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Robust Polymeric Microchannel Coatings for Microchip-Based Analysis of Neat PCR Products

Nicole J. Munro,[†] Andreas F. R. Hühmer,[†] and James P. Landers*[,‡,§]

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and Departments of Chemistry and Pathology, University of Virginia, Charlottesville, Virginia 22901

Several silica coatings have been evaluated for replicate PCR product analysis in capillaries and electrophoretic microchips. Silica coatings are an essential component to many electrophoretic separations, and this importance is magnified in microchips, where separation distances are minimized. Increasing the resistance of coatings to separation conditions improves the reproducibility and longevity of the coated microchip, which allows for the full potential of these devices (rapid separations, high throughput, and longevity) to be realized. In this study, several coating parameters have been evaluated experimentally and through the literature to produce a coating with high resistance to the separation conditions of interest, neat PCR product separations. Coating degradation induced under these conditions was tested for several coatings, and the influence of surface hydroxylation, surface hydration, silanization solvent, silanizing reagent, catalysis, endcapping, and polymerization procedure are discussed. Under the testing conditions, a coating (coating E) prepared by silanization with chlorodimethyloctylsilane in toluene with a polymer layer of poly(vinylpyrrolidone) attached by a hydrogen abstraction method [Srinivasan, K.; Pohl, C.; Avdalovic, N. ***Anal. Chem.*** **1997,** ***69,*** **2798−2805] was most resistant. This coating was tested for longevity on electrophoretic microchips and was compared to the traditional coating of polyacrylamide. The coatings produced similar resolution and efficiencies; however, coating E provided more reproducible migration times and had performed for 635 analyses when testing was terminated. This procedure provides a reproducible, resistant surface coating, thus allowing for replicate analysis of neat PCR product on microchips.**

Microfabricated devices hold great potential for clinical applications.[1,2] Although applications for enzyme assays[3], sequencing,[4,5] and thermocycling[6] have been demonstrated in the literature, perhaps the most exciting clinical application is in molecular diagnostics. This encompassing diagnostic tool probes genetic sequences, whether of human or infectious agent origin, for diagnostically relevant information. Three independent processes are fundamental components of molecular diagnostic assays: DNA purification, polymerase chain reaction (PCR), and DNA product separation. Attempts to integrate such processes into a single microfabricated device have been demonstrated (e.g., refs 6 and 7). Although the "lab-on-a-chip" or "micrototal analysis system" (μTAS) remains illusive, it is clear that the availability of such a device would revolutionize this area of clinical diagnostics.

Analysis of PCR-amplified products via microchip electrophoresis has typically required sample preparation prior to analysis to ensure separation efficiency and microchannel coating longevity. For the purposes of this report, coating longevity is defined as the ability of the coating to resist degradation when exposed to electrophoretic conditions (buffer components, ionic strength, pH). Coating stability is affected by alkali cations, which increase the solubility of silica,[8,9] the same Si−O−Si bond that typically attaches the coating to the silica surface. Buffer ions catalyze these hydrolysis reactions and deamidation by stabilizing the intermediate dipoles. A typical TBE separation buffer contains a minimal alkali cation concentration of ∼8 mM, whereas a typical PCR mixture contains ∼50 mM concentrations, increasing the dissolution rate from 5.1× to 8.8 × 10^{-12} mol/m^2s, a 72% increase (calculated using eq 16 in ref 8). Consequently, repeated PCR sample analysis would lead to accelerated coating degradation. It is for this reason that sample preparation, often involving desalting[10,11] or dilution,[12] has been used to reduce salt concentrations.

* Corresponding author: Phone: 804-243-8658. Fax: 804-243-8852. E-mail: jpl5e@virginia.edu.

[†] University of Pittsburgh.

[‡] Department of Chemistry, University of Virginia.

[§] Department of Pathology, University of Virginia.

(1) Jakeway, S. C.; de Mello, A. J.; Russell, E. L. *Fresenius J. Anal. Chem*. **2000**, *366* (6−7), 525−539.

(2) Dolnik, V.; Liu, S. R.; Jovanovich, S. *Electrophoresis* **2000**, *21* (1), 41−54.

(3) Hadd, A. G.; Raymond, D. E.; Halliwell, J. W.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem*. **1997**, *69* (17), 3407−3412.

(4) Salas-Solano, O.; Schmalzing, D.; Koutny, L.; Buonocore, S.; Adourian, A.; Matsudaira, P.; Ehrlich, D. *Anal. Chem*. **2000**, *72*, 3129−3137.

(5) Liu, S.; Shi, Y.; Ja, W. W.; Mathies, R. A. *Anal. Chem*. **1999**, *71*, 566−573.

(6) Khandurina, J.; McKnight, T. E.; Jacobson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M. *Anal. Chem*. **2000**, *72* (13), 2995−3000.

(7) Burns, M. A.; Johnson, B. N.; Brahmasandra, S. N.; Handique, K.; Webster, J. R.; Krishnan, M.; Sammarco, T. S.; Man, P. M.; Jones, D.; Heldsinger, D.; Mastrangelo, C. H.; Burke, D. T. *Science* **1998**, *282*, 484−487.

(8) Dove, P. M. *Am. J. Sci*. **1994**, *294*, 665−712.

(9) Dove, P. M.; Rinstidt, J. D. In *Silica: Physical Behavior, Geochemistry and Materials Applications*; Heaney, P. J., Prewitt, C. T., Gibbs, F. V., Eds.; Book Crafters, Inc.: Chelsea, MI 1994.

(10) Wooley, A. T.; Sensabaugh, G. F.; Mathies, R. A. *Anal. Chem*. **1997**, *69*, 2181−2186.

(11) Wooley, A. T.; Mathies, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11348−11352.

(12) Cheng, J.; Waters, L. C.; Fortina, P.; Hvichia, G.; Jacobson, S. C.; Ramsey, J. M. *Anal. Biochem*. **1998**, *257*, 101−106.

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In capillary and microchip electrophoresis, capillary/microchannel coatings have been traditionally based on polyacrylamide, as a result of its predominance in electrophoretic techniques; however, the limited lifetime of this coating and lack of reproducibility have generated research for a variety of alternative coatings.[13] Unfortunately, silica-coating literature remains contradictory as to optimal reagents, solvents, reaction times, etc.

Coating fused silica capillaries for electrophoresis typically involves two steps, silanization and polymerization. In the silanization step, most often an alkoxy or chloro organofunctional silane is reacted with the hydrated silica surface. A polymer layer is covalently bonded to this reagent, thus attaching it to the silica surface. The reproducibility and longevity problems known to plague these coatings arise from both steps. For the silanization procedure, reproducible performance is dependent on a number of effective parameters, including silica surface hydration, solvent hydration, and temperature. Polymerization reproducibility problems arise when polymerizing in situ, in which the typical parameters (O_2, temperature, flow rate, reaction time) become critical. Coating longevity is determined directly by the resistance of the coating to the conditions of analysis. Certain coatings tend to degrade more rapidly at higher pH because the Si−O−Si bond linking the polymer layer to the silica surface becomes increasingly susceptible to hydrolysis. As these bonds are hydrolyzed, the coating is anchored to fewer surface silanol groups. Various polymer layers reduce this attack to different degrees; however, the polymer itself may also possess hydrolyzable groups.

Several reports have described more stable coatings for electrophoresis. Gelfi et al.[14] described a propanol analogue of acrylamide that was found to exhibit greater stability and migration time reproducibility than polyacrylamide. This increased stability arises from the substituted amine in the hydrolyzable amide group in substituted acrylamide polymers, such as polydimethylacrylamide. With the aim of producing a stable, hydrophilic coating for protein separations, Srinivasan and co-workers[15] coated capillaries using a traditional silane reagent, but then they bound a preformed polymer using a hydrogen abstraction method. This polymer layer possessed characteristics that circumvented several of the difficulties experienced with capillary coatings. The reproducibility was improved over traditional methods, because the polymer was already formed and the affective polymerization conditions (O_2, temp, etc.) did not need to be as stringently controlled. This is important in light of our search for a coating for microchip-based DNA analysis, because the base hydrolysis of the amide bond (due to basic buffers) and susceptibility to increased salt concentration with PVP has been shown to be lower than that of polyacrylamide.[16,17]

In a previous report, we evaluated a number of capillary coatings for heteroduplex and DNA fragment analysis via capillary electrophoresis (CE),[18] identifying both the silanizing reagent and the polymer layer as important parameters for optimizing resolution. A combination of octyldimethylchlorosilane and PVP was found to be the optimal combination for attaining high resolution with those applications. In this manuscript, a stepwise evaluation and optimization was made of the parameters effecting these layers, with the ultimate goal being to generate a coating capable of robust performance in the presence of high-salt samples at relatively high pHs (pH 8.6), as in PCR product analyses. Conditions were initially tested on the capillary because of the automated capabilities and substrate availability. The optimized coating conditions were then translated to glass microchips for evaluation of longevity with respect to PCR sample analysis. Comparison to the commonly used microchip coating employing an in situ polymerized polyacrylamide[19] was found to be favorable.

