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Electrophoretic Concentration of Proteins at Laser-Patterned Nanoporous Membranes in Microchips

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Laser-patterning of nanoporous membranes at the junction of a cross channel in a microchip is used to integrate protein concentration with an electrokinetic injection scheme. Upon application of voltage, linear electrophoretic concentration of charged proteins is achieved at the membrane surface because buffer ions can easily pass through the membrane while proteins larger than the molecular weight cutoff of the membrane (>5700) are retained. Simple buffer systems can be used, and the concentration results constitute outward evidence that the uniformity of buffer ion concentration is maintained throughout the process. Local and spatially averaged concentration are increased by 4 and 2 orders of magnitude, respectively, upon injection with moderate voltages (70−150 V) and concentration times (100 s). The degree of concentration is limited only by the solubility limit of the proteins. The porous polymer membrane can be used repeatedly as long as care is taken to avoid protein precipitation.

The development of microfluidic devices during the past decade has facilitated improvements in biological and chemical analysis.[1,2] Incorporating sample preconcentration, mixing, reaction, separation, and detection functions into microchips can facilitate automation of labor-intensive sample analysis and reduce reagent consumption.

Sample preconcentration steps are ever-present components of analytical systems, since concentration of trace samples facilitates their detection. A sample preconcentration process concentrates sample analytes in a compact volume prior to detection and increases the signal-to-noise ratio, resulting in improved detectivity.

The numerous liquid-phase sample preconcentration techniques performed on microchips to date can be grouped roughly into three classes (surface techniques, electrokinetic manipulation in spatially varying electric field, porous filtering) based on the transport physics involved. Surface techniques use sample adsorption to surfaces for concentration and a surface or solvent change for elution. Solid-phase extraction[3−9] typically employs hydrophobic or electrostatic attraction and elutes using a change in buffer. Film electrode techniques[10] use electrostatic attraction at micropatterned electrodes to capture analytes and release with changes in voltage. Programmable surface techniques use surfaces with voltage- or temperature-addressable properties[11] and elute by changing the surface properties.

Electrokinetic manipulation in spatially varying electric fields has been used in a variety of forms to concentrate analytes.[12,13] The general property of these systems is that spatial variations in buffer properties lead to spatial variations in electric field and therefore concentration of analytes. Isoelectric focusing involves spatially varying electrokinetic transport in pH gradients.[14−16] Field-amplified sample stacking[17−23] and isotachophoresis[24,25] involve conductivity-induced spatial electric field variations in two- and

