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Technical Notes

Surface Plasmon Resonance Detection for Capillary Electrophoresis Separations

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A miniaturized surface plasmon resonance sensor has been used as an on-line detector for capillary electrophoresis separations. The capillary was modified slightly to shield the sensor electronics from the high voltages applied during the separation. A three-component mixture of high refractive index materials was separated and detected at the millimolar level by an untreated gold-sensing surface. A simple protein immobilization procedure was used to functionalize the surface for selective protein detection. A hybrid buffer system was developed, in which both the deposition of immobilized protein layers and the electrophoretic delivery of protein analytes were optimized. The detection system has a reproducibility of 15%, a dynamic range of 3 orders of magnitude, and a detection limit for IgG of 2 fmol.

Capillary electrophoresis (CE)[1,2] can achieve rapid and high-resolution separations of many different classes of analytes. It has been applied to a diverse range of analytical challenges, from the investigation of biomolecules and biological function,[3,4] to the screening of clinical and environmental samples,[5,6] to genome sequencing.[7] It is particularly powerful for biological applications, because it normally uses aqueous buffers and requires miniscule amounts of injected sample, enabling the chemical analysis of single biological entities and the screening of samples available in small amounts.

Although excellent detection methods exist for CE, many of these methods rely on the presence of inherent chromophores, fluorophores, or electrochemically active groups, or else they require that the molecule of interest be labeled in order to become spectroscopically or electrochemically accessible. Refractive index (RI) detection has the advantage of being universally applicable and nondestructive, and it has found wide use in high-performance liquid chromatography (HPLC) to detect otherwise elusive analytes such as carbohydrates.[8] The application of RI to microscale separation techniques such as μHPLC and CE has been limited by the challenges of miniaturizing a bulk property detector for nanoliter volumes. As a result, RI detection has not gained the extensive use in CE that it enjoys in HPLC, although RI detectors for CE have been developed,[9−11] and detection limits as low as 10^{-8} RIU have been achieved.[12]

Another detection scheme that relies on the sensing of refractive index is surface plasmon resonance (SPR) spectroscopy. In SPR the refractive index of a dielectric medium (such as an aqueous solution) is monitored indirectly by measuring the angle at which the resonant excitation of a surface plasmon wave in a metal adjacent to the dielectric occurs. The principles of SPR have been extensively reviewed.[13−15] By immobilizing molecules on the metal surface with affinity for the analyte(s) of interest, a sensitive and selective sensor can be created. In this way, SPR has been fruitfully applied to biological interaction analysis. This technology has been made commercially available in the BIAcore system (Biacore AB, Uppsala, Sweden).[16] More recently, a miniaturized and integrated SPR sensor has been developed (Spreeta, Texas Instruments, Dallas, TX).[17,18] Using either BIAcore or laboratory-built instrumentation, SPR has been used as a detector for flow injection analysis (FIA) and HPLC[19−24] as well as a means for

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measuring the refractive index of various complex liquids.[25,26] FIA-SPR and HPLC-SPR have most frequently been applied to the detection of carbohydrates and glycopeptides and have been used both for on-line sensing and for the postcolumn analysis of collected fractions. The use of SPR as a CE detector, however, appears not to have been reported.

In this work, we investigate the application of a commercially available SPR sensor as a CE detector. Using unmodified gold surfaces, the separation and detection of high refractive index solutions has been realized. We use a simple protein immobilization scheme based on biotin−streptavidin and protein A−immunoglobulin (IgG) interaction to customize a plain gold-sensing surface for the detection of small amounts of human IgG that we deliver to the sensor electrophoretically. In what follows, we describe the experimental setup and present results that allow us to assess its performance.

EXPERIMENTAL SECTION

Materials. Benzyl alcohol, 2-chlorophenol, and *p*-nitrophenol were purchased from Sigma (St. Louis, MO) and used without further purification. Biotinylated bovine serum albumin (BSA−biotin), streptavidin, and biotinylated protein A (protein A−biotin) were purchased from Pierce (Rockford, IL), reconstituted, aliquotted, and stored according to the vendor's instructions. Polyclonal human IgG was purchased from Sigma and stored in aliquots at −20 °C.