MATERIALS AND METHODS

Materials. For the coating procedures, chlorodimethyloctylsilane (OCT), chlorotrimethylsilane (TMS) and molecular sieves 4A were obtained from Acros Chemicals (Fair Lawn, NJ). Octyltrichlorosilane (OTCS) and sodium hydrogen sulfate were purchased from Aldrich Chemicals Co., Milwaukee, WI, and α-methacryloxypropyltrimethoxysilane (MET) was obtained from Sigma Chemicals, St. Louis, MO. Toluene and diethylamine were obtained from J. T. Baker (Phillipsburg, NJ). *N*,*N*,*N*′,*N*′ tetramethylethylenediamine (TEMED) and ammonium persulfate were obtained from Biorad (Hercules, CA). Poly(vinylpyrrolidone) (PVP; M_w, 1 000 000) and linear polyacrylamide (LPA; M_w, 700 000−1 000 000) were purchased from Polysciences, Inc. (Warrington, PA). Polyimide-coated capillaries (50-μm i.d. × 360-μm o.d.) were purchased from Polymicro Technologies (Phoenix, Arizona).

For the EOF measurements, sodium borate was obtained from Acros Chemicals. Dimethyl sulfoxide (DMSO) was purchased from Aldrich Chemical Co. 1X TBE was prepared from Trizma base (89 mM, Sigma), boric acid (89 mM, Acros), and ethylenediamine tetraacetic acid (2 mM, EDTA, Sigma) at pH 8.6. The PCR mixture was prepared from magnesium chloride (Fisher), Triton X-100 (Sigma), potassium chloride (Mallinckrodt), dNTP solutions (Gibco BRL, Rockville, MD), and Taq polymerase (Perkin-Elmer, Branchburg, NJ). 1X PCR mixture concentrations followed the Promega protocol: 10 mM Tris−HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM of each dNTP, and 0.03 units/μL Taq polymerase. The PCR mixture was prepared in 1X TBE. A 1X TBE/PCR buffer was also prepared by mixing 2X TBE with 2X PCR mixture as described above but omitting the dNTPs and Taq polymerase.

DNA standard analysis was performed using pBR322 *Hae*III digest (Sigma). Hydroxyethylcellulose (HEC; M_w ∼250 000; Aldrich) and poly(vinylpyrrolidone) (M_w 360 000, Acros) were used to prepare the sieving matrixes. 1% HEC or 1.5% PVP, as indicated in the text, was added to heated 1X TBE during stirring as previously described.[20] A fluorescent intercalator (Yo-Pro, Molecular Probes, Eugene, OR) was added to the sieving matrix at a concentration of 1 μL dye/1 mL buffer for all applications except for DNA analysis on the coating E microchip, for which 0.5 μL dye/1 mL buffer was used.

(13) Dougherty, A. M.; Cooke, N.; Shieh, P. In *Handbook of Capillary Electrophoresis*, 2nd ed.; Landers, J. P., Ed.; CRC Press: New York, 1997.
(14) Gelfi, C.; Curcio, M.; Righetti, P. G.; Sebastiano, R.; Citterio, A.; Ahmadzadeh, H.; Dovichi, N. J. *Electrophoresis* **1998**, *19* (10), 1677−1682.
(15) Srinivasan, K.; Pohl, C.; Avdalovic, N. *Anal. Chem.* **1997**, *69*, 2798−2805.
(16) Gelfi, C.; de Besi, P.; Alloni, A.; Righetti, P. G. *J. Chromatogr.* **1992**, *608*, 333−341.
(17) Conix, A.; Smets, G. *J. Polym. Sci.* **1955**, *15*, 221−229.
(18) Tian, H.; Brody, L. C.; Mao, D.; Landers, J. P. Anal. Chem. **2000**, *72* (21), 5483−5492.
(19) Hjertén S. *J. Chromatogr.* **1985**, *14*, 191−198.
(20) Munro, N. J.; Snow, K.; Kant, J. A.; Landers, J. P. *Clin. Chem.* **1999**, *45* (11), 1906−1917.

Table 1. Performance of Capillary Coating Variations

coating	silanizing reagent	solvent[a]	silanizing reagent 2	solvent 2	polymer[b]	EOF (cm^2/Vs)	% RSD	% degradation
A	MET	H_2O/AA			acylamide	5.48×10^{-6}	40.16	89.17 ± 11.22
B	OCT	tol	TMS	tol	PVP_b	1.96×10^{-5}	14.84	9.04 ± 32.99
C	MET	H_2O/AA			PVP_b	1.54×10^{-5}	13.10	48.87 ± 12.08
D	OCT	tol	TMS	tol	LPA_b	6.91×10^{-5}	14.07	−11.76 ± 11.57
E	OCT	tol			PVP_b	1.85×10^{-5}	27.01	2.76 ± 12.69
F	OCT	EtOH			PVP	1.75×10^{-6}	17.15	49.74 ± 42.71

[a] Abbreviations: tol, toluene; EtOH, ethanol; AA, acetic acid. [b] Polymers with b subscript were prepared in buffered solution.

PCR products for the replicate analyses on the microchip were clinical samples testing for the hepatitis C virus (HCV). DNA extracted from cerebrospinal fluid was used for the amplification, as previously described.[21]

Silica-Coating Procedures. Capillary pretreatment involved rinsing with 5:1:1 water:ammonium hydroxide:hydrogen peroxide for 5 min. The capillary was then placed in a boiling bath of the solution for 10 min (RCA Clean 1, developed by RCA Company). Capillaries were then rinsed with water for 1 h, ensuring that the elutant was neutral. The capillaries were then blown dry with N_2 and stored in a ∼50% (49−56% humidity) humidity environment produced by a saturated solution of sodium hydrogen sulfate in a desiccator[22] (with no desiccant) until use (> 24 h). The coatings for the capillaries and microchips are summarized in Table 1. The various coatings for testing were prepared in at least two batches.

Coating A was produced as previously described.[19] The silanization step was achieved by rinsing the capillary with a solution of 0.4% (v/v) MET in water brought to a pH of 3.7 with acetic acid. This was allowed to react at room temperature for 1 h. The capillary was then rinsed thoroughly with water. For the polymerization step, the capillary was filled with a 3.5% (w/v) acrylamide solution that had been deaereated and then added to 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulfate. The reaction was allowed to proceed for 30 min, after which the capillary was rinsed for 15 min with water.

Coating B silanization involved rinsing the capillary with a solution of 4% (v/v) OCT in dry toluene (stored over molecular sieves). The reaction was allowed to sit for 2 h. When coating the microchip, the access holes were sealed using Teflon seals (Fisher) and binder clips to prevent evaporation. The capillary was rinsed thoroughly with toluene then was filled with a solution of 3% (v/v) TMS in dry toluene. After a 2 h reaction time, the capillary was rinsed with toluene, then acetone, and blown dry. For the polymerization step,[15] the capillary was filled with a deaereated 4% (m/v) PVP solution in 50 mM phosphate buffer at pH 8 to which 0.1% (v/v) TEMED and 0.1% (m/v) ammonium persulfate were added immediately prior to filling. The capillary/channel was sealed with Teflon seals and allowed to react at 80 °C for 18 h.

Coating C was produced using the silanization procedure from coating A with the polymerization procedure from coating B. Coating D utilized the silanization procedure from coating A and followed the polymerization procedure for coating B, with the exception of using 2% LPA instead of 4% PVP. Coating E utilized the OCT silanization procedure as for coating B. The polymerization step was as in coating B.

