Robert J. Meagher[1]
Jiehyun Seong[2]
Paul E. Laibinis[3]
Annelise E. Barron[1]

[1]Department of Chemical Engineering, Northwestern University, Evanston, IL, USA
[2]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA
[3]Department of Chemical Engineering, Rice University, Houston, TX, USA

A very thin coating for capillary zone electrophoresis of proteins based on a tri(ethylene glycol)-terminated alkyltrichlorosilane

We describe the use of a tri(ethylene glycol)-terminated alkyltrichlorosilane to create a very thin, protein-resistant "self-assembled monolayer" coating on the inner surface of a fused-silica capillary. The same compound has been demonstrated previously on flat silica substrates to resist adsorption of many proteins. As a covalently bound capillary coating, it displays good resistance to the adsorption of cationic proteins, providing clean separations of a mixture of lysozyme, cytochrome *c*, ribonuclease A, and myoglobin for more than 200 consecutive runs. Electroosmotic flow (EOF) was measured as a function of pH; the coated capillary retains significant cathodal EOF, with roughly 50% of the EOF of an uncoated capillary at neutral pH, making this coating promising for applications requiring some EOF. The EOF was reasonably stable, with a 2.9% relative standard deviation during a 24 h period consisting of 72 consecutive separations of cationic proteins. Efficiencies for cationic protein separations were moderate, in the range of 190 000–290 000 theoretical plates per meter. The coating procedure was simple, requiring only a standard cleaning procedure followed by a rinse with the silane reagent at room temperature. No buffer additives are required to maintain the stability of the coating, making it flexible for a range of applications, potentially including capillary electrophoresis-mass spectrometry (CE-MS).

Keywords: Capillary coating / Electroosmotic flow / Poly(ethylene glycol) / Protein analysis / Self-assembled monolayer
DOI 10.1002/elps.200305714

1 Introduction

Capillary electrophoresis (CE) has emerged as a powerful technique for analytical separations of biomolecules, including peptides, proteins, and nucleic acids. Proteins are particularly notorious for adsorbing, sometimes irreversibly, to many different surfaces, including the inside of fused-silica capillaries [1, 2]. Basic (positively charged) proteins present a particularly difficult challenge in CE due to strong electrostatic interactions between the protein and the negatively charged silica surface, although all proteins may be somewhat prone to adsorption due to hydrophobic or van der Waals forces. In capillary electrophoresis, the effects of protein adsorption are manifested in poor peak shape, irreproducible migration times, irregular electroosmotic flow (EOF), and in the most severe cases, total adsorption resulting in failure of a protein to elute. Thus, a major objective of capillary coating protocols is to modify or mask the silanol groups on the capillary surface by presenting a more inert surface.

A second major reason for treating capillary surfaces is to control EOF. EOF arises from the collection of positive ions in the double layer near the negatively charged silanol surface. In a tangentially applied electric field, these positive ions migrate toward the negative electrode. The migration of these ions drag the surrounding solvent molecules, leading to a bulk flow with an approximately plug-flow velocity profile [3, 4]. EOF is undesirable in some applications, like DNA separation or sequencing, where the analytes' electrophoretic mobility is toward the positive electrode, in opposition to the EOF. EOF also tends to be unstable in uncoated capillaries, leading to irreproducible migration times. Thus, many coatings have been aimed at almost total suppression of EOF. However, EOF can be a useful "pump" in some applications, sweeping positive, neutral, and negative analytes to

Correspondence: Professor Annelise E. Barron, Northwestern University, Department of Chemical Engineering, 2145 Sheridan Road, Room E136, Evanston, IL 60208, USA
E-mail: a-barron@northwestern.edu
Fax: +847-491-3728

Abbreviations: EG, ethylene glycol; **EG_3-silane**, methyl[(1-trichlorosilyl)undec-11-yl]triethylene glycol; **SAM**, self-assembled monolayer

the detector, and speeding the analysis times for analytes with weak electrophoretic mobilities. In the burgeoning field of microspray CE-MS, EOF is useful for introducing the analytes from the capillary into the MS [5, 6]. In addition, CE-MS requires buffers with a minimum of low-volatility interfering components such as salts [7]. For such applications, a coating that would provide stable, reproducible EOF with a minimum of interfering buffer additives, and yet resist analyte adsorption, would be beneficial.