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(1) Reyes, D. R.; Isossifidis, D.; Auroux, P.-A.; Manz, A. *Anal. Chem.* **2002**, *74*, 2623−2636.
(2) Auroux, P.-A.; Isossifidis, D.; Reyes, D. R.; Manz, A. *Anal. Chem.* **2002**, *74*, 2637−2652.
(3) Broyles, B. S.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **2003**, *75*, 2761−2767.
(4) Ro, K. W.; Chang, W.-J.; Kim, H.; Koo, Y.-M.; Hahn, J. H. *Electrophoresis* **2003**, *24*, 3253−3259.
(5) Jemere, A. B.; Oleschuk, R. D.; Ouchen, F.; Fajuyigbe, F.; Harrison, D. J. *Electrophoresis* **2002**, *23*, 3537−3544.
(6) Sato, K.; Tokeshi, M.; Kimura, H.; Kitamori, T. *Anal. Chem.* **2001**, *73*, 1213−1218.
(7) Yu, C.; Davey, M. H.; Svec, F.; Fréchet, J. M. J. *Anal. Chem.* **2001**, *73*, 5088−5096.
(8) Dodge, A.; Fluri, K.; Verpoorte, E.; de Rooij, N. F. *Anal. Chem.* **2001**, *73*, 3400−3409.
(9) Oleschuk, R. D.; Shultz-Lockyear, L. L.; Ning, Y.; Harrison, D. J. *Anal. Chem.* **2000**, *72*, 585−590.
(10) Lin, Y.-C.; Ho, H.-C.; Tseng, C.-K.; Hou, S.-Q. *J. Micromech. Microeng.* **2001**, *11*, 189−194.
(11) Huber, D. L.; Manginell, R. P.; Samara, M. A.; Kim, B.; C., B. B. *Science* **2003**, *301*, 352−354.
(12) Lichtenberg, J.; de Rooij, N. F.; Verpoorte, E. *Talanta* **2002**, *56*, 233−266.
(13) Chien, R.-L. *Electrophoresis* **2003**, *24*, 488−497.
(14) Tan, W.; Qiu, C. X.; Ricco, A. J.; Gibbons, I. *Electrophoresis* **2002**, *23*, 3638−3645.
(15) Catherine, R. C.; Yager, P. *Electrophoresis* **2001**, *22*, 355−362.
(16) Wen, J.; Lin, Y.; Xiang, F.; Matson, D. W.; Udseth, H. R.; Smith, R. D. *Electrophoresis* **2000**, *21*, 191−197.
(17) Kutter, J. P.; Ramsey, R. S.; Jacobson, S. C.; Ramsey, J. M. *J. Microcolumn Sep.* **1998**, *10*, 313−319.
(18) Li, J.; Wang, C.; Kelly, J. F.; Harrison, D. J.; Thibault, P. *Electrophoresis* **2000**, *21*, 198−210.
(19) Lichtenberg, J.; Verpoorte, E.; de Rooij, N. F. *Electrophoresis* **2001**, *22*, 258−271.
(20) Yang, H.; Chien, R.-L. *J. Chromatogr., A* **2001**, *924*, 155−163.
(21) Jung, B.; Bharadwaj, R.; Santiago, J. G. *Electrophoresis* **2003**, *24*, 3476−3483.
(22) Quirino, J. P.; Terabe, S. *Anal. Chem.* **2000**, *72*, 1023−1030.
(23) Palmer, J.; Burgi, D. S.; Munro, N. J.; Landers, J. P. *Anal. Chem.* **2001**, *73*, 725−731.
(24) Vreeland, W. N.; Williams, S. J.; Barron, A. E.; Sassi, A. P. *Anal. Chem.* **2003**, *75*, 3059−3065.

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three-buffer systems, respectively. Temperature gradient focusing[26] uses temperature gradients to establish steady-state ionic strength gradients.

Porous filtering techniques concentrate analytes at semipermeable interfaces. Interfaces used at a microchip level include a silicate layer deposited between a cover and substrate of a microchip,[27,28] and an etched capillary.[29] Porous filtering is inherently different from other concentration techniques in that the concentration is specific to certain size classes of analytes.

Although the above techniques have been shown to concentrate analytes by factors ranging from 1 to 5 orders of magnitude, their application can be limited by fabrication complexities, buffer handling challenges, or physical uncertainties. Several of the techniques (solid-phase extraction, isoelectric focusing, field-amplified sample stacking, isotachophoresis) require spatial or temporal buffer variations. Others (film electrodes, programmable surfaces, temperature gradient focusing) require microfabrication complexity that might not always be justified.

In well-defined systems, porous filtering is a straightforward and intuitive concentration technique. Porous interfaces that are permeable to buffer ions but impermeable to sample analytes can be used to stack analytes at the interface upon application of voltage. When electrophoretic sample transport is used, the increase of sample concentration should be linearly proportional to the product of applied voltage and the length of time over which it is applied (as long as the geometry remains constant). These methods are simple to carry out, but results presented to date have shown that the increase of sample concentration is a nonlinear function of sample injection time and voltages for unknown reasons.[27,28]

This paper presents a porous membrane sample preconcentration system that is straightforward to use with pinched electrokinetic injections,[30] allows concentration up to the protein solubility limit, and affords a linear response to the time−voltage product. We have implemented a technique for in situ fabrication of nanoporous polymer membranes on a microchip, used previously for microchip dialysis of proteins.[31] In the present studies, a membrane is laser-patterned at the junction of a cross channel in microchips. By implementing the electrokinetic pinched injection, sample analytes (charged proteins) concentrate at the surface of membrane and the concentrated sample can be eluted downstream. In the following sections, the fabrication method and membrane characteristics are presented, and the performance of the preconcentration system is explored in detail.