Coating F followed the procedure described by Srinivasan and co-workers[15] in which silanization was achieved using 2% (v/v) OCT in ethanol (95%) with a 24 h reaction time. The capillary was thoroughly rinsed with water and then polymerized as described for coating B.

Capillaries testing silanizing reagents without a polymer layer were rinsed with the silanizing solution and allowed to sit for 2 h. The capillaries were rinsed with toluene, then acetone, and then blown dry. The silanizing solution for OCT-coated capillaries was 4% (v/v) OCT in dry toluene, as described for coating B. For OTCS, a 4.15% (v/v) OTCS solution in dry toluene was used, and the capillary was cured (4 h at 150 °C) after drying with N_2. The amine-catalyzed silanization utilized a 1.6% (v/v) OCT solution in dry toluene with 0.1% (v/v) diethylamine.[23]

All coated capillaries were blown dry with N_2 for storage. Control EOF measurements for coatings A through F were collected in triplicate on a bare silica capillary pretreated as described above for the capillaries to be coated. The control capillaries for the capillaries testing silanizing reagents were also pretreated; however, they underwent the same treatment as the capillaries being coated (toluene, acetone, drying, and curing), omitting the silanizing reagents and diethylamine.

Capillary Electrophoresis. A Beckman P/ACE System 2210 (Beckman Instruments, Fullerton, CA) was used for CE analysis. Absorbance detection at 200 nm was employed for EOF measurements. For DNA standard analyses, a LIF detector was used that excites at 488 nm and collects the scattered fluorescence at 520 ± 10 nm. Instrument control and data collection were performed using an IBM 486 computer utilizing System Gold software (version 8.1).

CE separations were performed using 24 cm × 50 μm capillaries (effective length, 17 cm). Freshly coated capillaries for PCR buffer endurance tests were initially rinsed with water for 10 min. EOF measurements were performed by prerinsing with nonelectrophoresed separation buffer (50 mM borate buffer at pH 8.6) for 5 min, then a 2-s pressure injection (0.5 psi) of neutral marker (DMSO) followed by a 2-s pressure injection of separation buffer. Separation followed at 500 V/cm using normal polarity (inlet anode). Capillary temperature was maintained at 20 °C.

Degradation analysis was initiated by measuring the EOF in triplicate following the procedure outlined above. The capillary

(21) Hofgartner, W. T.; Hühmer, A. F. R.; Landers, J. P.; Kant, J. A. *Clin. Chem.* **1999**, *45* (12), 2120−2128.
(22) Weast, R. C., Ed. *The Handbook of Chemistry and Physics*; The Chemical Rubber Company: Cleveland, 1973; pp E−46.
(23) Blitz, J. P.; Shreedhara Murthy, R. S.; Leyden, D. E. *J. Colloid Interface Sci.* **1988**, *126*, 381−392.

was then rinsed with the PCR mixture in 1X TBE for 5 min and then electrophoresed at 350 V/cm for 10 min using the 1X TBE/PCR buffer in the inlet and outlet vials. The EOF was measured again. These steps were repeated, with the electrophoresis time for the PCR mixture being increased from 10 min to 30, 60, then 120 min in each successive iteration.

DNA standard analysis was achieved using 1% HEC in 1X TBE. A 5.38 mg/mL pBR322 *Hae*II digest in 10 mM Tris (pH 8.6) was used. Capillaries were preconditioned by rinsing them with water and then the sieving matrix for 10 min each. Injection involved a 2-s injection of water at 3 kV (125 V/cm), a 5-s injection of DNA standard at 6 kV (250 V/cm), and a 2-s injection of water at 3 kV. Separation was performed with reversed polarity (outlet anode) at 4.8 kV (200 V/cm). Separations were performed in triplicate with rinses of water (3 min) and sieving matrix (5 min) between analyses.

DNA Analysis via Microchip Electrophoresis. Instrumentation used for microchip voltage control and fluorescence detection has been described previously.[20] Electrophoretic microchips were fabricated by the Alberta Microelectronics Center (μchips for Research Applications, Edmonton, AB, Canada) and employed a simple cross-channel layout, as described previously.[21]

Microchip sample injection was performed by applying a 400 V (333 V/cm) potential across the sample and sample waste reservoirs, with the sample at ground for 45 s (DNA standard) or 30 s (PCR product). Subsequent separation involved placing the sample and sample waste to ground, and applying −300 V to the inlet and 2800 V to the outlet (375 V/cm). An effective separation length of 5.5 cm was used, and fluorescence was collected at 18 Hz. Grams/386 was used to analyze the electropherograms.

RESULTS AND DISCUSSION

Design and Durability Testing of Different Channel Coatings. There are many variables to consider when optimizing a silica channel coating for electrophoretic purposes, including the silanizing conditions, polymer, and polymerization procedure. The literature describes the optimization of many of these parameters. The covalent attachment of silanizing reagents to silica surfaces has been studied extensively with respect to its application for self-assembled monolayers (SAM). Although SAM formation is not necessary for capillary coatings, the literature on this topic can be used to optimize conditions conducive to silanization of as many silica surface silanol groups as possible. SAMs on silica are formed by utilizing solutions with a dry, organic solvent on a clean, hydrated surface or by vapor deposition (not relevant to our application).[24] The reaction is thought to occur through three steps:[24] hydrolysis of the silanizing reagent by the water layer weakly adsorbed on the silica surface or by water in the bulk solution; hydrogen bonding to the silanol groups on the silica surface; and finally, covalent bonding to the surface silanols. There are a number of parameters that affect the efficiency of these reactions. These include silica surface hydroxylation, surface hydration, silanizing reagent (leaving group, alkyl chain length), solvent, reaction time, and temperature. Information available in the literature provides a good starting point for many of these parameters.

(24) Vansant, E. F.; Van Der Voort, P.; Vrancken, K. C. *Characterization and Chemical Modification of the Silica Surface*; Elsevier: New York, 1995; Chapters 3, 4.

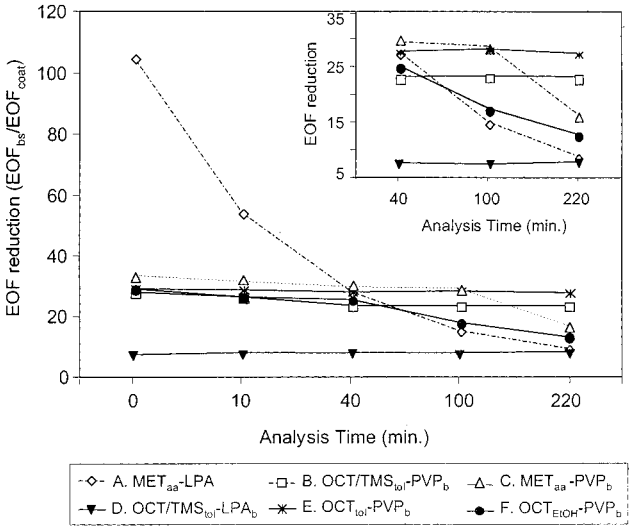

Figure 1. Resistance testing of coatings. Capillaries were coated with coatings A−F as indicated in the text and Table 1. Coating testing involved measuring the EOF and then exposing the capillary to a 1X TBE/PCR mixture under electrophoretic conditions for the analysis time indicated. The EOF was measured between each analysis time and used to calculate the EOF reduction (measured EOF/EOF of a bare silica capillary). Three capillaries of each coating were tested from at least two different coating batches. For capillary 1, the EOF was measured once per analysis time, and for capillaries 2 and 3, the EOF was measured three times. The pooled standard deviation for the EOF measuring procedure used was calculated as 3.65×10^{-6}, a relative standard deviation of 9.49%. The averages of the three capillaries for each coating and exposure time are shown; however, the error bars have been eliminated for clarity.