The velocity of the EOF is given by the following equation (from Hjertén) [8]:

$$\mu_{EOF} = \frac{\varepsilon}{4\pi}\int_0^{\zeta}\frac{1}{\eta(x)}\,d\psi(x) \quad (1)$$

where x is the distance normal to the surface, $\eta(x)$ is the viscosity at position x, and $\psi(x)$ is the electrical potential at position x. ζ is the zeta potential, and ε is the dielectric constant of the buffer. Thus, EOF is seen to depend on the zeta potential (related to the surface charge and the ionic strength of the medium), and the viscosity within the double layer. Wall coatings or treatments for CE typically modify one or both of these parameters. Neutral, hydrophilic polymers, either covalently bound or physically adsorbed, create a thick region of very high viscosity that extends beyond the double layer, effectively eliminating EOF [9]. Covalently bound polymers such as polyacrylamide [8, 10–14] and its derivatives [15–18] are frequently polymerized *in situ*, but polymer chains can also be bound to the wall fully formed [19–22]. A wide variety of neutral hydrophilic polymers have also been used as physically adsorbed coatings for protein separations; these include dextran, hydroxyethylcellulose, hydroxypropylmethylcellulose, poly(vinyl alcohol) [23–25], polyethylene oxide [26, 27], and acrylamide-based polymers such as poly(acrylamide-*co*-allyl-β-D-glucopyranoside-*co*-allyl glycidyl ether) (epoxy poly(AG-AA)) [28], epoxy-poly (dimethylacrylamide) (EPDMA) [29], and poly(*N*-hydroxyethylacrylamide) (PHEA, trade name polyDuramide™) [30]. Both approaches have been quite successful for analysis of proteins and other biomolecules but are not useful in cases when EOF is desired. Many effective wall coatings and treatments for protein separations have recently been reviewed by Doherty *et al.* [31], Horvath and Dolník [32], Rodriguez and Li [33], and Chiari *et al.* [34].

For protein separation applications where EOF is desired, other approaches are required. A number of charged polymers have been used to modify surface charge as well as to reduce analyte adsorption. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (hexadimethrine bromide, HDB, trade name Polybrene™) has been widely used to create a cationic surface and reverse the direction of EOF [35–37], as have polyarginine [38], polyethyleneimine [39–41], and poly(diallyldimethylammonium chloride) (PDADMAC) [42]. Polyelectrolyte multilayers, or successive multiple ionic layers (SMILs) have also been used to create both cationic and anionic surfaces using two or more alternating layers of a cationic polymer such as Polybrene or PDADMAC and an anionic polymer such as dextran sulfate or polystyrene sulfonate [43–45]. The commercial CElixer™ kit (Microsolv Technology, Long Branch, NJ, USA) uses a variation of this approach to create an anionic surface with essentially pH-independent EOF. The SMIL approach of Katayama *et al*. [44] was demonstrated to be compatible with CE-MS, whereas CElixer, which incorporates anionic polymer in the running buffer, is not.

Small molecules, either as buffer additives or as coatings, have also been effective for reducing adsorption and modifying EOF. Alkylamines are believed to interact strongly with silanol groups at the capillary surface, modifying the charge at the surface, and potentially reversing EOF [46–50]. Various types of surfactants, including cationic [40, 51, 52], anionic, zwitterionic [53–55], nonionic [54], and fluorosurfactants [56] have been used as buffer additives to suppress adsorption and tune EOF, and can allow separation of both cationic and anionic proteins in a single run. The double-chained cationic surfactant didodecyldimethylammonium bromide (DDAB) [57] and the double-chained, zwitterionic phospholipid 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC, C_{12}) [58] have found utility as semipermanent coatings that remain stable on the capillary surface with no surfactant present in the running buffer.

