faces, whereas the TIR face was polished to a flatness of $\lambda/2$. Excitation of the cavity at 538 nm was performed with pulses (0.5−1 mJ) from a Quanta-Ray PDL-3 pulsed dye laser with coumarin 152, pumped by the 355 nm output (third harmonic) of a Quanta-Ray Nd:YAG laser (Spectra-Physics, Mountain View, CA) at a repetition rate of 10 Hz and a pulse duration of 5 ns. In these experiments, the EW probes the sample layer closest to the surface approximately 100 times. After each laser pulse, the light recorded by a photomultiplier tube (Hamamatsu, Shimokanzo, Japan) and a fast sampling oscilloscope of 1 GHz analogue bandwidth (Tektronix 510 5GS/s) was fitted to a monoexponential decay function. From the ring-down time τ_0 in the absence and τ in the presence of analyte, the absorbance (in 10-base absorbance units, AU) is calculated as

$$\varepsilon C_{\text{eff}} l = \left(\frac{\eta_{\text{quartz}} L_{\text{quartz}} + \eta_{\text{air}} L_{\text{air}}}{2.303c}\right)\left(\frac{1}{\tau} - \frac{1}{\tau_0}\right) \qquad (1)$$

where ε is the molar extinction coefficient at 538 nm in M^{-1} cm^{-1}, C_{eff} is the effective concentration at the surface layer in M, l is the effective depth of the evanescent wave calculated as 919

nm in this setup,[19] c is the speed of light, η_{quartz} is the refractive index of the prism (1.461), η_{air} is the refractive index of air (1.0008), L_{quartz} is the path length through the prism (9.4 mm), and L_{air} is the path length through the air (59 mm). For nonhomogeneous samples, the total measured absorbance is the sum of the absorbance by the bulk solution over the effective depth l and the absorbance by surface-adsorbed analytes. The latter will be discussed in more detail in the Results and Discussion. With the 70° prism, obtainable τ_0 ring-down times were between 25 and 40 ns, and these decreased to 15−20 ns for the highest concentrations of absorber used in this study.

A 14 μL sized Teflon flow cell was clamped leak-tight on the TIR face of the prism. Cyt c adsorption experiments were performed using a stopped-flow approach: 100 μL of a cyt c solution was injected in a continuous flow (0.1 mL/min) of 10 mM potassium phosphate buffer (pH 7.4) delivered by a LC pump. When the injection plug completely filled the flow cell, the flow was stopped. The absorbance signal was monitored for a period of 10−15 min, after which the flow was restarted and the cyt c flushed away with clean buffer solution.

Coating of the Prism. Single-layer PB and triple-layer PB−DS−PB coatings were used in this study. For applying a coating layer on the prism, 100 μL of aqueous polymer solutions was injected and left to react with the surface for 10 min by stopping the flow. For the PB−DS−PB coating, the flow cell was flushed with running buffer for several minutes between application of the polymer layers to clean the flow cell of nonbound PB or DS. Irreversibly bound cyt c could successfully be removed by injecting 100 μL of 0.1 M sulfuric acid in a flow of 0.05 mL/min. However, in the case of the PB coating, this treatment resulted in degradation of the layer, necessitating the complete removal and fresh application of the PB layer. The PB and the PB−DS−PB layers could be removed by injecting, respectively, one or several 500 μL plugs of acetonitrile at a flow of 0.05 mL/min.

CE System. CE experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument equipped with a UV detector (Beckman Coulter, Fullerton, CA). Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ), having a total length of 30 cm (effective length, 20 cm) and an internal diameter of 50 μm. Hydrodynamic injections were performed using a pressure of 0.5 psi for 4 s. The separation voltage was −30 kV, the capillary temperature was 20 °C, and UV absorbance detection was performed at 214 nm. New bare fused-silica capillaries were rinsed with 1 M sodium hydroxide for 30 min at 20 psi, and water for 15 min at 20 psi. After this treatment, the capillaries were coated with the procedure described below. Electropherograms were analyzed using 32 Karat Software, version 7.0 (Beckman Coulter). The electroosmotic mobility (μ_{EOF}) and apparent mobility of cyt c (μ_{app}) were calculated as:

$$\mu_{\text{EOF}} = \frac{L_{\text{tot}} L_{\text{det}}}{V t_{\text{EOF}}} \qquad (2A)$$

$$\mu_{\text{app}} = \frac{L_{\text{tot}} L_{\text{det}}}{V t_{\text{cyt}c}} \qquad (2B)$$

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where L_{tot} and L_{det} are the total length (30 cm) and length to the detector (20 cm) of the CE capillary, respectively, V is the applied voltage (30 or -30 kV), and t is the migration time (s) of an EOF marker (for μ_{EOF}) or cyt c (for μ_{app}). The effective mobility of cyt c was subsequently calculated as:

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \qquad (3)$$

The effective mobility (μ_{eff}) may differ from the true electrophoretic mobility due to the contribution of adsorption to the overall mobility.