In the first part of this study, a number of coating parameters were altered in the coating procedure, and the coated capillaries were tested for their resistance to repeated 1X TBE/PCR buffer exposure. The ultimate goal was to produce a more robust surface for the repeated analysis of neat PCR products on microchips. Table 1 details the various silanizing reagent/polymer combinations that were evaluated for covalently modifying the capillary surface. It is clear from the reduction in EOF, calculated as the ratio of the bare silica capillary EOF to the coated capillary EOF (uncoated EOF/coated EOF), that both parameters affect the EOF reduction, as is well-referenced in the literature.[13]

Following the initial EOF measurements, the resilience of the coating to conditions encountered with DNA analysis was tested by exposing the capillary to a 1X TBE/PCR mixture for extended periods of time. To mimic electrophoretic conditions as best as possible,[14] a potential was applied during exposure to the 1X TBE/PCR mixture, and analysis capillary temperature and electric field strength were matched to real analysis conditions. The measure of resistance to the conditions is maintained EOF reduction. Each coating was tested on three capillaries that were coated in at least two different batches. Comparisons of the effect of the 1X TBE/PCR mixture on EOF over time with each of the coatings is shown in Figure 1, where data obtained with each of the coatings is displayed. The trends in these data indicate that different combinations of silanizing and polymerization conditions provide coatings with varying resistance to the electrophoretic conditions. For example, it can be seen that coating A appeared to be the least resistant to degradation, and coating E appeared to have the greatest stability. The overall trend for the coatings that were

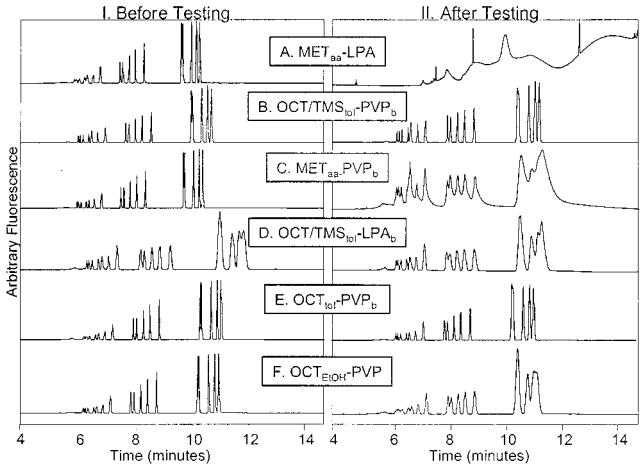

Figure 2. DNA standard separations employing freshly coated capillaries and those exposed to electrophoretic conditions (see Figure 1). DNA standard electrpherograms obtained in capillaries coated with coatings A−F, (I) before and (II) after testing. Injection: 2-s injection of water at 3 kV (125 V/cm), 5-s injection of DNA standard at 6 kV (250 V/cm), and 2-s injection of water at 3 kV. Separation was performed with reversed polarity (outlet anode) at 4.8 V (200 V/cm). Sample: 5.38 μg/mL pBR322 *Hae*III digest in 10 mM Tris, pH 8.6. Buffer: 1% HEC in 1X TBE buffer, pH 8.6.

tested was a decrease in EOF reduction over time (except for coating D, as discussed below).

Although the EOF increase over the time course for testing (i.e., the inability to maintain reduced EOF) is a significant marker for coating degradation, the initial reduction in EOF does not necessarily reflect the resolution attainable with the capillary.[18] This is exemplified in Figure 2, which displays a separation of the fragments in a DNA standard (pBR322 *Hae*III digest) as an indicator of the resilience of each coating to extensive exposure to the 1X TBE/PCR mixture under electrophoretic conditions. For example, the initial reduction in EOF with coating A is significantly lower than coating B (Table 1); however, the electrophoretic profiles obtained with the DNA standard before testing show similar resolution (Figure 2IA and 2IB, respectively). Comparing the resolution after testing (Figure 2IIB and 2IIA, respectively), clearly shows that the loss of reduced EOF (i.e., an increase in EOF) during exposure of coating A to the 1X TBE/PCR mixture (Figure 1) appears to correlate with a significant loss of resolution. This and other significant aspects of the information conveyed through Figures 1 and 2 are best discussed in the context of the multiple coating parameters known to be critical to the effective covalent modification of silica surfaces. These parameters include silica surface hydroxylation, surface hydration, solvent, silanizing reagent, catalyst, endcapping, and polymerization. Although there is an abundance of literature available from many disciplines (SAM formation, surface science, HPLC, etc.) discussing the parameters important for silanization of silica surfaces, the logic underlying the choice of conditions for capillary coatings in CE is often not discussed. Consequently, it is appropriate to discuss the data in Figures 1 and 2 in light of the relevant literature addressing the various capillary-coating parameters. Within this framework, it will become clear why the specific conditions tested and those ultimately employed were chosen to generate a coating procedure that is resilient for performing replicate PCR product analysis on microchips.

Surface Hydroxylation. An important, yet often ignored, factor in producing capillary coatings is pretreatment of the silica surface.[24] Silica surface chemistry has been studied extensively; however, due to its complexity, it remains a poorly understood area. Although the differences in surface chemistry between capillary (fused silica) and glass microchips (borofloat glass) must be explored for a complete understanding of the separation system, it is beyond the focus of this manuscript. Therefore, only the literature pertinent to silica surface preparation for silanization is discussed. Readers are directed to refs 9, 24, and 25 for more extensive information on silica surface chemistry.

Silica surfaces are prepared for silanization by increasing the number of reactive groups (silanols) on the surface, in addition to cleaning the surface. A number of preparation methods for silica have been reported and include 10 min in 3:7 H_2O:sulfuric acid at 150 °C,[26] 30 min exposure to 5% NaOH followed by chromic acid (50 °C),[27] a 12-[28] or 160-h[29] immersion in water, or 20 min of boiling in a mixture of water:ammonium hydroxide:hydrogen peroxide (5:1:1) followed by 24 h in water.[30] It is difficult to judge the efficiency of these methods without knowing the exact history of the substrate, especially when the hydroxylation is not verified experimentally.

One published study compared eight of the common methods used for cleaning and preparing silica for silanization.[31] Preparation efficiency was tested by measuring the hydrophilicity of the surface, which would not only provide a measure of cleanliness, but also indicate the hydroxylation status, because siloxane bonds are hydrophobic.[25] Successful surface preparation was obtained by either a 30-min rinse with 1:1 MeOH:HCl or a 5-min exposure to a mixture of boiling water:ammonium hydroxide:hydrogen peroxide (5:1:1, RCA Standard Clean 1). Insufficiently prepared surfaces were created by 10% KOH in 2-propanol at 80 °C, piranha wash (1:2, H_2O_2:H_2SO_4), and 1 M NaOH followed by piranha wash. Ironically, the least effective treatment was found to be a 20-min rinse with 1 M NaOH, the method of choice (with 0.1−1.0 M NaOH) for regenerating capillary surfaces in CE. The authors attribute this to NaOH's being an aggressive etchant, which eventually generates surface roughness; however, other authors report that 12 M NaOH does not increase surface roughness for glass but does cause a substantial increase in the surface roughness of quartz.[32] In addition, 37% HCl was also found to increase surface roughness for both glass and quartz substrates. The RCA clean method for 10 min apparently does not increase surface roughness.[33]

On the basis of this information, it is clear that although a variety of methods can be used, many do not provide optimal surface preparation for silanization. Unfortunately, due to the

(25) Iler, R. K. *The Chemisty of Silica*; John Wiley and Sons: New York, 1979; Chapter 6.
(26) Brzoska, J. B.; Azouz, I. B.; Rondelez, R. *Langmuir* **1994**, *10*, 4367−4373.
(27) McGovern, M. E.; Kallury, K. M. R.; Thompson, M. *Langmuir* **1994**, *10*, 3607−3614.
(28) Angst D. L.; Simmons G. W. *Langmuir* **1991**, *7*, 2236−2242.
(29) Duchet, J.; Chabert, B.; Chapel, J. P.; Gerard, J. F.; Chovelon J. M. Faggrezic-Renault, N. *Langmuir* **1997**, *13*, 2271−2278.
(30) Opila, R. L.; Legrange, J. D.; Markham, J. L.; Heyer, G.; Schroeder, C. M. *J. Adhesion Sci. Technol.* **1997**, *11(1)*, 1−10.
(31) Cras, J. J.; Rowe-Taitt, C. A.; Nivens, D. A.; Ligler, F. S. *Biosens. Bioelectron.* **1999**, *14*, 683−688.
(32) Eske, L. D.; Galipeau, D. W. *Colloids Surf. A* **1999**, *154*, 33−51.
(33) Couillard, J. G.; Ast, D. G.; Umbach, C.; Blakely, J. M.; Moore, C. B.; Fehlner, F. P. *J. Non-Cryst. Solids* **1997**, *222*, 429−434.

varying compositions of glasses and the resulting differences in chemical reactivity, it is difficult to draw on the literature references for any effects the cleaning reagent may also have on the silica surface. Consequently, treatment with a mixture of boiling water:ammonium hydroxide:hydrogen peroxide (5:1:1) for 10 min was used to hydroxylate the silica surface for the capillaries and microchips coated in the current work, because it has been shown to adequately prepare silica surfaces for silanization.