A charged surface may be undesirable if proteins of both positive and negative charge are to be analyzed with a single capillary, while surfactants and other buffer additives can potentially interfere with certain analyses and detection methods such as CE-MS. An alternative approach for retaining EOF is a thin covalent coating of neutral polymers or small molecules. In very thin coatings, the high-viscosity coating region does not span the entire electrical double layer, and significant EOF is retained. As early as 1989, Bruin *et al.* [59] attached short poly(ethylene glycol) (PEG) chains (M_w = 600 g/mol) to a capillary that had been silanized with a bifunctional silane reagent. A similar approach was also employed by Nashabeh and El Rassi [60], who created coatings of interlocked or linear PEG chains. These coatings retained EOF in a manner related to the size of the PEG groups attached to the wall, and thus could be used roughly to "tune" EOF, or to create step changes in EOF in coupled capillaries [61, 62]. More recently, König

and Welsch [63] have created a thin oligourethane coating, beginning with a urethane-terminated silane, and flushing alternately with a diisocyanate and a diol or polyol.

Silane reagents have also been used directly to create very thin wall coatings. In some cases, simple alkylsilanes have been used to create a hydrophobic surface as a foundation for adsorbed polymers or surfactants [2, 64]. Another approach, which has been employed in the current study, is to use functionalized silanes to create coatings of monomolecular thickness that are thinner than the electrical double layer and retain significant EOF. Shao *et al.* [65] have reported silanizing a capillary wall with 3-glycidoxypropyltrimethoxysilane, with no further modification, to create a thin, diol-bonded phase that was resistant to protein adsorption and retained 20% of the original EOF. 3-Aminopropanesilane has also been used to create a very thin, positively charged coating with reversed EOF [5, 66], although this type of coating is most useful at low pH, presumably because residual silanol groups on the surface are less highly charged at acidic buffer conditions.

Alkanethiols on gold surfaces are well-known to spontaneously assemble into oriented, densely packed monolayer films ("self-assembled monolayers" or "SAMs"). The Whitesides group has demonstrated, beginning in 1991, that self-assembled monolayer films consisting of oligo(ethylene glycol)-terminated alkylsilanes on gold surfaces exhibit excellent resistance to adsorption of a variety of proteins [67–69]. Interestingly, only a few ethylene glycol units were needed to suppress adsorption, as opposed to the longer PEG chains that are sometimes grafted to capillaries and other surfaces [59, 60]. A more systematic study of approximately 50 different end-groups has been performed using both single-component SAMs and mixed SAMs [70, 71]. The end-groups of protein-resistant SAMs showed some structural similarities: they tended to be hydrophilic, containing groups that were hydrogen-bond acceptors but not hydrogen-bond donors, and overall electrically neutral. In addition to oligo(ethylene glycol), good protein resistance was observed in coatings terminating in permethylated sorbitol and oligo(sarcosine), which also exhibit these structural properties.

For glass and metal oxides, including silica, trifunctional organosilane reagents can be deposited from organic solvents to create dense, SAM films [72, 73]. It is believed that a thin film of water strongly adsorbed to the silica surface hydrolyzes trichlorosilane reagents, forming a two-dimensional polymeric network, which is bonded only partially to the silica surface. "Conventional" films composed entirely of octadecyltrichlorosilane (C18) result in only partially cross-linked layers, with a surface density of about 5 μmol/m^2, whereas films composed of a mixture of C18 with C1 or C3 trichlorosilanes are able to cross-link more extensively, with surface coverage of 8 μmol/m^2 or greater [74, 75]. Surface charge density is reduced relative to bare silica, but due to incomplete cross-linking and incomplete reaction of surface silanol groups, surface charge is not eliminated entirely.