EXPERIMENTAL SECTION

Microchip Fabrication and Surface Functionalization. The fabrication procedures are based on previous work and are summarized briefly here. Fused-silica (Corning 7980) microchips with cross channels were fabricated in-house using standard photoresist, UV patterning, and isotropic HF wet-etch steps.[31] Following thermal bonding, the silica channel surfaces were prepared for covalent attachment of polymers by coating with 3-trimethoxysilylpropyl acrylate through acid-catalyzed hydrolysis and condensation.[32] Channels were first conditioned by 30-min flushes with 1 M HCl and then 1 M NaOH, then rinsed with deionized water, and dried. The microchannels were then incubated for 30 min with a solution consisting of a 2:2:1 (v/v/v) mixture of deionized water, glacial acetic acid, and 3-trimethoxysilylpropyl acrylate. The channel was then rinsed thoroughly with deionized water and 1-propanol.

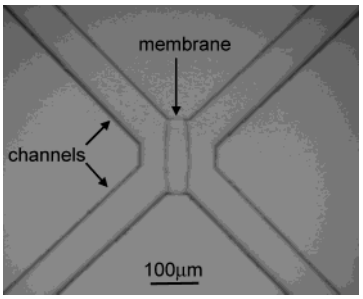

Figure 1. Cross channel with laser-patterned porous polymer membrane used for the present studies. The channel width and depth are 80 and 20 μm. The membrane thickness is roughly 50 μm.

Membrane Fabrication. Zwitterionic polymer membranes with molecular weight cutoff <5700 were fabricated inside microchannels using laser-patterning techniques specified previously[31] and summarized briefly here. A 355-nm laser beam was used to excite a photoinitiator in a monomer/solvent solution, leading to polymerization and phase separation of a thin membrane that was covalently bonded to the acrylate-functionalized silica channel surface. The irradiated region was defined by shaping the beam into a 40 μm × 300 μm laser sheet by imaging an adjustable slit with cylindrical and spherical optics. The resulting membranes were 50 μm thick (Figure 1). Laser exposure time was typically 3.5 min. After polymerization, the channels were flushed thoroughly with water.

Electroosmosis was suppressed in the silica channels by coating with linear polyacrylamide[32] after membrane fabrication. Channels were coated by placing the microchip 0.5 in. above a 30-W, 365-nm-cutoff UV lamp (model EW-97605-00, Cole-Parmer, Vernon Hills, IL) for 30 min while the channel was incubated with a solution of 5 mg/mL acrylamide in deionized water containing 200 ppm hydroquinone (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/mL 2,2′-azobis(2-methylpropanimidamide) dihydrochloride (Wako Chemicals, Richmond, VA). The channel was then rinsed thoroughly with water. The entire coating process, including filling and rinsing the channel, was repeated twice.

Analytes and Instrumentation. Two Alexa Fluor 488-labeled proteins were used as model analytes: 0.5 μM bovine serum albumin (MW 66 000, pI = 4.9, Molecular Probes) in a pH 7.5, 10 mM phosphate buffer and 0.5 μM phosphorylase *b* (MW 94 000, pI = 6.3, Sigma-Aldrich) in a pH 9.3, 10 mM borate buffer. Protein concentration increases were quantified with a fluorescence microscope (Olympus, model IX70) and 12-bit, mono, cooled CCD