One procedure not employed in this work that has been reported to provide more reproducible silica surfaces for capillary electrophoresis is leaching with dilute acid,[43] which removes metal impurities from the surface of the silica. In capillary experiments, reproducible EOF measurements were achieved with different capillaries only after treatment with 0.1 M HCl for 2 h at 70 °C.[43] Although not performed in this work, it is advisable for the most reproducible coating to follow the leaching procedures outlined above.

Surface Hydration. There is much discrepancy in the literature with respect to the amount of water needed at the silica surface for monolayer formation. It is generally agreed that some amount of water is needed for the silanization reaction, whether it is in the solvent or on the silica surface. The water reacts with the silanizing reagent to form a silanol derivative that will ultimately react with the surface silanol groups;[24] however, it is thought that an excessively thick layer of water on the silica surface causes the silane to condense above the water layer.[34] This is obviously detrimental, because bonding to the silica surface would not occur, and the unstable layer is easily removed.

To control the amount of water in the system, substrates are often hydrated by exposure to an environment of specific humidity[23,35] or by spiking otherwise dry organic solvents with a specified amount of water.[27,28,34,35] With the latter, a suitable water content for certain organic solvents has been estimated to be, for example, 0.15 mg of H_2O for 100 mL toluene,[27] 6×10^{-3} M H_2O in CCl_4,[34] or 2 drops of H_2O in CCl_4/Isopar G.[28,35] The alternative method ensures reproducible hydration through exposure of the silica to a controlled humidity environment; 50% humidity has provided efficient coupling, as reported by several groups.[23,36] A systematic study by Fairbank and Wirth[36] showed that equilibration in an atmosphere with 50% humidity for several hours (13 H_2O/nm^2) provided the correct hydration of the silica surface for the reaction of trichlorosilanes. They determined that 50% humidity provided the stoichiometric amount of water needed for reaction with the trichloro reagents. If this were comparable to the reaction with a monochloro reagent, the humidity would need to be lowered to approximately 30% (4.2 H_2O/nm^2) according to their data. However, use of the monochloro reagents eliminates the problem that is observed at higher humidities, where trichloro silanizing reagents have a proclivity for vertical polymerization.

The technique involving equilibration in an environment of specific humidity is simpler and more easily reproduced when used in conjunction with a dry solvent, as opposed to hydrating solvents. Because water contents of 50% were not large enough to prohibit silane attachment,[36] a humidity of 50% was used in this study to ensure optimized reaction.

Solvent. Several organic solvents have been used for the silanization process, in addition to aqueous systems. Only organic systems have been shown to produce monolayer coverage (the most efficient coverage) with silanizing reagents.[24] In aqueous systems, silanizing reagents hydrolyze before becoming miscible; however, they then begin to polymerize at a rate dependent on the nature of the organofunctional group. Under commonly employed conditions, acetic acid is used as a catalyst (pH 3) in an aqueous solvent. The degree of silanization achievable under these conditions is reduced because of low surface adsorption rates of the silane in aqueous solvents[37] and the relative stability of the silane reagents at this pH.[38] For organic solvents, it is hypothesized that the optimal solvents are more efficient at drawing off the extended water layer from the silica surface (the layers not hydrogen-bonded directly to the surface).[34] It has been proposed that this water hydrolyzes the silane in the bulk solution. McGovern et al.[27] found benzene (followed by toluene) to be the optimal solvent, because of its more efficient extraction of the water from the silica surface, as compared to the other solvents that were tested. Conversely, it has been reported that carbon tetrachloride[34] promoted better coverage, because it is less polar and does not hydrogen bond to the silica surface and restrict silanol sites.[29] Both toluene[27,29,39] and carbon tetrachloride[27,29] appear to be suitable solvents for the silanization procedure. Toluene was chosen for the experiments carried out in the current study because of its higher boiling point (110.6 °C vs 76.7 °C) and more environmentally friendly nature.

Reaction times and temperatures must also be considered for silanization reactions. These parameters were not explored in the current study; the available literature was used to obtain values that have been used with success. The reaction times needed for complete silanization, as reported in the literature, have been highly variable (100 min,[39] 15 min,[29] 1 hr,[27,31] 30 min[28,35,30], 18 hr[36]); however, reaction times of < 2 h have frequently been used and determined to be sufficient for the silanization reaction employed here. It has been shown that temperature does affect the coverage observed;[26] therefore, silanization was performed at room temperature, which was ensured to be between 22 and 25 °C for consistent coverage.

As discussed above, aqueous systems are commonly used for silanization, even though organic systems can provide a more consistent, comprehensive coverage.[24] The effect of silanization using toluene versus ethanol (95%) as the solvent can be seen in Figure 1, where capillaries were coated with OCT in dry toluene (Figure 1E) and OCT in ethanol (Figure 1F).[15] A PVP layer was covalently linked to the silanized surface, and the coating was tested for robust performance by analysis of a PCR mixture. The choice of silanization procedure resulted in a large effect on the coating stability, as evidenced by the coating produced by the toluene system's being much more resistant to the analysis

(34) Silberzan, P.; Leger, L.; Ausserre, D.; Benattar, J. J. *Langmuir* **1991**, *7*, 1647−1651.
(35) Le Grange, J. D.; Markham C. R.; Kurkjian, J. L. *Langmuir* **1993**, *9*, 1749−1753.
(36) Fairbank, R. W. P.; Wirth, M. J. *J. Chromatogr. A* **1999**, *830*, 285−291.
(37) Engelhardt, H.; Cuñat-Walter, M. A. *J. Chromatogr. A* **1995,** *716*, 27−33.
(38) Plueddemann E. P. *Silane Coupling Agents*, 2nd ed; Plenum Press: New York, 1991; Chapter 3.
(39) Banga, R.; Yarwood, J. *Langmuir* **1995**, *11*, 4393−4399.
(40) Pawlenko, S. *Organosilicon Chemistry*; Walter de Gruyter: New York, 1986; Chapter 1.
(41) Tripp, C. P.; Hair, M. L. *Langmuir* **1995**, *11*, 149−155.
(42) Angst, D. L.; Simmons, G. W. *Langmuir* **1991**, *7*, 2236−242.
(43) Cifuentes, A.; Canalejas, P.; Ortega, A.; Diez-Masa, J. C. *J. Chromatogr. A* **1998**, *823*, 561−571.

conditions. These data are summarized in Table 1 and Figure 1G, where the percent degradation in EOF during the testing of coating E ($OCT_{tol}-PVP_b$) was only 2.76%, as compared to 49.74% for coating F ($OCT_{EtOH}-PVP$). This was verified by comparing the electrophoresis of DNA standards in a freshly coated capillary with that of a coated capillary exposed to PCR buffer. The new capillaries for coating E (Figure 2IE) and coating F (Figure 2IF) provided similar resolution for the DNA standard; however, there was a significant loss of separation efficiency after extensive exposure to the separation conditions (Figure 2IIE,F).

The toluene system was also compared to the traditional method, which utilizes MET silanization in an aqueous solvent with an acetic acid catalyst[19] (Figure 1C). This coating was also produced by Srinivasan and co-workers[15] for their protein analysis. When compared to the toluene system employed here (Figure 1E), the toluene system appears to provide a much more stable coating, as reflected by the DNA standard profile displayed in Figure 2. Coatings C ($MET_{H_2O}-PVP_b$) and F ($OCT_{EtOH}-PVP$) were tested by Srinivasan et al.[15] and were shown to function equally well for their protein separations. For our analysis, these coatings also performed in a similar manner, with an overall degradation of 48.87% for coating C and 49.74% for coating F (Table 1). Figure 2 shows the significant loss of resolution with the DNA standard after PCR mixture-testing with both coatings (Figure 2IIC,F).

The coating stability differences observed here with different silanizing reagents under different silanizing conditions contradicts the findings of Engelhardt and Cuñat-Walter,[37] who found varying degrees of EOF reduction for different silanizing reagents, but who also reported finding no difference between the reagents with respect to coating stability. This study, however, was performed at pH 10, where hydrolysis of the polyacrylamide amide bond is more significant and could dominate the findings.