In 1998, Lee and Laibinis [76] reported an approach to creating protein-resistant monolayer coatings for glass and metal oxide surfaces by synthesizing alkyltrichlorosilanes terminated with 2 or 3 ethylene glycol units, in direct analogy to the ethylene glycol-terminated alkanethiols explored by the Whitesides group. These reagents had the general structure $CH_3O(CH_2CH_2O)_n(CH_2)_{11}SiCl_3$ with n = 2 or 3 ethylene glycol (EG) units. The reagents were deposited on flat silicon wafers with a hydrated SiO_2 surface using a straightforward solution-phase adsorption process, analogous to that used for a conventional alkyltrichlorosilane monolayer. Ellipsometry was used to confirm monolayer films of thickness 18–20 Å, depending on the number of EG units. The coated surfaces were exposed to a variety of proteins ranging from 10 to 400 kDa, and results mirrored those for alkanethiols on gold: the EG-capped surfaces resisted the test proteins well, with the exception of fibrinogen which adsorbed to a minor extent. The tri(ethylene glycol) film displayed greater protein resistance than the di(ethylene glycol) film.

In this study, we report the use of the tri(ethylene glycol)-terminated alkyl silane reagent, $CH_3O(CH_2CH_2O)_3(CH_2)_{11}SiCl_3$, or "$EG_3$-silane" to create a very thin, protein-resistant coating inside a fused-silica capillary. The excellent protein resistance and uniformity of the EG_3-silane coating on flat silica substrates make it an attractive candidate for a protein-resistant capillary coating, and it is believed that the films formed inside the capillary retain many of the desirable features previously reported for flat substrates [76]. The coated capillary retains substantial EOF and is useful for rapid, high-resolution separation of basic proteins and peptides. No buffer additives are required to maintain the coating, making it suitable for a wide range of applications. The coating procedure is attractive due to its simplicity, requiring only a simple cleaning followed by a single silanization step to produce the coating. This distinguishes it from other thin coatings of PEG or other polymers, which at a minimum require separate silanization and grafting steps. Results are particularly interesting in that an EG chain of only three monomer units is required to confer protein resistance, as opposed to most previously reported polymeric wall coatings, where much longer chains of PEG or other polymers were required.

2 Materials and methods

2.1 Materials

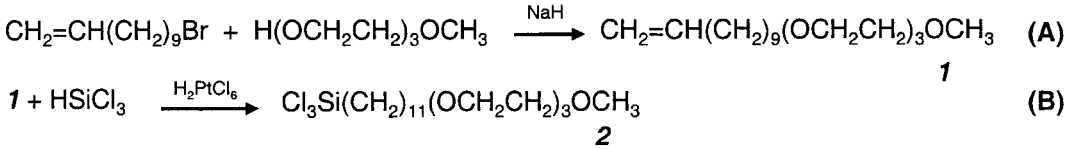
All of the chemicals used in the synthetic protocol for the EG_3-silane reported below were obtained from Aldrich (Milwaukee, WI, USA) unless noted otherwise. 11-Bromo-1-undecene (99%) was from Pfaltz & Bauer (Waterbury, CT, USA). Sodium hydride (NaH) was received from Aldrich as a 60% dispersion in mineral oil and used without purification. Fused-silica capillary (360 μm OD × 50 μm ID) with a polyimide outer coating was purchased from Polymicro Technologies, LLC (Phoenix, AZ, USA). Methanol, phosphoric acid, monobasic and dibasic sodium phosphate, acetic acid, and sodium hydroxide were from Fisher Scientific (Fair Lawn, NJ, USA). Sodium dodecyl sulfate was from GibcoBRL (Grand Island, NY, USA). Anhydrous toluene and benzyl alcohol were purchased from Aldrich (Milwaukee, WI, USA). Anhydrous toluene was used as supplied, without additional removal of water. 200-proof ethanol was from Pharmco Products (Brookfield, CT, USA). Fluorinert FC-77 was purchased from 3M (St. Paul, MN, USA). Tris was from Amresco (Solon, OH, USA). Bicine, lysozyme (chicken egg white), cytochrome *c* (horse heart), ribonuclease A (bovine pancreas), and myoglobin (horse skeletal muscle) were purchased from Sigma (St. Louis, MO, USA). Water was purified and deionized to a resistivity of at least 17.8 MΩ·cm with a Barnstead E-Pure system (Boston, MA, USA). The pH of buffer solutions was measured with an Orion Model 370 pH meter (Beverly, MA, USA), calibrated daily using pH 4.00, 7.00, and 10.00 standards from Fisher Scientific.