Coating of the Capillary. Capillaries were coated with a single PB layer by rinsing for 6 min with 10% (w/v) PB solution at 5 psi followed by 3 min with deionized water at 10 psi. For the PB−DS−PB coating, the capillary was successively rinsed 6 min with 10% (w/v) PB solution at 5 psi, 3 min with deionized water at 10 psi, 6 min with 3% (w/v) DS solution at 5 psi, 3 min with deionized water at 10 psi, 6 min with 10% (w/v) PB solution at 5 psi, and 3 min with deionized water at 10 psi. After coating and between runs, capillaries were flushed with fresh BGE for 1 min at 10 psi. Overnight, capillaries were left filled with BGE, with both ends immersed in vials containing BGE.

RESULTS AND DISCUSSION

EW-CRDS Measurements. In a previous publication, it was demonstrated that EW-CRDS can be used to monitor cyt c adsorption on silica surfaces.[16] Adsorption from protein solutions with concentrations as low as 1 μM could be detected reliably. In the present work, to evaluate the effectiveness of coatings for adsorption prevention, surfaces were challenged with much higher protein levels. As a first experiment, the adsorption of cyt c to bare silica from a solution of 100 μM in phosphate buffer (pH 7.4) was determined. A typical profile of this EW-CRDS experiment is shown in Figure 2A. Immediately after the cyt c solution had reached and filled the flow cell, the flow was stopped, and the absorbance signal increased up to a practically constant level due to a combination of absorbance by cyt c in the bulk solution and by cyt c adsorbed to the surface. Starting the flow of neat buffer through the flow cell removed the unadsorbed cyt c and potentially desorbs reversibly bound cyt c. The absorbance measured 4 min after restarting the flow was presumed to be caused by irreversibly adsorbed protein. The observed absorbance signal of 7×10^{-4} AU (Figure 2A) agreed well with results on bare silica obtained previously with this system.[16] In addition, as no decrease of the absorbance signal was observed during the measurement, photodegradation of cyt c could be excluded.

The absorbance measured with EW-CRDS could be used to calculate the amount of adsorbed protein on the surface. For the conditions used in our experiments, a maximum cyt c saturation surface coverage of 18 pmol/cm^2 has been reported,[6,20] which corresponds with 1.1−1.2 pmol present in the laser-illuminated spot of 6−7 mm^2. Assuming a protein extinction coefficient of 1.0×10^4 M^{-1} cm^{-1} at 538 nm, the absorbance of a monolayer would be 1.8×10^{-4} AU in a conventional transmission geometry at normal incidence. The protein extinction coefficient was determined in house for a cyt c solution in the same buffer using a Cary 50 UV−vis spectrometer. However, a
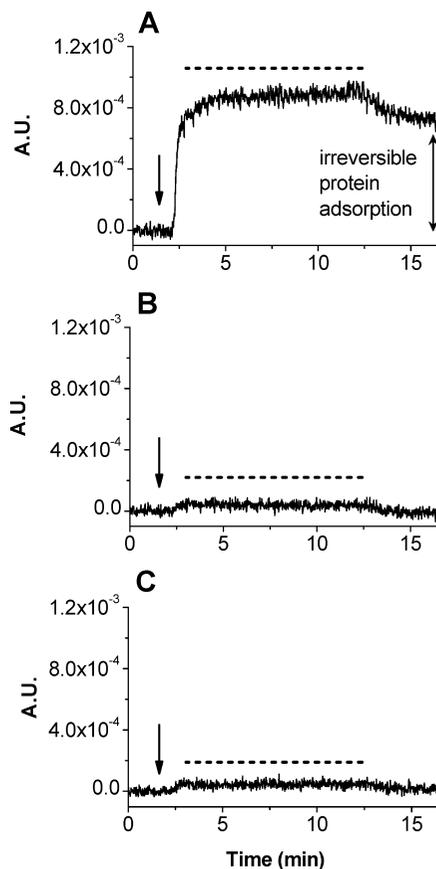
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Figure 2. EW-CRDS profiles of a (A) bare, (B) PB-coated, and (C) PB−DS−PB-coated silica surface upon exposure to a solution of 100 μM cyt c. Arrows indicate the time of cyt c injection, and the dashed lines indicate the time interval in which the flow was stopped. AU, absorbance units. For further conditions, see Experimental Section.