In the current work, optimal coating coverage was achieved using an organic solvent, toluene. Toluene seems to increase the surface coverage of silanizing reagents or, at least, seems to promote more complete surface coverage and avoid premature inactivation of reactive silane functional groups through condensation. Therefore, subsequent hydrolysis of these reactive groups is substantially reduced, and more consistent PVP coverage is promoted.

Silanizing Reagent. There are several important parameters for silanizing reagent optimization: leaving group, organofunctional group, and degree of substitution. Chloro and alkoxy groups are commonly used as the reactive moiety on silanizing reagents. The reactive group is hydrolyzed in the presence of water, where the chloro groups are generally more reactive;[40] the choices for the organofunctional groups are more diverse. Through investigation of several silanizing reagents used for producing capillary coatings, Tian et al.[18] determined that OCT provided the highest resolution for DNA fragment separations; therefore, a chlorinated octyl silane was employed in the current work.

Another important factor when considering silanizing reagents is the number of functional groups on the silicon. There is, again, a discrepancy in the literature regarding which reagent produces the most effective coverage.[41,42] Trifunctional reagents are used most commonly and can cross-link, thus forming a "net" on the silica surface. It has been suggested in several reports that this net is bonded to the silica surface only at points (approximately 1 in 5 attached groups);[34,41,35] however, vertical and horizontal polymerization can occur with these trifunctional reagents, which can lead to an inconsistent and irreproducible surface. This process is even more pronounced in an aqueous solvent.[24] It is also necessary to cure the trichloro version to cross-link the silanol reagents and increase silanol linkages to the silica surface.[41,42] Acording to Hair and Tripp,[41] the reaction kinetics for actually forming a linkage to the silica surface favor the monofunctional reagents, because of the longer bond length for the Si−OH derivative.

As a result of these discrepancies, the monochloro (OCT) and trichloro octyl (OTCS) reagents were tested for coating efficiency. The silanized capillaries were analyzed without the polymer layer, and additionally, the OTCS capillaries were cured at 150 °C for 4 h, as was reported to be necessary in the literature.[41,42] The monochloro reagent showed an average ($N = 3$) reduction in EOF of 1.37 ± 0.29, but the ratio for the trichloro reagent was 0.90 ± 0.23 (data not shown). The reduction in EOF was on the same order as those determined by Cifuentes and co-workers[43] for silanated capillaries (when comparing their calculated θ values to those for this system). The actual increase in EOF experienced in the OTCS-coated capillaries may arise because only one silanol group on the trisilanol derivative of OTCS has reacted with the silica surface. If the other two groups are not cross-linked during curing due to the proximity of other reagents, the free silanols will regenerate the EOF. Considering this possibility, we found it was difficult to determine which reagent provided the higher surface coverage; however, it was readily determined that the monochloro reagent more efficiently reduced the number of silanol groups on the surface. In light of these data, the monochloro reagent was employed, as in some of the work by Srinivansan et al.,[15] who used both monofunctional (OCT) and trifunctional (MET) reagents.

Catalytic Effect on Silanization Efficiency. It has also been suggested that the most efficient method for coupling silane reagents to silica surfaces to form SAMs is through an amine catalyst;[24,23,44] however, it is very difficult to control this reaction because of rapid oligomerization of the silane in solution.[24] For our purposes, a complete monolayer is not necessary for the capillary coating. Consequently, the catalyzed method was tested to see if it induced an increased coupling of the silane to the surface in comparison with the noncatalyzed approach. Many amines have been used as catalyst for reaction of silanizing reagents with silica surfaces. Blitz and co-workers[23] determined ethylamine to be the most efficient in toluene, but due to convenience, diethylamine was used here, which they found to provide similar efficiency. This catalyzed method provided a reduction in EOF of 1.47 ± 0.02, as compared to 1.42 ± 0.01 without the catalyst, for the same capillary batch (data not shown). The catalyst provided a slightly larger surface coverage, as evidenced by the lower EOF; however, because of the reportedly lower control over the reaction,[24] the catalytic effect was not considered significant enough to employ.

Endcapping Effect on Coating Longevity. A common step in the preparation of silica coatings for HPLC stationary phases is endcapping. Backfilling has been shown to increase surface

(44) Kinkel, J. N.; Unger, K. K. *J. Chromatogr.* **1984**, *316*, 193−200.

coverage when using silanizing reagents with bulky organofunctional groups as the main silanizing reagent.[45] A trimethyl version of the silanizing reagent is commonly used in attempts to react remaining, accessible silanol groups. We hypothesized that the surface of the microchannel would be rougher than the capillary surface because of the fabrication procedures. If the surface was rougher, an endcapping step would, in theory, aid in reacting with silanol groups located in more sterically hindered positions.

To test the effect of endcapping on the coating, capillary B was coated with OCT and then TMS in toluene. A comparison of B and E of Figure 1, illustrates the effect of the additional endcapping layer which, surprisingly, appears to lead to a slightly higher degradation (Table 1). Electrophoresis of the DNA standard before and after testing with each coating (coating B, Figure 2IB and 2IIB; coating E, Figure 2IE and 2IIE) showed that similar profiles resulted; however, the overall degradation was ~6% higher for coating B (Table 1). Standard deviations associated with the percent degradation values were relatively high (Table 1), which is not surprising because of the initial EOF standard deviations and the inherently variable nature of the coating process (discussed further below). If one considers these standard deviations, coating B and E performed very similarly.

Polymer Layer Effects on Coating Stability. The bulk of the silanol masking in capillaries stems from the polymer layer that is attached to the silane reagent. This is evidenced by the reduction in EOF calculated for the silanized versus the silanized/polymerized capillaries (1−1.4 vs 20−100). As described by Hjertén,[19] the additional EOF reduction is obtained by increasing the viscosity in the capillary surface double layer. The polymer layer also acts to protect the bond between the silica surface and the silanizing reagent, which is known to be hydrolyzable. The requirements for the polymer layer for the purposes of the current study were 3-fold. The layer should be reproducible with a minimum number of parameters to control. Additionally, the layer formed should be consistent and as smooth as possible. These characteristics have been shown to improve resolution over a rougher, inconstant version of the same type of coating.[46] Finally, the polymer layer should be inert to the separation conditions, that is, degradation and loss of performance over time should be minimized.

For CE coatings, the polymerization step is almost always performed in a nonbuffered solution.[47] Barberi and co-workers[47] found this had an adverse effect on the polymer formation because of the high pH (pH 10−11) induced by TEMED addition. At this alkaline pH, the silanol reagent linkage to the silica surface is more hydrolyzable, and linkages are lost as polymerization proceeds. Linear polyacrylamide is commonly used as the polymer for capillary coatings, because of its hydrophilic nature (when compared to alternative polymers) and its ease of formation. However, at the pH existing with unbuffered TEMED containing polymerizing solutions, the polyacrylamide will hydrolyze to form polyacrylate.[47] Because of the negative charge on this polymer, the chain will elongate from its neutral collapsed conformation, leaving the polymer and the silanol linkages more open to hydrolyzing reagents. The authors found that by buffering the solution at pH 7.0, a complete, homogeneous coating could be formed; therefore, a buffered polymerization solution was employed for coatings B−E.

As discussed earlier, the polymer layer can be either polymerized in situ or attached as a preformed polymer. Srivansan et al.[15] used a hydrogen abstraction method to covalently bond the polymer to the silane linker while simultaneously cross-linking the polymer. This coating provided greater stability because of the highly cross-linked nature of the polymer, which makes the silane linkage less susceptible to hydrolytic attack. In this study, poly(ethylene oxide), LPA, and PVP were used as the coating polymers for comparison. Theoretically, a PVP layer should be more resistant to hydrolysis than LPA,[16,17] because it has been reported to be less susceptible as a result of the tertiary nitrogen, as compared to the primary nitrogen in LPA. This increased electron density on the nitrogen reduces the dipolar character of the amide group, making the carbonyl carbon more stable against hydrolytic attack. Using the hydrogen abstraction method with PVP will form a highly cross-linked polymer layer that should reduce attack of the siloxane linkages, while being more resistant itself to hydrolytic attack.[15] In addition, the preformed polymer increases reproducibility by eliminating the affective polymerization parameters for in situ polymer formation.