Miniaturized SPR Sensor and Flow Cells. SPR measurements were performed using a Spreeta sensor (Texas Instruments) that has been described in detail by its manufacturers.[17,18] We recently characterized the use of the Spreeta for biomolecular interaction analysis.[27] Briefly, the Spreeta instument combines a near-infrared light-emitting diode (LED), a gold-sensing surface, and an angle-sensitive diode array detector in an integrated device with dimensions 1.5 × 3.0 × 4.0 cm. Light from the LED is filtered to select the p-polarized component, which is then directed though a plastic prism and experiences total internal reflection at the prism−metal interface. The evanescent component of the light excites a surface plasmon wave in the gold film that extends ∼400 nm beyond the metal. For a given set of conditions, there will be a particular incident angle, the resonance angle, at which light will most efficiently excite the plasmon wave. The position of the resonance angle, which is reported by the diode array detector, is highly sensitive to the refractive index of the material in contact with the gold. Therefore, the SPR sensor is a sensitive detector for changes in refractive index, whether they result from the presence of a nonbinding high refractive index solution or the immobilization of layers of protein molecules to the gold.

We controlled the delivery of solutions to the gold-sensing surface using a small-volume flow cell attached to the face of the sensor. A rectangle cut into a piece of silicon rubber defined the flow channel. By varying the channel dimensions and the thickness of the rubber, flow cells of different volumes can be created. For the CE-SPR experiments, we used either a 3.5-μL volume flow cell that was designed and built in-house or a 400-nL volume flow cell designed and built by Prolinx (Bothell, WA). For the delivery of large volumes (100 μL) of protein solution, we used a two-position, six-port Cheminert switching valve (VICI, Houston, TX). A flow rate of 20 μL/min was achieved with pressure from a Harvard Apparatus model 11 syringe pump (Holliston, MA). The temperature of the sensor was not actively controlled.

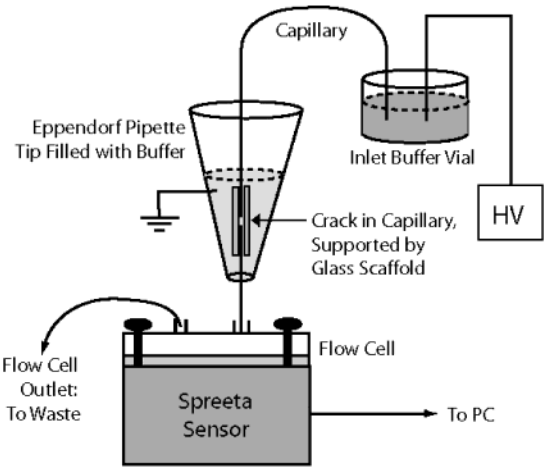

Figure 1. Schematic diagram of the capillary electrophoresis−surface plasmon resonance sensor separation and detection system. The capillary is cracked and grounded above the outlet to isolate the detection electronics in the SPR sensor from the separation voltage. Solution leaving the capillary enters a small-volume (400 nL or 3.5 μL) flow cell that is attached to the face of the sensor. The sensor reports on the refractive index of the solution adjacent to the gold-sensing surface. Refractive index monitored as a function of separation time produces an electropherogram.

Capillary Electrophoresis. Figure 1 presents a schematic representation of a CE-SPR experiment. Sample was injected onto the capillary by gravity; the capillary inlet was immersed in the sample vial that was then elevated to 10 cm for 10 s, resulting in an injected plug volume of 270 nL. This injection method was employed because of its simplicity, but it has the drawback of larger variations in injected volume. The separation voltage (+ 6 kV) was applied with a high-voltage supply (Glassman High Voltage, Inc., Whitehouse Station, NJ). Because of the sensitive electronics necessary for accurate SPR sensing, it was not possible to ground the separation voltage at the capillary outlet. Instead, we used a modification of a design used previously in our laboratory to perform CE separations with living cell detectors.[28] Briefly, a small crack was made in the capillary ∼5 cm from the outlet. The cracked region is very fragile and was supported by small pieces of pipet glass that were glued together with epoxy, forming a scaffold. The cracked region was bathed in a 1-mL volume of run buffer, and the buffer solution was connected to ground through a metal wire. Unmodified fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). The capillaries used were 363-μm o.d., 150-μm i.d., and 50-cm total length (45 cm from inlet to ground). All CE experiments used 100 mM borate buffer (pH 9) as the run buffer. Separations were performed at ambient temperature. Cracked capillaries were prepared for use each morning by flushing with run buffer at a flow rate of 0.5