2.2 Synthesis of EG_3-silane reagent

Scheme 1 shows the two-step synthesis used for the preparation of the adsorbate; the individual steps are based on direct modifications of the previously reported procedures [67, 76]. (A) 8.7 mmol of NaH was dispersed in 30 mL of anhydrous dimethylformamide (DMF). Subsequently, 26.0 mmol of $H(OCH_2CH_2)_3OCH_3$ was mixed with NaH/DMF dispersion for 30 min, and then 8.6 mmol of $CH_2{=}CH(CH_2)_9Br$ was added dropwise. After being stirred at room temperature under a nitrogen atmosphere for 7 h, the reaction mixture was extracted four times with hexane, and the collected extracts were concentrated by a rotary evaporator. The purified product **1** was obtained by flash chromatography on silica gel (eluent: hexane, followed by hexane/ethyl acetate 9:1, and lastly 5:5) as a colorless oil in an isolated yield of ~ 80%. (B) 50 mg of 0.12 M H_2PtCl_6 in anhydrous tetrahydrofuran (THF) was mixed with 5.8 mmol of **1** and 17.4 mmol of $HSiCl_3$ in a N_2 glove box and then stirred for 2 h. The final product **2**, EG_3-silane, was obtained in > 95% yield after removal of unreacted $HSiCl_3$ under the vacuum. The EG_3-silane was stored in sealed glass ampoules with an N_2 atmosphere until ready to use. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.2–1.5 (*m*, 16 H), 1.56 (*m*, 4 H), 3.38 (*s*, 3 H), 3.45 (*t*, 2 H), 3.5–3.75 (*m*, 12 H).

2.3 Capillary coating procedure

The following procedure was performed on a 4 m length of fused-silica capillary. Liquids and nitrogen were introduced into the capillary using a home-built stainless steel "bomb", pressurized to 3 MPa with nitrogen gas. The capillary was pretreated by rinsing for 10 min with a 5:1:1 v/v/v mixture of water, ammonium hydroxide, and 30% hydrogen peroxide at 80°C (maintained by immersing the capillary in a hot water bath, with the solution flowing continuously). Next, the capillary was rinsed for 20 min with flowing water at room temperature, followed by 5 min with methanol, and 5 min with nitrogen. The capillary was then equilibrated for 5 min with flowing toluene. Meanwhile, the sealed ampoule containing the silane reagent was opened, and the contents were dissolved in toluene to give a final concentration of 8 mg/mL. This solution was then immediately flushed through the capillary for 15 min. The flow was stopped, and the capillary was left to sit overnight at room temperature, with the ends of the capillary immersed in sealed vials (with septa) containing the toluene-silane solution. After sitting for 16 h, the capillary was rinsed with ethanol for 5 min, and dried with flowing nitrogen for 10 min. The capillary was stored at 4°C with the ends capped.



Scheme 1. Synthesis of methyl[(1-trichlorosilyl)undec-11-yl]tri(ethylene glycol) (EG_3-silane).

2.4 Capillary electrophoresis

Electrophoresis was performed on two identical BioFocus 3000 capillary electrophoresis instruments (BioRad Laboratories, Hercules, CA, USA), with a ± 30 kV power supply and a deuterium lamp and photodiode detection with multiwavelength scanning capability for monitoring UV absorbance. Capillaries with lengths ranging from 25 to 37 cm were cut from the 4 m section of coated capillary as needed. Detection windows were created by scraping off a few millimeters of the outer polyimide coating with a razor blade; this was done to prevent possible heat-induced damage to the inner silane coating that may result from burning off the polyimide coating. Capillary cartridges were thermostated at 20° or 25°C with circulating liquid coolant (either Fluorinert FC-77 or 80/20 v/v water/methanol). Sample carousel temperature was controlled at 8°C during extended runs to minimize denaturing of protein samples. The typical field strength was 300 V/cm, leading to currents ranging from 10–24 μA, depending on buffer pH and composition. EOF was measured by observing the migration time of a neutral marker (benzyl alcohol), at a dilution of approximately 1:20 000 in the samples. Protein samples were prepared as stock solutions at 10 mg/mL in 25 mM sodium phosphate buffer, pH 7.0, stored at 4°C, and diluted to final concentrations of 0.2–0.3 mg/mL in deionized (DI) water prior to use. Samples were introduced into capillaries by pressure injection, with a typical injection time constant of 20.7 kPa·s (3 psi·s). Coated capillaries were simply flushed with buffer for 60–90 s at 690 kPa (100 psi) between runs. Uncoated capillaries were treated prior to the first run by flushing with 1 M sodium hydroxide for 10 min, followed by 0.1 M sodium hydroxide for 10 min, followed by water for 5 min. Between runs, uncoated capillaries were reconditioned by flushing with 0.1 M sodium hydroxide for 3 min, followed by water for 1 min, followed by buffer for 1 min at 690 kPa (100 psi). Buffers were typically replaced after 3–4 runs (~1 h of electrophoresis).