characteristic of evanescent waves is that the absorbance close to the surface is strongly enhanced. For a thin layer with thickness d ($d \ll$ penetration depth l of the evanescent wave), an enhancement factor[21] comes into play, which was calculated to be 7.4 for the present experimental conditions. Taking this factor into account, the theoretical EW absorbance of a cyt c layer was approximately 13×10^{-4} AU. So, the observed absorbance signal of 7×10^{-4} AU on the bare prism corresponded to approximately one-half of the saturation surface coverage, indicating that 0.6 pmol of cyt c was irreversibly adsorbed at the probed surface spot.

Subsequently, the effect of positively charged coatings on protein adsorption was assessed. A PB single layer and a PB−DS−PB triple layer coating were applied as described in the Experimental Section. Using this procedure, a coating layer thickness of the triple layer will be at most 2 nm,[22,23] which is very small with respect to the effective penetration depth of 919 nm of the EW-CRDS detection. In other words, the layer will not interfere with the EW-CRDS measurement. Figure 2B and C show, on the same absorbance scale as the bare silica measurement,

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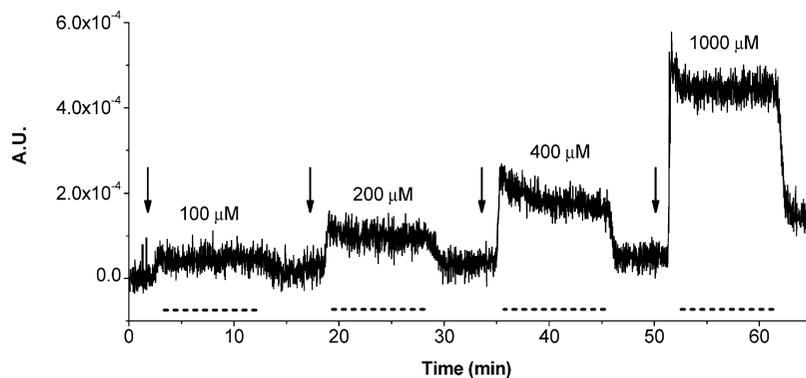(23) Liu, X.; Erickson, D.; Li, D.; Krull, U. J. *Anal. Chim. Acta* **2004**, *507*, 55−62.

Figure 3. EW-CRDS profile of a PB−DS−PB-coated silica surface obtained upon successive injections of 100 μL of cyt c solutions of increasing concentration. Arrows indicate times of cyt c injection, and dashed lines indicate time intervals in which the flow was stopped. AU, absorbance units. For further conditions, see Experimental Section.

Table 1. Irreversible Protein Adsorption As Measured with EW-CRDS on a PB and PB−DS−PB-Coated Prism Exposed to Different Cyt c Concentrations

concentration cyt c (μM)	PB coating				PB−DS−PB coating			
	absorbance $(10^{-4}$ AU$)^a$	SD $(10^{-4}$ AU$)^a$	% of saturation coverage^b	adsorbed amount (pmol)^b	absorbance $(10^{-4}$ AU$)^a$	SD $(10^{-4}$ AU$)^a$	% of saturation coverage^b	adsorbed amount (pmol)^b
100	0.025	0.24	<2.0	<0.02	0.15	0.21	<2.0	<0.02
200	0.15	0.36	<2.0	<0.02	0.33	0.38	<2.0	<0.02
400	0.27	0.17	2.1	0.02	0.44	0.29	3.4	0.04
1000	7.1	0.69	55	0.6	1.5	1.1	12	0.1

^{a} Average absorbance of three measurements; SD, standard deviation. ^{b} 100% saturation coverage corresponds to a signal of 13×10^{-4} AU and 1.15 pmol of cyt c in the laser-illuminated surface spot.