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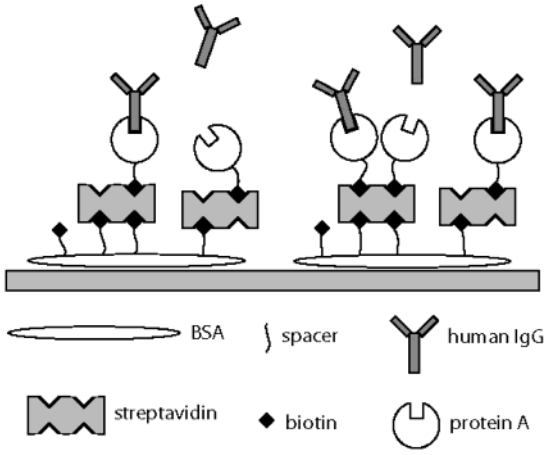

Figure 2. Schematic of the procedure for immobilizing proteins on the gold-sensing surface. The surface was layered sequentially with BSA−biotin (20 μg/mL), streptavidin (50 μg/mL), and protein A−biotin (40 μg/mL). These solutions were delivered as 100-μL plugs using a flow rate of 20 μL/min and were in a PBS solution. After this layering was complete, the separation capillary was affixed above the surface. Human IgG of varying concentrations in borate solution was introduced into the capillary and delivered electrophoretically to the surface, where it was immobilized as a result of the interaction between its Fc fragment and protein A.

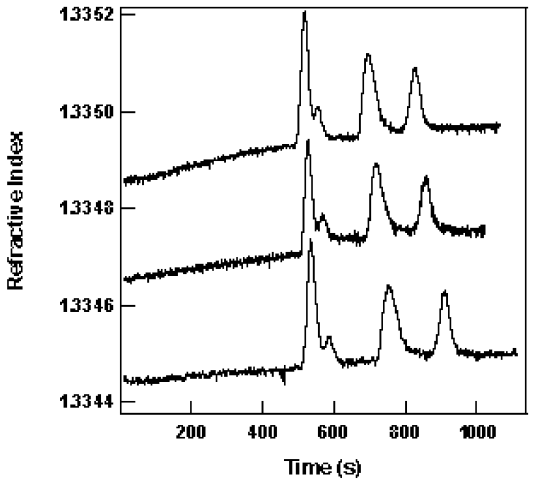

Figure 3. Electropherograms resulting from the CE separation and SPR detection of a three-component mixture of high refractive index materials that do not bind to the sensing surface. The analytes were benzyl alcohol (0.2%), 2-cholorophenol (0.2%), and *p*-nitrophenol (10 mM) in 100 mM borate buffer. Separation voltage was +6 kV. The minor peak at ~575 s results from an impurity in 2-chlorophenol. The two top traces have been offset vertically from the bottom trace for clarity. The variation in baseline drift is caused by the lack of temperature control.

mL/h for 60 min but were not otherwise treated or conditioned. The capillary outlet was surrounded by a piece of polyethylene tubing, which fit into the inlet of the SPR flow cell. As solution eluted from the end of capillary, it was directed into the flow cell where it was interrogated by the Spreeta sensor. The resulting record of refractive index as a function of time produced an electropherogram.