3 Results and discussion

3.1 Optimization of coating procedure

Two variations of the coating procedure were attempted; the version presented in Section 2.3 gave the most stable and inert surface. The earliest attempt employed a "piranha" wash (70/30 v/v mixture of concentrated H_2SO_4 and 30% H_2O_2) at room temperature as a surface pretreatment, instead of the "RCA Clean 1" mixture (5:1:1 water/NH_4OH/30% H_2O_2) described in Section 2.3. Compared to the piranha treatment, the RCA Clean 1 procedure was judged to be marginally safer, easier to implement, and less corrosive to laboratory equipment. Based on the results of Cras *et al*. [77], who studied different methods of surface pretreatment, and also following the extensive review and experiments of Munro *et al*. [78], it is likely that the RCA Clean 1 procedure better prepares the surface for silanization than either the piranha wash or a simple rinse with NaOH or KOH. Pretreatment with a 1:1 methanol-HCl wash should also be sufficient for preparing the surface; Cras *et al.* [77] also recommend rinsing with concentrated H_2SO_4 following this step. Leaching with dilute acid may also help remove metal impurities from the capillary surface, reducing the acidity of the surface [79], although this step was not incorporated into the coating procedure for this study.

The second variable that appears to have affected the quality of the silane coating was moisture. The initial attempt to generate the coating was done with care to eliminate as much water as possible. The capillary was dried for several hours in flowing nitrogen at 100°C, and the silane solution was prepared in fresh, anhydrous toluene in a dry glove box. The silanization reaction does, however, require some moisture (see Munro *et al.* for an excellent review of the sometimes contradictory literature on this point [78]). In subsequent attempts, the capillary was dried only briefly with room-temperature nitrogen, and the silane solution was prepared simply in a fume hood under flowing nitrogen. Fairbank and Wirth [75, 80] report that 50% relative humidity is optimal for dense horizontal cross-linking of trichlorosilanes on fully hydroxylated silica. The optimal amount of water depends on the silanol surface concentration; insufficient water leaves unreacted Si-Cl groups, whereas excessive water leads to excess polymerization [75]. No attempt was made to control the humidity inside the capillary specifically at 50% or any other level, but it is assumed that, without a high-temperature drying step, a significant amount of water remained bound to the surface at the time that the silane reagent was introduced. In summary, coatings generated using the RCA Clean 1 pretreatment, and without excessive drying of the capillary surface, resulted in coatings that were more resistant to protein adsorption, with more stable EOF, than capillaries prepared with a piranha pretreatment and drier conditions.

3.2 Measurement of EOF

EOF was measured over a range of pH in both coated and uncoated capillaries, with results shown in Fig. 1. Below pH 3, both the coated and uncoated capillaries show approximately equal EOF mobilities. At higher pH, the two diverge, with EOF in the silane-coated capillary dimin-