the EW-CRDS profile as obtained for the PB-coated and PB−DS−PB-coated prisms, respectively, after injection of 100 μL of 100 μM cyt c in buffer. From the EW-CRDS signal, it was clear that the adsorption of cyt c to the prism surface is reduced significantly by coating the fused silica surface with a positively charged layer. After the flow was stopped, the absorbance signal only marginally increased to approximately 0.8×10^{-4} AU on both the PB and PB−DS−PB-coated prism. Taking the effective penetration depth and cyt c extinction coefficient into account, an absorbance signal of 0.9×10^{-4} AU could be expected due to bulk absorbance, that is, in the absence of protein adsorption. When the buffer flow was started again, the measured absorbance returns to baseline level as the cyt c leaves the flow cell. So it can be concluded that no measurable amount of cyt c was irreversibly adsorbed. Considering the uncertainty (noise) in the absorbance baseline, this implies that with the polymer coatings the protein surface coverage was below 2% when exposed to 100 μM cyt c.

A solution of 100 μM cyt c was also injected after the prism was coated with a bilayer of PB and DS, which presents a negatively charged surface. After incubation, the buffer flow was started to remove unadsorbed protein from the flow cell. A very high absorbance signal of approximately 4×10^{-3} AU remained, indicating a strong irreversible adsorption (data not shown). Actually, the EW-CRDS signal indicated that the protein adsorption was 5−6 times higher on the PB−DS bilayer than on bare silica. Apparently, the positively charged protein adsorbed readily to the negatively charged DS. The absorbance signal suggested that the surface was covered with two to three layers of cyt c. This value was a rough estimate because the

ring-down times became as short as 15 ns and, therefore, could no longer be fitted reliably.

The PB and PB−DS−PB coating were successively exposed to increasing cyt c concentrations (100, 200, 400, and 1000 μM), and the increase in surface absorbance (expressed in AU) upon each injection was determined by EW-CRDS. Figure 3 shows a typical example of the resulting EW-CRDS profile obtained for the triple layer coating. The experiment was performed in triplicate for both coatings, and the average amount of adsorbed protein was calculated (Table 1). Protein concentrations up to 200 μM did not result in measurable irreversible protein adsorption. Significant cyt c adsorption was observed only for very high protein concentrations (400 and 1000 μM). At a cyt c concentration of 400 μM, protein adsorption was similar on the single and triple layer coatings. In this case, approximately 2−4% of the surface was covered, which corresponds to 0.02−0.04 pmol of protein adsorbed onto the prism surface. At a cyt c concentration of 1000 μM, there is a clear difference between the two coatings. On the triple-layer coating, a surface coverage of 12% is obtained, corresponding to 0.1 pmol of irreversibly adsorbed protein. The PB single layer yields a surface coverage of 55% (0.6 pmol), which is similar to that observed after injecting 100 μM cyt c on bare silica.

When the single- and triple-layer-coated silica surfaces were repetitively exposed to 100 and 200 μM cyt c over a period of more than 2 h, no measurable irreversible protein adsorption was observed (data not shown). From this result, we also conclude that prolonged exposure to protein solutions up to 200 μM does not seriously affect the stability of the PB and PB−DS−PB coatings. Irreversibly bound protein, as obtained during exposure
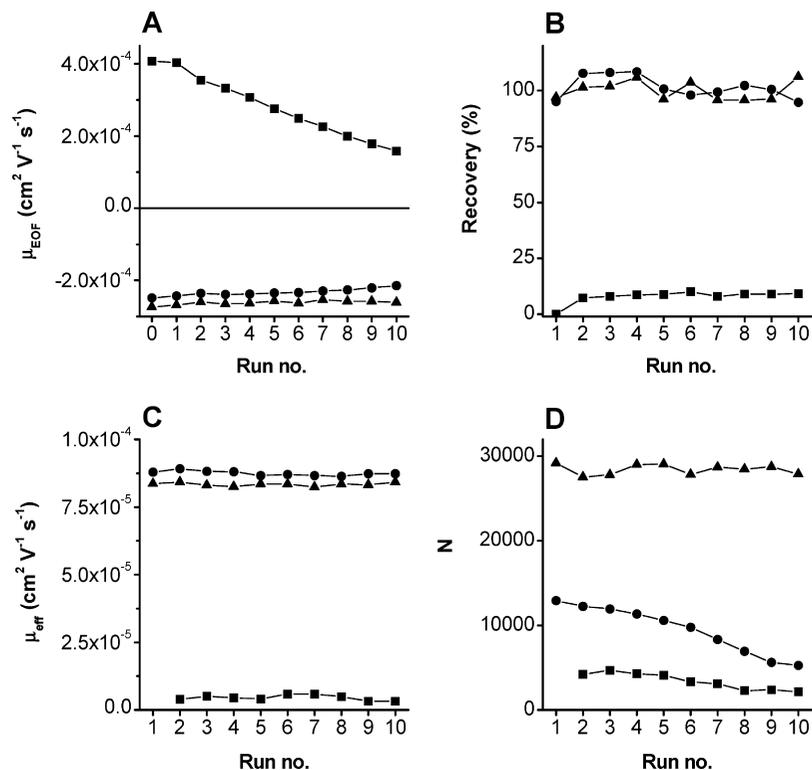
Figure 4. (A) EOF mobility, and (B) recovery, (C) effective mobility, and (D) plate number of cyt *c* obtained during CE-UV analyses of 0.2 v/v% formamide (run no. 0) or a mixture of 0.2 vol % formamide and 16 μM cyt *c* (runs no. 1–10) using a (■) BFS capillary, and a (●) PB and (▲) PB–DS–PB-coated capillary. With BFS, no cyt *c* was detected during run no. 1. Voltage, 30 kV (BFS) and −30 kV (coated capillaries). For further conditions, see Experimental Section.