Protein Deposition. To perform SPR detection on electrophoretically delivered protein, we functionalized the surface to recognize and interact with the protein of interest. We chose to perform CE-SPR on human IgG by taking advantage of its high-affinity interaction with protein A, a surface receptor from *Staphylococcus aureus*.[29] Figure 2 illustrates the general scheme for protein layer formation. The immobilization began with a clean, unmodified gold surface. The surface was cleaned between uses by gently wiping with a cotton swab or Kimwipe soaked in 1% sodium dodecyl sulfate (SDS) detergent solution, followed by extensive rinsing with Milli-Q water. The surface was allowed to dry in air. Once the sensor was dry, the flow cell was set up, and solution was pushed through the flow cell by the syringe pump. Protein solution was loaded into the 100-μL sample loop on a switching valve and injected into the flow stream. BSA−biotin was injected first, and then streptavidin, a tetramer that forms a very stable complex with biotin, leaving additional biotin-binding sites available. Protein A−biotin was then injected, producing a stable, bound layer of protein A available to interact with IgG. During the protein-layering steps, the solution flow rate was 20 μL/min, resulting in protein contact times of 5 min. Once the protein A layer was deposited, the solution delivery line from the switching valve was removed, and the cracked capillary assembly was inserted into the flow cell in its place.

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RESULTS AND DISCUSSION

CE-SPR of High Refractive Index Solutions. A solution of three high refractive index materials—benzyl alcohol (RI = 1.54), 2-chlorophenol (RI = 1.55), and *p*-nitrophenol (RI = 1.57)—in 100 mM borate buffer (RI = 1.335) was injected onto a capillary and separated by CE. As solution left the separation capillary, it was directed into a flow cell where it was continually monitored by SPR. Although these three analytes do not bind to the gold surface of the SPR sensor, each has a higher refractive index than the buffer, and therefore, their migration off the column and into region adjacent to the sensing surface shifts the surface plasmon resonance curve. Figure 3 shows three electropherograms resulting from the separation and SPR detection of the mixture of these high refractive index materials. Three major peaks are apparent in Figure 3 along with a minor peak that results from an impurity in 2-chlorophenol. For these electropherograms ($n = 3$), the mean peak area for benzyl alcohol was $(7.6 \pm 0.4) \times 10^{-3}$ RI × s, and the mean width (full width at half the maximum signal, fwhm) was 22 ± 2 s (uncertainty determined as one standard deviation of the mean). For 2-chlorophenol, the mean peak area was $(6.5 \pm 0.5) \times 10^{-3}$ RI × s, and the fwhm was 35 ± 4 s. The mean peak area for *p*-nitrophenol was $(3.7 \pm 0.5) \times 10^{-3}$ RI × s, and the fwhm was 25 ± 1 s. For all three analytes, the standard deviation of peak area and width is ~10% of the mean. Variation in amount of analyte detected can be attributed to variation in the injection process, which was not optimized for precision. The peaks are longer in time than peaks usually observed in a CE separation. This phenomenon results from the fact that an analyte band, which is tightly focused on the column, is diluted considerably upon introduction into the flow cell (flow cell volume is 400 nL for the separations shown in Figure 3). Smaller volume flow cells would help to narrow the peaks in the electropherogram. It should be noted, however, that the surface-sensitive SPR detection method is best suited for analytes that can be bound to the sensing surface.

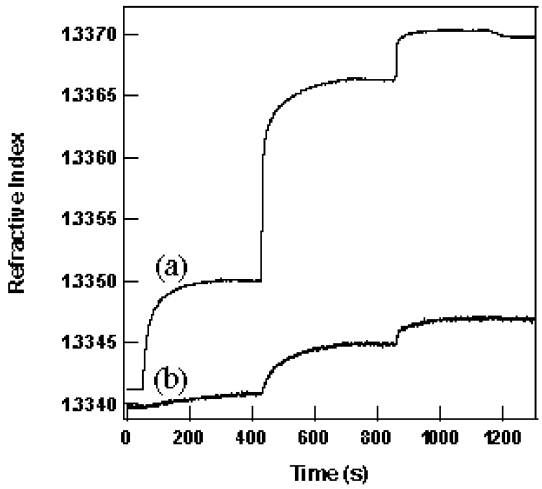

Figure 4. Sensorgrams showing the sequential binding of three proteins, BSA−biotin, streptavidin, and protein A−biotin, (a) in PBS buffer and (b) in borate buffer. Protein concentrations and delivery conditions are the same as in Figure 2. BSA−biotin was injected at 20 s, streptavidin was injected at 400 s, and protein A−biotin was injected at 830 s.