The effect of the polymer for capillary coating was tested by reacting PVP and LPA on OCT silanated capillaries. Figure 1D shows the results of testing of the LPA-coated capillary (coating D). This coating displayed a relatively low reduction in EOF, indicating low polymer coverage or inconsistent coverage. This is reflected in the analysis of the DNA standard, where low resolution was obtained for both the new (Figure 2ID) and PCR mixture tested (Figure 2IID) capillaries. The similar resolution is surprising because, under these conditions, the LPA would be expected to degrade. However, due to the inefficient polymer coverage, it is difficult to draw conclusions regarding stability to PCR mixture exposure. Srivansan et al.[15] obtained similar efficiencies for capillaries coated with both PVP and LPA; however, for the LPA, only MET was used as the silanizing reagent. The higher reactivity of this reagent may be necessary for efficient coupling with the LPA during the radical reaction.

The results of polymerizing the LPA in situ versus reacting preformed PVP on MET-coated capillaries can be seen by comparing A and C of Figure 1. The noncross-linked LPA layer formed in situ provides the largest reduction in EOF of all of the coating versions tested. This coating appears to provide the highest viscosity in the region of the double layer.[19] It has also been reported that the long polyacrylamide chains can help cover neighboring uncoated regions, which would also aid in eliminating residual EOF.[47] However, these chains leave the Si−O−Si bond open to hydrolytic attack and are hydrolyzable themselves, as evidenced by the rapid increase in EOF. Coating C degraded less rapidly (as seen in Figure 1) and in the DNA standard profiles in Figure 2IIA,C for coatings A and C, respectively.

Coating Reproducibility. Reproducibility can be a problem with capillary coatings because of any one or a combination of the parameters discussed above. Table 1 shows the relative standard deviations for the initial EOF of the freshly coated capillaries tested here. Coating A exhibited the highest degree

(45) Mathauer, K.; Frank, C. W. *Langmuir* **1993**, *9*, 3446−3451.
(46) Cifuentes, A.; Diez-Masa, J. C.; Fritz, J.; Anselmetti, D.; Bruno, A. E. *Anal. Chem.* **1998**, *70*, 3458−3462.
(47) Barberi, R.; Bonvent, J. J.; Bartolino, R.; Roeraade, J.; Capelli, L.; Righetti, P. G. *J. Chromatogr. B.* **1996**, *683*, 3−13.

of variation. Notably, this was the only coating polymerized in situ, a procedure requiring stringent control of all the parameters. Even with careful control of solution degassing, reagent concentration, and reaction time, the coating was still variable.

The coating showing the least degradation during testing was coating E; however, this coating showed a percent RSD in initial EOF of 27.01%, ~2 times that of coatings B, C, and D. Coating B and E differ only in the endcapping step (TMS silanization), which seems to provided a more reproducible coating.

Capillary Coating Summary. Overall, coating E showed the most resistance to the PCR mixture electrophoretic analysis conditions. There was only a 2.76% increase in EOF after exposure. For the silanization procedure, a monofunctional reagent was found to provide more coverage than a trifunctional reagent, and a noncatalyzed reaction performed within 3% of the amine-catalyzed reaction. Toluene (coating E) as a solvent was found to promote a more resistant coating than ethanol (coating F) or aqueous solvents (coating C). For the polymerization step, the reaction mixture was buffered to reduce hydrolysis during the polymerization and a PVP layer (coating C) reduced coating degradation over an in situ polymerized LPA layer (coating A).

Effectiveness of Coatings for PCR Product Analysis on Microchips. Having determined the coating parameters for optimum resistance to PCR product separation conditions in a capillary (coating E), we translated the method to a microchip, for which a performance comparison was made with the traditional coating (coating A). Coating longevity for microchip analysis was determined by repeated PCR product and DNA standard analyses. This involved performing 10 electrophoretic analyses of a DNA standard followed by 40 analyses of a PCR product. This process was repeated until the coating failed, as indicated by loss of resolution and significant peak broadening, or when 635 analyses were completed.

DNA Standard Analysis. Figure 3I shows the electrophoretic profile of a DNA standard on glass microchips coated with coatings A and E. The profiles observed were consistent throughout the course of experimentation, with subtle shifts in migration times, resolution, and efficiency (discussed below). For Coating A (Figure 3I), analysis 303 is shown, which follows 240 PCR product analyses; the DNA standard profile for coating E was obtained after 320 PCR sample analyses. Partial resolution of the 123 and 124 bp fragments was still observed in both profiles, even after this significant number of analyses.

PCR Product Analysis. Figure 3II shows typical profiles for the analysis of PCR products amplified from DNA extracted from cerebrospinal fluid specimens obtained from patients suspected of a hepatitis C viral (HCV) infection. These samples are representative of the PCR products typically found in a clinical laboratory setting and are, therefore, well-suited for testing the coating longevity toward PCR products. In preliminary experiments, PCR samples evaluated for T-cell lymphoma,[20] B-cell lymphoma,[20] herpes simplex virus,[21] and mutations in BRCA1[48,49] were also used as representative PCR product samples. Figure 3II displays the HCV PCR product profiles where amplification of a 250 bp fragment of the viral DNA is indicated by the presence

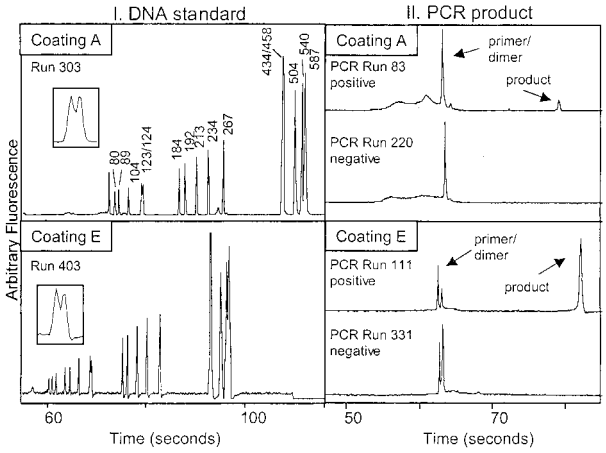

Figure 3. Representative DNA standard and PCR product profiles on microchips. (I) DNA standard profiles for coatings A and E on microchips. Inset: enlargement of the 123 and 124 bp fragments. (II) PCR standard analyses for coatings A and E. Positive samples yield profiles with a product peak at ~80 s; negative samples have no peak at ~80s. Injection: 400 V sample waste, and sample grounded (333 V/cm) for 45 s (30 s for PCR products). Separation: sample and sample waste grounded, inlet at −300 V, outlet at 2800 V. L_{eff} = 5.5 cm. Sample is 5.38 μg/mL pBR322 *Hae*III digest in 10 mM Tris, pH 8.6.

of a product peak. This correlates with infection (a positive result), for which negative samples do not produce the 250 bp product. For coating A, analysis 83 (positive) and 220 (negative) are displayed but for coating E, analysis 111 (positive) and 331 (negative) are shown. These profiles are representative of those seen throughout the 380 separations performed on the coating A microchip and the 635 separations performed on the coating E microchip.

Resolution, Efficiency and Reproducibility of the Microchip Coatings. Analysis of the DNA standard after every 40 PCR sample analyses allows for coating viability to be assessed using three measures: resolution, efficiency, and reproducibility. Resolution was calculated for DNA fragments 80/89 bp, 123/124 bp, and 234/267 bp. Figure 4 graphs the resolution for the 80/89 bp fragments and the 234/267 bp fragments against analysis number for the two coatings on the microchips. The peak separation (migration time difference/migration time average) for the 123 and 124 bp fragments is also shown, because the peaks were only partially resolved. Similar resolution is displayed for both coatings until analysis 350, when coating A began to fail (between 350 and 400 analyses). In contrast, coating E continued to provide acceptable resolution beyond 630 analyses (500 PCR/130 DNA standard) when testing was arbitrarily terminated. The true longevity (number of analyses) for this coating has not been determined.

Another measure of coating viability is the separation efficiency (number of theoretical plates). Figure 5 displays the efficiency, as gauged by three DNA fragments in the standard, versus number of separation experiments for the two coatings. Panel A shows the efficiency for the 104 bp fragment, where similar efficiency is observed for both coatings. The longer migration times exhibited when coating A was employed (Figure 3I) affect these results, because peak widths for the 104 bp fragment are 0.189 ± 0.020 and 0.177 ± 0.028 for coatings A and E, respectively.