to 400 and 1000 μM cyt *c*, was removed by injection of 0.1 M sulfuric acid into the flow cell. This treatment also removed the single PB layer as was clear from the significant protein adsorption observed when the surface was subsequently exposed to 100 μM cyt *c*. Interestingly, the PB–DS–PB coating did not deteriorate upon protein removal with sulfuric acid. The EW-CRDS absorbance signal returned to baseline, whereas the effectiveness against protein adsorption of the triple-layer coating was found to be the same as before the sulfuric acid treatment. As a consequence, the same triple layer could be used during the various adsorption experiments without noticeable deterioration. The increased chemical stability of the PB–DS–PB coating with respect to the PB coating was also found by others[17] and may be attributed to stronger interactions between the oppositely charged polymer layers as compared to the silanol–PB interaction.[24]

CE-UV Measurements. To further investigate the performance of the coatings for preventing protein adsorption, CE-UV measurements were carried out. Bare fused-silica (BFS) capillaries were compared to PB and PB–DS–PB-coated capillaries. The BGE was 10 mM potassium phosphate (pH 7.4), that is, the same as with the EW-CRDS experiments, whereas solutions of 16 μM cyt *c* were injected. First, a measurement with only formamide (EOF marker) was performed to determine the magnitude of the EOF on the three capillaries (Figure 4A, run no. 0). Note that the positively charged coatings resulted in an anodic EOF as opposed to the cathodic EOF that is obtained with BFS.

Ten successive CE analyses of a mixture of EOF marker and cyt *c* were performed on the BFS and both of the coated capillaries. On BFS, the EOF decreased upon each injection of protein (Figure

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4A). The decrease can be explained by irreversible protein adsorption of the positively charged protein with the negatively charged capillary wall, which alters the zeta potential at the capillary wall and causes a change of the EOF velocity.[4] Next to the magnitude of the EOF, irreversible protein adsorption was also reflected in the protein recovery. For each cyt *c* peak measured on the coated and noncoated CE capillaries, the recovery was determined relative to the migration time-corrected peak area of cyt *c* as obtained during the first run on the PB–DS–PB-coated capillary (Figure 4B). On BFS, during the first run, no cyt *c* peak could be observed, whereas the other runs resulted in a cyt *c* recovery of less than 10%. In addition, cyt *c* was also repetitively injected on a capillary that was coated with a PB–DS bilayer. No protein peaks were observed, whereas the EOF decreased upon each protein injection (data not shown). Obviously, significant irreversible binding of the protein occurs on both the BFS capillary and the PB–DS-coated capillary as was also observed with EW-CRDS.