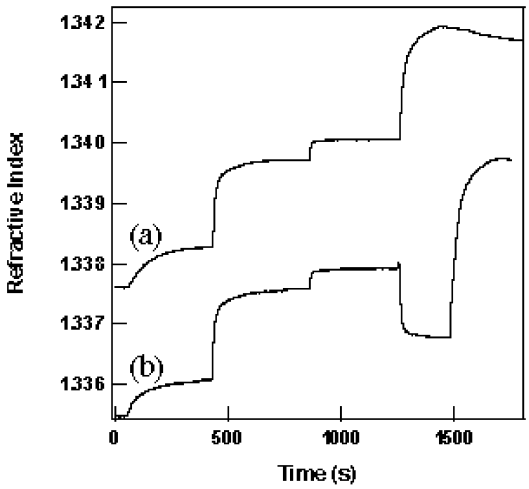

Figure 5. Sensorgrams showing the binding of BSA−biotin, streptavidin, protein A−biotin, and human IgG in (a) PBS buffer and (b) with a hybrid buffer system. All four proteins were delivered as 100-μL plugs using 20 μL/min flow from a syringe pump. The concentrations and binding conditions for the first three proteins are the same as described in Figure 2. The concentration of human IgG was 100 μg/mL. In sensorgram b, the first three proteins were loaded in PBS buffer. At ~1200 s, the buffer was switched to 50 mM borate. After the RI signal reached a new level (at a lower RI because the borate buffer was of lower refractive index than PBS), IgG was injected. The top trace has been offset vertically from the bottom trace for clarity, and a segment of sensorgram b corresponding to buffer switching and equilibration has been removed.

The detection limit for high refractive index materials that are sensed in solution but not bound to the surface is therefore poor. The present system is able to detect ~10 mM concentration of the phenols present. In contrast, the use of phenol-specific receptors has been reported by Wright and co-workers[30] in an SPR assay, and they were able to detect phenols at the 0.1 mM level.

CE-SPR of Proteins. Successful pairing of CE with SPR for the separation and detection of proteins requires the use of buffer conditions that favor both the reproducible migration of protein analytes through the separation column and the binding of protein layers to the gold-sensing surface. SPR investigation of protein binding is usually performed using high-salt buffers, such as phosphate-buffered saline (PBS), at physiological pH. PBS is not commonly used as a CE buffer, however, because its high ionic strength necessitates the use of very low separation voltages, resulting in increased analysis times. In addition, it is often desirable to perform CE of proteins at high pH because at pH > pI both the capillary wall and the protein will be negatively charged, and electrostatic interaction between them will be minimized.

Experiments on a commercial CE instrument with UV absorbance detection showed that, at low separation voltages (+6−8 kV), 100 mM borate buffer at pH 9 gave reproducible migration times for human IgG, the protein of interest (data not shown). Therefore, the deposition of proteins in borate onto the gold surface was investigated. Figure 4 shows two sensorgrams resulting from the binding of the three proteins—BSA−biotin, streptavidin, and protein A−biotin—that are used to prepare the surface for IgG binding. The protein solutions were delivered to the gold surface with a syringe pump at a flow rate of 20 μL/min. In sensorgram a, the proteins were dissolved in PBS. In sensorgram b they were in a borate solution. For replicate measurements (n = 3) in PBS solution, RI changes caused by protein binding were (8.1 ± 0.9) × 10^{-4} for BSA−biotin, (1.7 ± 0.1) × 10^{-3} for streptavidin, and (3.7 ± 0.4) × 10^{-4} for protein A−biotin. The binding of proteins in borate was significantly smaller; mean RI changes (n = 2) caused by protein binding were (9 ± 1) × 10^{-5} for BSA−biotin, (4.05 ± 0.06) × 10^{-4} for streptavidin, and (1.8 ± 0.1) × 10^{-4} for protein A−biotin.

To take simultaneous advantage of the larger amount of protein binding in PBS and the attributes of borate as a CE buffer, we developed a hybrid buffer scheme. In this scheme, the gold surface was given the desired functionality (protein A) by deposition of three protein layers (BSA−biotin, streptavidin, protein A−biotin) in PBS solution, using a syringe pump to deliver the proteins. The functionalization of the gold surface is important because it binds in the ideal case to only the protein of interest. As a result, it concentrates the analyte of interest within the sensor area. To understand whether nonspecific binding is compromising these studies, we introduced a protein of the same size and general nature, an IgG of different specificity. We found that the nonspecific binding was only 15−25% of the full specific binding.