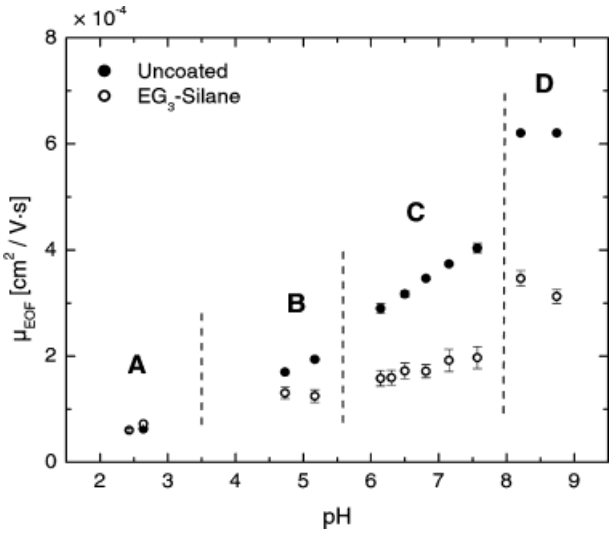


Figure 1. Electroosmotic mobility in EG3-silane coated and uncoated fused-silica capillaries. The buffers used were: (A) 25 mM sodium phosphate, (B) 25 mM sodium acetate, (C) 25 mM sodium phosphate, and (D) 25 mM Tris, 25 mM Bicine. Uncoated capillaries were flushed with 0.1 M NaOH between measurements. Error bars for coated capillary represent the RSD for at least four measurements, except below pH 3. RSD for EOF in uncoated capillaries was within the size of the data points.

ished by ~ 50% at pH 7.15. The EOF data are somewhat discontinuous around pH 8; this is due to the very different ionic strength of the sodium phosphate buffer used below pH 8, and the Tris-Bicine buffer used at higher pH.

The Si-O-Si bond of silane coatings is prone to base-mediated hydrolysis, so a sparse or poorly cross-linked coating will be unstable at high pH. The EOF was stable in the EG_3-silane coated capillary for at least 10 h between pH 8.2 and 8.74 (roughly 6 h at pH 8.21 and 4 h at pH 8.74), indicating good stability in this pH range. The test was not extended beyond 10 h, so the lifetime between pH 8–9 was not determined. An upper limit of pH for stability of the coating was also not determined.

The results are broadly consistent with other researchers who have created thin coatings that retain EOF. Huang *et al.* [81] measured EOF in capillaries after derivatizing with alkylsilane chains of varying lengths, and demonstrated that EOF suppression is directly related to the length of the alkyl chain, *e.g.*, a C_3 alkyl chain suppressed EOF by 24%, while a C_8 alkyl chain suppressed EOF by 65%. Shao *et al.* [65] reported an 80% decrease in EOF after silanizing with 3-glycidoxypropyltrimethoxysilane to create a thin, diol coating, while Nashabeh *et al*. [60] report ~ 40–70% suppression of EOF near pH 7 with their "fuzzy" and "interlocking" PEG coatings of different molecular weights. The EOF suppression reported here is similar in magnitude to that reported by Nashabeh *et al*. for the coatings formed from PEG-200 and PEG-600 bound to a thin silane layer. The actual extent of EOF suppression for these types of coating should depend on coating thickness and density and the extent of reaction of surface silanols, as well as buffer pH and ionic strength. The EG_3-silane coating is, in principle, more regular and uniform in thickness than others based on grafting polydisperse polymers to a preformed silane monolayer, and this uniformity should lead to more regular EOF.

3.3 Protein separations

Separation of basic proteins at neutral pH is a significant challenge for many coatings, and so to present a "difficult" test, separations of cationic proteins were performed extensively. A mixture of four proteins – lysozyme (p*I* 11), cytochrome *c* (p*I* 10.7), ribonuclease A (p*I* 9.5), and myoglobin (p*I* 7.0, 7.4) – were separated by CE in buffers at pH $<$ 7, such that all of the proteins had net positive charge. Figure 2A shows electropherograms for a series of runs with a single capillary using 25 mM sodium phosphate at pH 6.7. The peaks are well-resolved for over 200 consecutive runs, with only a short flush with buffer between runs. Following elution of the proteins, the baseline returns to essentially its initial value, suggesting relatively low adsorption of proteins on the detection window. A small decline in the rate of EOF over the course of the first 50 runs may be due to slow accumulation of proteins on the surface, although the largest change in EOF occurred following an overnight shutdown after approximately 30 runs. The peak shape deteriorates somewhat after approximately 150 runs, with more noticeable tailing of the lysozyme and cytochrome *c* peaks, and the appearance of a "shoulder" shortly ahead of the myoglobin peak.