In contrast to both negatively charged capillaries, a stable EOF was obtained after application of a positively charged coating to the silica wall (Figure 4A). This indicated that no noticeable irreversible cyt *c* adsorption occurred, most probably due to the electrostatic repulsion between the positively charged coatings and protein molecules. This conclusion is confirmed by the cyt *c* recovery on the coated CE systems, which was found to be within 95–110%. The EOF and recovery measurements are in line with the results of the EW-CRDS experiments showing no measurable irreversible protein adsorption at the PB and PB–DS–PB coatings. The magnitude of the EOF is slightly lower with the PB coating than with the PB–DS–PB coating, which might point at

incomplete coverage of the silica surface by the coating, resulting in sites for reversible protein adsorption (see below). It should be noted that based on CE results only, it is not possible to fully exclude the possibility of irreversible protein adsorption as recoveries had to be determined relatively (i.e., with respect to the PB−DS−PB coating assuming no protein adsorption). In contrast, EW-CRDS allowed the direct monitoring of (the amount of) adsorbed protein on the silica surface.

Whereas the stability of the EOF and protein recovery reveal the occurrence of irreversible adsorption, the proteins' migration time and peak width can yield information on reversible adsorption.[2] The effective mobility (μ_{eff}) will differ from the true electrophoretic mobility when protein adsorption occurs. As shown in Figure 4C, the effective mobility of cyt c was approximately 20 times lower on BFS than on the coated capillaries, indicating considerable retention and, thus, reversible protein adsorption.[25] A comparison between the PB and the PB−DS−PB-coated capillaries reveals a small but significant difference in the effective mobility of cyt c (Figure 4C). Because cyt c migrated slower than the EOF when using positively charged coatings, the somewhat higher mobility obtained with the single PB layer indicates retention and, therefore, reversible adsorption.

Determination of the plate number of the cyt c peak over 10 runs on the three systems (Figure 4D) showed that the lowest plate numbers are obtained on BFS. This is evidently caused by the significant reversible adsorption of cyt c to the capillary wall, which leads to extra band broadening due to the resistance to mass transfer.[26] Indeed, cyt c plate numbers for the coated capillaries were higher than for BFS. Still, the cyt c plate number for the PB single layer was lower than for the PB−DS−PB coating, and a decrease in time was observed. This might be due to the partial detachment of PB during the successive runs, although it should be noted that the recovery (and thus irreversible adsorption) did not significantly change during the successive injections of cyt c. The PB−DS−PB triple layer coating clearly provides the highest and most stable plate numbers. This was also previously found by us[18] and others,[17] and could be contributed to the more effective suppression of reversible protein adsorption by the triple-layer coating.

CONCLUSIONS

The effectiveness of charged noncovalent polymer capillary coatings to prevent adverse adsorption of basic proteins to a silica surface was studied by EW-CRDS and CE-UV. EW-CRDS is particularly useful for direct probing of irreversible protein adsorption. The EW-CRDS results showed that for cyt c concentrations up to 200 μM, PB and PB−DS−PB coatings effectively suppress irreversible adsorption. The triple layer coating appeared to be most stable when exposed to high protein concentrations and sulfuric acid. CE-UV results on EOF and cyt c peak area confirmed that the PB and PB−DS−PB coatings strongly reduce irreversible adsorption of basic proteins. It should be noted that, in contrast to EW-CRDS, CE-UV provides a relative measure for irreversible adsorption. On the other hand, monitoring of electrophoretic protein mobilities and plate numbers as determined with CE-UV allows the evaluation of reversible protein adsorption, which is not easy to assess with EW-CRDS. It follows that especially the BFS capillary, but also the PB-coated capillary, exhibits reversible adsorption, whereas this phenomenon is most effectively minimized by the PB−DS−PB coating. This led to the most optimum plate numbers for cyt c on the triple layer coating. Overall, from this study it can be concluded that EW-CRDS and CE-UV provide complementary information about the nature and magnitude of basic protein adsorption, and show good potential for the evaluation of silica coating materials.

Further improvement of the EW-CRDS setup would allow detection of lower amounts of adsorbed protein. Enhancement of the sensitivity could, for example, be achieved by employing the Soret band instead of the Q-band of cyt c by using a laser in the blue region of the spectrum in combination with the appropriate mirrors. At the moment we are studying this option. For other types of proteins that do not have a strong absorption in the visible range, there are also options to carry out CRDS measurements in the UV range, although at very short wavelengths currently the mirror quality often still is the limiting factor.[27] We also will use EW-CRDS and CE-UV for the study of the adsorption of acidic proteins on bare and coated silica surfaces.

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