(25) Wainright, A.; Williams, S. J.; Ciambrone, G.; Qifeng Xue; Wei, J.; Harris, D. *J. Chromatogr., A* **2002**, *979*, 69−80.
(26) Ross, D.; Locascio, L. E. *Anal. Chem.* **2002**, *74*, 2556−2564.
(27) Khandurina, J.; McKnight, T. E.; Jacobson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **2000**, *72*, 2995−3000.
(28) Khandurina, J.; Jacobson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **1999**, *71*, 1815−1819.
(29) Wei, W.; Yeung, E. S. *Anal. Chem.* **2002**, *74*, 3899−3905.
(30) Jacobson, S. C.; Hergenroder, R.; Koutny, L. B.; Warmack, R. J.; Ramsey, J. M. *Anal. Chem.* **1994**, *66*, 1107−1113.
(31) Song, S.; Singh, A. K.; Shepodd, T. J.; Kirby, B. J. *Anal. Chem.* **2004**, *76*, 2367−2373.
(32) Kirby, B. J.; Wheeler, A. R.; Zare, R. N.; Fruetel, J. A.; Shepodd, T. J. *Lab Chip* **2003**, *3*, 5−10.

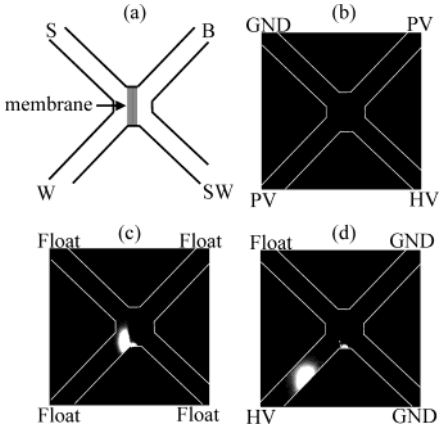

Figure 2. Membrane schematic and background-corrected images of protein concentration. Microchannel edges have been drawn for clarity: HV, high voltage; PV, pinch voltage (PV = 0.45 HV); GND, ground. (a) Schematic. S denotes sample, W waste, B buffer, and SW sample waste. (b) Before loading. (c) After loading. (d) During injection.

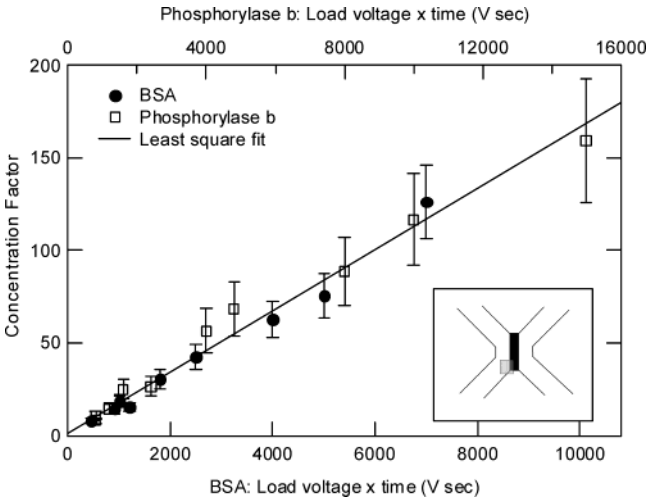

Figure 3. Linear increase in concentration of both BSA and phosphorylase *b* with the voltage−time product. The increase can be by over 2 orders of magnitude. See Experimental Section for details. "Concentration factor" denotes measured protein concentrations, normalized to the 0.5 μM initial concentration. Measurement uncertainties (±16% for BSA and ±21% for phosphorylase *b*) are dominated by uncertainty in the low signal level of the unconcentrated sample. Concentration measurements were averaged over a measurement window specified in gray in the inset, chosen to be comparable to the channel width and size of injected sample.

camera (CoolSNAPHQ, Roper Scientific, Inc., Tucson, AZ) at 20-ms exposure time. Background signal from dark current and ambient light was subtracted before analysis. Photobleaching was minimized through the choice of fluorescent label and by illuminating the field of view only briefly at each measurement point. To inject and elute analytes by electrophoresis, voltage (20−150 V) was applied across the membrane using a high-voltage source[33] (μChemLab, Sandia National Laboratories, Livermore, CA). The detailed voltage configuration is described in the following sections.