After functionalizing the gold surface, the buffer was switched to borate, and human IgG was delivered to the surface. To evaluate the efficacy of the two-buffer system, human IgG delivery was accomplished initially by using large volumes of protein solution and pressure from a syringe pump. Figure 5 shows two sensorgrams resulting from the binding of BSA−biotin, streptavidin, protein A−biotin, and human IgG. Each protein solution was injected onto a 100-μL loop on a two-position, six-port switching valve and was then delivered to the gold-sensing surface at a flow rate of 20 μL/min. In sensorgram a, all four proteins were in PBS, whereas in sensorgram b, only the first three were in PBS. Buffer

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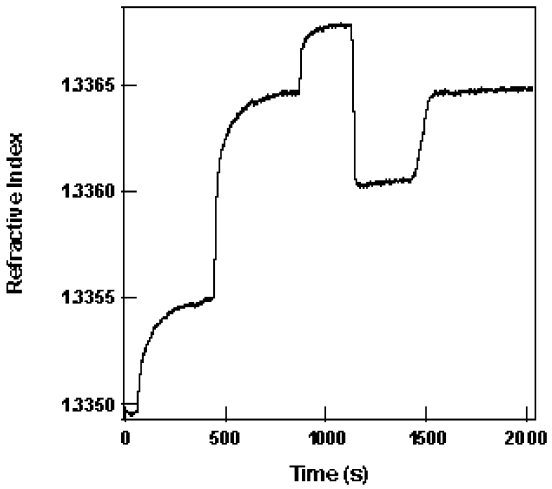

Figure 6. Sensorgram showing the pressure-driven delivery of BSA−biotin, streptavidin, and protein A−biotin in PBS followed by the electrophoretic delivery of human IgG in 100 mM borate. A 270-nL plug of 100 mg/mL IgG was injected, and the separation voltage (+ 6 kV) was applied at 1270 s.

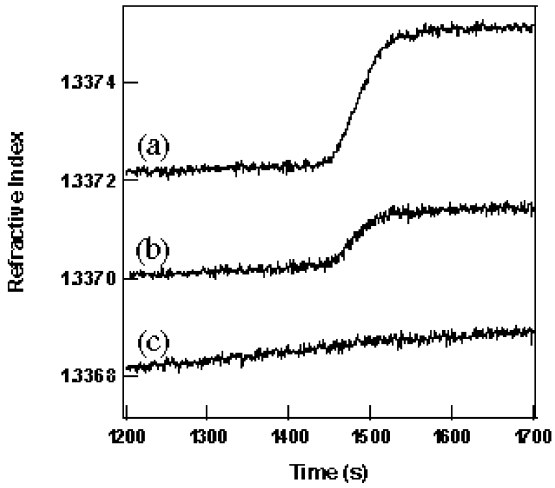

Figure 7. Three electropherograms showing the delivery of a 270-nL plug of (a) 100 μg/mL IgG, (b) 50 μg/mL IgG, or (c) run buffer. The time scale has been truncated to only show the electrophoretic part of the sensorgram; prior to 1200 s, the surface had been functionalized with protein A as in Figure 5.

exchange (removal of PBS and introduction of borate) occurs at ~1300 s, as indicated by the decrease in bulk refractive index. Introduction of human IgG in 100 mM borate (which occurs at 1510 s) results in a sizable binding event. For replicate measurements ($n = 5$), the mean RI change caused by the binding of a 100-μL plug of IgG in borate was $(2.0 \pm 0.3) \times 10^{-3}$, which is essentially identical to the binding in PBS, for which the mean RI change was $(1.77 \pm 0.06) \times 10^{-3}$.