(48) Tian, H.; Brody, L. C.; Landers, J. P. *Genome Res.* **2000**, *10* (9), 1403−1413.
(49) Tian, H.; Jaquins-Gerstl, A.; Munro, N.; Trucco, M.; Brody L. C.; Landers, J. P. *Genomics* **2000**, *63*, 25−34.

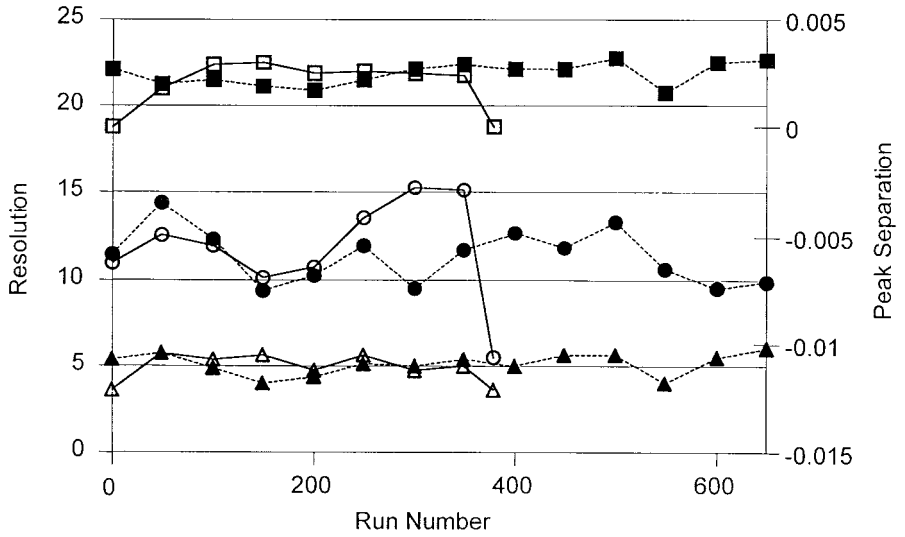

Figure 4. Resolution of the 80/89, 123/124, and 234/267 bp fragments. Resolution for the DNA standard fragments 80/89 bp and 234/267 bp for the two microchip coatings A, E. The peak separation of the 123/124 bp fragments is also shown. The standard profile was evaluated approximately every 50 analyses (40 PCR/10 standard). Coating A: Δ, 80/89; □, 123/124; ○, 234/267. Coating E: ▲,80/89; ■,123/124; ●, 234/267.

The longer migration times offset the slightly wider peak widths so that similar efficiency is observed. For the 267 and 504 bp fragments, the efficiency is similar except for the region spanning the 350−400-run range, where coating A begins to fail. The efficiency exhibited by coating A for the 250−350 bp fragments was higher than that observed with coating E. This can be attributed to the increase in migration time before failure of the coating; however, peak widening did not increase as quickly, thus producing an overall increase in efficiency. In general, similar efficiency was obtained on both coatings until coating A began to rapidly deteriorate after 350 analyses.

Variation in migration time is also an important consideration for the reliability of a capillary coating. Table 2 provides details on the average coefficient of variance (CV) values for eight DNA fragments in the DNA standard (80, 89, 104, 123, 124, 234, 267, and 504 bp) on the two coated microchips. Variation in migration times were calculated for 350 separations for coating A and 600 separations for coating E (standard evaluated every 40 PCR product separations). Coating E provided lower cv (3.79%) than coating A (4.89%), indicating improved migration time reproducibility, even over the 600 analyses. This is consistent with the increase in migration time discussed above for coating A that affected the efficiency. The percent CV was also calculated for sets of 20 PCR sample separations performed in one channel filling. Table 2 shows the increase in percent CV for coating A with separation number; however, the percent CV for analyses on coating E remains relatively constant over 600 analyses. The ability to achieve reproducible analysis of neat PCR products eliminates the need to microfabricate new elements into the microchip for on-line sample desalting.[50] In addition, the coating longevity achieved with coating E is ideal for extended use for rapid analysis of PCR-derived samples.

A microchip coated with coating B was also tested using 1.5% PVP as the separation matrix. This sieving matrix provided a

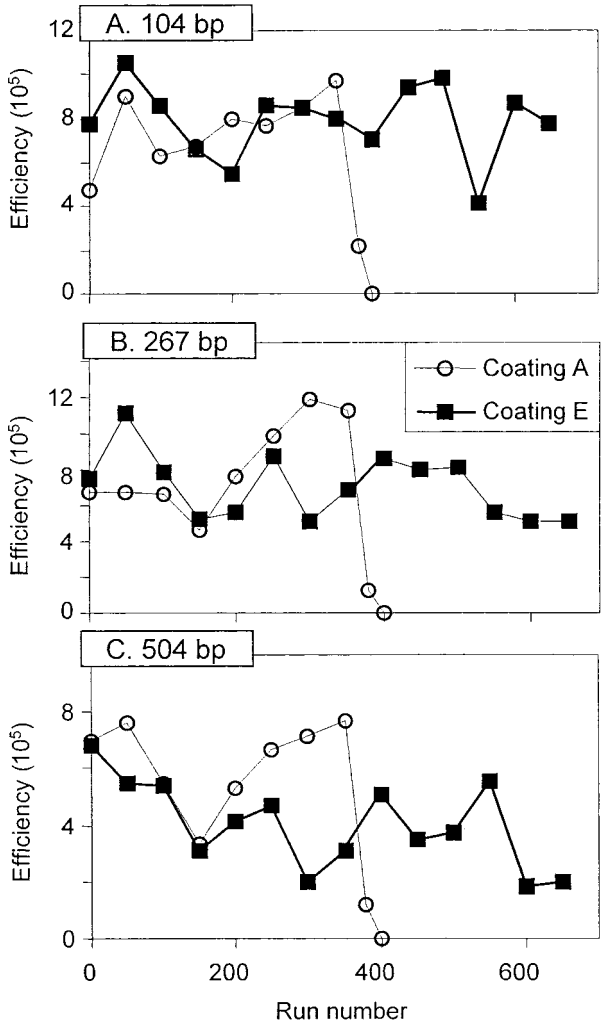

Figure 5. Efficiency measurements for microchip analysis of the DNA standard. Efficiency (number of theoretic plates) was calculated for the (A) 104, (B) 267, and (C) 504 bp fragments, as evaluated on the microchips coated with coatings A, E. The standard profile was evaluated approximately every 50 analyses (40 PCR/10 standard).

(50) Khandurina, J.; Jacabson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **1999**, *71*, 1815−1819.

Table 2. Migration Time Variances for Microchip Coatings

	% CV for 8 DNA standard fragments expt range, 0−635[a]	% CV for PCR analyses (expt range)				
		71−90	211−230	331−350	361−380	611−630
coating A	4.89	1.14	1.24	2.62	3.43	
coating E	3.79	1.57	1.26	1.59	1.92	1.81

[a] or 0−380 for coating A.

higher efficiency for the smaller fragments on this coating than the HEC (data not shown). This coating also performed well for 500 separation experiments, at which point testing was terminated. Microchips coated by procedure E and B have been used in this laboratory for the analysis of a variety of neat PCR products including HSV amplicons[21] and BRCA1 amplified DNA for heteroduplex analysis (HDA)[48] and single-strand conformation polymorphism (SSCP)[49] analysis. These references provide additional data for the verification of this coating through its use on different microchips under a variety of separation conditions for varying PCR products by several operators[21,48,49].

CONCLUSIONS

Several silica-coating parameters were tested to produce a coating with the greatest resistance to replicate neat PCR product analysis. The coating that provided the highest resistance to PCR mixture exposure consisted of a covalently linked octylsilane layer to which a poly(vinylpyrrolidone) layer had been grafted and cross-linked (coating E). Degradation of this coating over the testing period (220 min) was only 2.76%. This coating was compared to the traditional polyacrylamide coating for longevity and migration-time reproducibility on electrophoretic microchips. It was observed that although resolution and efficiency were similar, the migration time reproducibility was higher for coating E (3.79% vs 4.89%). Coating E performed well for >635 separations (when testing was terminated), as compared to 380 separations for the traditional coating. This coating has been verified for a variety of clinical PCR samples with several different sieving matrixes on multiple microchips by several operators.

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