Sample Loading, Concentration, and Injection. A cross channel is used with a membrane laser-patterned at the intersection (Figure 2a). Each channel is connected to its own reservoir. All the channels and reservoirs are filled with the same buffer, but the buffer to the left of the membrane also contains the fluorescently labeled proteins. To load proteins, voltage (15−150 V) is applied for 20−100 s between the sample and sample waste reservoirs (Figure 2b), and proteins above the membrane MWCO are retained at the surface of the membrane. A pinch voltage (0.45 HV) is applied to the buffer and waste channels during this step to define the sample plug shape and to minimize diffusional loss of the proteins away from the membrane. Finally, 100 V is applied to the waste reservoir to inject analytes for downstream analysis (Figure 2d), while the sample reservoir is allowed to float and the other reservoirs are grounded.

To evaluate the performance of the sample preconcentration system, sample analyte concentration was measured near the membrane for various injection times and voltages. Images of the sample analytes stacking at the membrane surface were recorded, and a spatially averaged concentration factor was calculated by integrating over a measurement window placed near the membrane as shown in the inset of Figure 3. Since the protein is localized to a thin region adjacent to the membrane during the loading step, this measurement was performed after allowing the proteins to diffuse away from the membranes for a prescribed time interval (7 s). The integration window is a 21 by 21 pixel square whose size is comparable to the channel width—spatially averaged concentration factors in this window are thus representative of the effective sample concentration observed downstream as compared to an electrokinetic injection alone without concentration. Further, allowing the sample to diffuse eliminated self-quenching and CCD saturation errors. The pixel intensity in the window was averaged and compared to a similar average before concentration. As will be noted later, local protein concentration values are greatly in excess of the spatially averaged value.

RESULTS AND DISCUSSION

Membrane Characteristics. Mechanical and species diffusion characteristics of the polymer membrane have been examined extensively. The molecular weight cutoff corresponding to the pore size of the membrane used in this work has been estimated to be below 5700, by demonstrating that insulin (5700) and a number of proteins do not cross the membrane while small tracer species (<500) do.[31] The membranes also maintain their properties upon exposure to a wide range of solvent conditions such as pH (2−9.3) and polarity (water, acetonitrile, C_1−C_3 alcohols). The membrane shown in Figure 1 has been used in more than 120 realizations over three months without any observable changes in properties. The zwitterionic and hydrophilic polymer membrane shows no measurable protein adhesion after flushing with water and isotonic phosphate-buffered saline. The membranes have been tested with differential pressures up to 1 bar, so vacuum pressure can be applied to manipulate fluids as part of the fabrication or testing processes.

The electrical attributes of the membrane confirm that the charge-carrying ions in solution freely pass through the membrane and that no electric breakdown or ion depletion effects are present. The electrical resistances of equivalent channels with and without

(33) Renzi, R. F.; Stamp, J. F.; Crocker, R. W.; Wiedenman, B. J.; Ferko, S. M.; Horn, B. A.; Yee, D.; VanderNoot, V. A.; West, J. A.; Fruetel, J. A.; Fintschenko, Y.; Stoddard, M. C. μTAS 2002; Babu, Y., Shoji, S., Berg, A. v. d., Eds.; Nara, Japan, November 3−7, 2002; pp 703−705.

the laser-patterned membrane are equal within measurement accuracy (5%), consistent with the assertion that the membrane is permeable to molecules below MW 500.[31] Furthermore, currents through the system are temporally steady and linear with voltage.

Electrophoretic Concentration of Proteins. The mechanical and electrical properties of the membranes allow simple electrophoretic concentration and injection of proteins (Figure 2) to be performed with a single buffer as part of a pinched electrokinetic injection, as described in the Experimental Section. Electrophoresis stacks proteins against the semipermeable membrane while the charge-carrying ions pass essentially unimpeded. The negatively charged proteins concentrate at the lower corner of the membrane (Figure 2c) because the lower corner is at a higher potential. By placing the membrane at an angle to the equipotential lines of the system and thereby inducing an asymmetric protein distribution, the majority of the protein sample is injected onto the running column (W in Figure 2d) and only a small amount is pulled back into the sample channel when voltages are switched. Protein retention at the membrane is minor.