Figure 6 shows the refractive index change resulting from the functionalization of a gold surface by selective protein deposition and subsequent interaction with an electrophoretically delivered nanoliter volume of human IgG. For all CE-SPR experiments using IgG, the surface was prepared with layers of proteins terminating in the protein A functionality, as described in the previous paragraph. The SPR sensor, with its attached flow cell, was then removed from the solution delivery line (from the syringe pump and switching valve), and the separation capillary was inserted into the flow cell (this event occurs at 1200 s in Figure 6). High voltage (+6 kV) was applied briefly to verify that the current was stable, that the voltage did not interfere with the functioning of the sensor, and that electoosmotic pumping was delivering solution through the flow cell. The voltage was then switched off, human IgG was injected, and the voltage was reapplied (at 1270 s in the sensorgram shown). In contrast with the nonbinding analytes shown in Figure 3, human IgG binds very tightly to the surface as a result of its high-affinity interaction with protein A ($K_d = 62.5$ nM).[29] High-affinity binding enhances the detection limit for the protein in contrast with a nonbinding analyte and is responsible for the characteristic step shape of the binding event. Figure 7 shows three electropherograms resulting from the delivery of solution to a protein A-functionalized surface. Electropherogram a shows the delivery ($n = 15$ replicate measurements) of a 270-nL plug of 100 μg/mL IgG, in electropherogram b a 50 μg/mL IgG solution ($n = 8$) is detected, and electropherogram c is a control in which protein-free buffer was injected and delivered to the sensor surface. For replicate trials ($n = 8$), the RI change resulting from binding of the 50 μg/mL solution was $(1.3 \pm 0.2) \times 10^{-4}$. With the injection volumes used, this analyte concentration corresponds to the detection of 90 fmol of IgG. In this case, the standard deviation is 15% of the mean, indicating a reproducibility comparable to that achieved in the pressure-driven delivery of proteins to the surface. In other words, the complexities of protein deposition and binding, and not the use of CE to deliver proteins to the surface, sets the limit on the reproducibility of CE-SPR as a separation and detection method.

At low concentrations of IgG (below 100 μg/mL), the sensor response is linear as a function of IgG concentration (data not shown). If we assume that a linear relationship continues at higher concentrations (which is reasonable up to the limit of saturation, for a high-affinity binding interaction) and that the sensor response elicited from a 100-μL plug of 100 μg/mL IgG represents surface saturation, we can then calculate that the surface saturates at 1 pmol of IgG. We have previously shown that, with a protein immobilization scheme similar to that described here, the Spreeta sensor has a detection limit for antibody of 2 fmol,[27] suggesting that this detector has a dynamic range of 3 orders of magnitude. Different surface preparations with different densities of binding sites will alter this dynamic range and the minimum detectable amount of protein. The affinity of the analyte for the immobilized reaction partner also affects the detection limit and the shape of the electropherogram.

CONCLUSIONS

We have demonstrated the use of a miniaturized SPR sensor as a postcolumn detector for CE separations. Because of its relatively low cost, small size, and experimental simplicity, this separation and detection method could be of use in the screening of small samples for particular proteins of interest in clinical applications. The flexibility of the protein immobilization procedure used here—in which IgG molecules of any specificity can be immobilized through protein A—means that the sensor can be customized to respond to any protein against which an antibody is available. The dynamic range of response and the detection sensitivity seem adequate for many purposes, but the reproducibility of ~15% is likely to need improvement before this technique is widely adopted.

We suggest that several aspects of the CE-SPR setup described here could be further optimized. Two major sources of irreproducibility are injection errors and lack of temperature control. Each of these are planned to be improved in future studies. In particular, active temperature control is important for achieving good CE separations, by minimizing the effects of Joule heating. More significantly, the refractive index of an aqueous solution is very sensitive to temperature, with RI changes on the order of 10^{-4}/°C. Controlling the temperature of the capillary and the flow cell is therefore expected to reduce RI signal drift and increase the signal-to-noise ratio. The version of the Spreeta that we used for these studies did not contain a resistive heating thermostat for temperature control, but other models do.

The sample dilution caused by the discrepancy between the volume of analyte bands on the capillary and the volume of the currently available flow cells is another point at which sensitivity could be improved. Finally, the protein detection described here uses a high-affinity interaction to produce RI steps when the analyte is detected. The use of low-affinity antibodies against the molecule of interest would result in an electropherogram with peaks rather than steps, as has been previously demonstrated for an HPLC-SPR detection of carbohydrates.[24]

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