The spatially averaged concentrations of both BSA and phosphorylase *b* observed in the measurement window can be increased by over 2 orders of magnitude (Figure 3). The maximum concentration factors demonstrated are about 130 for BSA and 160 for phosphorylase *b*. The injection time varied between 20 and 100 s for both cases. The voltage applied to the sample waste reservoir ranged from 15 to 70 V for BSA and from 40 to 150 V for phosphorylase *b*. The corresponding electric field strengths ranged from 7.5 to 35 V/cm for BSA and from 20 to 75 V/cm for phosphorylase *b*.

The concentration increases linearly with the time−voltage product (Figure 3). Since the proteins are carried toward the membrane by electrophoresis and then retained at the membrane surface, the rate at which proteins are concentrated (i.e., the slope of the concentration factor vs time−voltage product) relates directly to their electrophoretic mobility (μ_{ep}). The concentration rates inferred from the slopes of the linear fits to the BSA and phosphorylase *b* data ($r^2 = 0.92$) are 16.7 and 11.3 kV^{-1} s^{-1}, respectively, leading to a ratio of the concentration rates of ∼1.5. From an approximate calculation of the charge states of BSA and phosphorylase *b* in the experimental buffer systems,[34] combined with the relation $\mu_{ep} \sim (q/M)^3$ ($0.33 < s < 0.67$; *q*, charge; *M*, mass),[35] the ratio of electrophoretic mobilities of BSA and phosphorylase *b* is estimated to be 1.35−1.52, which is consistent with the experimentally observed ratio of concentration rates.

The linear response of this system is in contrast to the data presented for other porous filtering concentration techniques, which have shown strongly nonlinear response to date. The direct benefit of linear response is that the effect of concentration can straightforwardly be quantified without calibration. Further, the linear response of the system indicates that the charge-carrying buffer ions pass unhindered through the membrane and that uniform buffer−ion distributions due to the use of a single buffer are unaffected by the loading process. This means that separations or other electrokinetic techniques will be unaffected by the concentration technique. While other porous filtering concentration techniques[27,28] have shown the ability to concentrate sample, the nonlinear response raises questions about the possible presence of ion depletion/concentration or electric breakdown effects.

The technique used to quantify concentration here (averaging over a diffuse window) was chosen because it is representative of the signal expected downstream. However, local concentration of protein is much more dramatic than the average values reported in Figure 3. We observed protein precipitation beyond 7000 V·s for BSA and 15 000 V·s for phosphorylase *b*. When the precipitation occurs, system currents change and the concentration rate slows. From the solubilities of the proteins, it can be inferred that, in both cases, the protein concentration has been increased locally by at least 4 orders of magnitude.

CONCLUSIONS

A simple protein preconcentration system has been presented using a 50-μm-thick nanoporous polymer membrane fabricated in the junction of a cross channel of microchips. The membrane is used to trap proteins using a pinched electrokinetic injection and a single buffer. Moderate voltages (70−150 V) and concentration time (100 s) lead to increases in local and spatially averaged protein concentrations by 4 and 2 orders of magnitude, respectively. Unlike other porous filtering concentration techniques presented to date, the response of this system is linear with the voltage−time product up to the point of protein precipitation. This linearity enables precise control of concentration level in a system whose buffer and ion distribution are unchanged by the concentration technique. The membrane contributes no additional electrical resistance to the system and can be used repeatedly. The present paper shows results specifically for two specific proteins, and this system would work generally for any charged proteins or samples such as particles or cells, as long as they are larger than the ∼1-nm pore size of the membrane.

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(34) Messana, I.; Rossetti, D. V.; Cassiano, L.; Misiti, F.; Giardina, B.; Castagnola, M. Peptide Analysis by Capillayer (Zone) Electrophoresis. *J. Chromatogr. B* **1997**, *699*, 149−171.

(35) Adamson, N. J.; Reynolds, E. C. *J. Chromatogr., B* **1997**, *699*, 133−147.