In an attempt to clean or regenerate the coating surface, the capillary was flushed with a 2 wt% solution of SDS for 10 min [82], followed by a 10 min rinse with water. As illustrated in Fig. 2B, the first few separations after this procedure show increased EOF and slightly broader peaks, most likely due to residual SDS slowly desorbing from the surface. Within 10 runs after the SDS treatment, "normal" performance has been restored, with electropherograms resembling those from runs 100–150 prior to the SDS treatment. Tailing of the lysozyme peak is still evident, while the shoulder ahead of the myoglobin peak has been eliminated.

Peak efficiencies are in the neighborhood of N = 60 000–90 000 theoretical plates (190 000–290 000 plates per meter) for the cationic proteins. This is moderate compared to some other "thin" coatings and treatments,

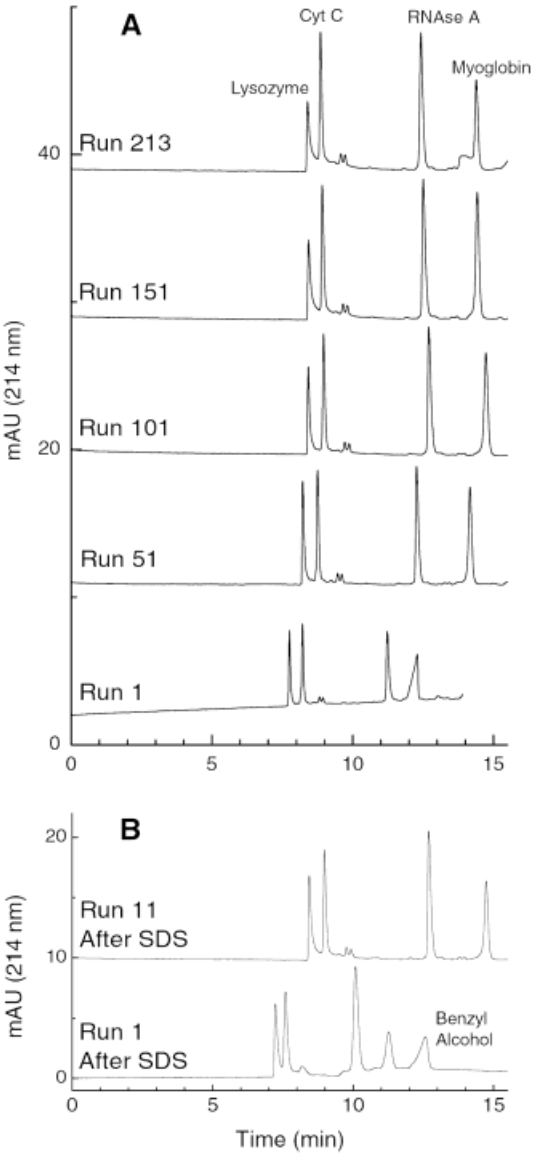


Figure 2. Electrophoresis of cationic proteins lysozyme, cytochrome *c*, ribonuclease A, and myoglobin in EG_3-silane coated capillary. Peaks were identified by spiking one component at a time. Capillary, 50 μm × 36 cm long (31.4 cm effective length); buffer, 25 mM sodium phosphate, pH 6.7; field strength, 300 V/cm. (A) Selected electropherograms from 213 consecutive separations in a single capillary. (B) Electropherograms from the same capillary following a 10 min rinse with 2% SDS.

for which efficiencies in excess of 500 000 to 1 000 000 plates per meter can be achieved [55, 57, 58]. However, the efficiencies (in plates per meter) for the EG3-silane coating are quite similar to those reported for previous thin grafted PEG coatings [59, 60]. The moderate efficiency is in part related to the short analysis time, which results from both the use of a fairly short capillary and the relatively rapid EOF that prevailed. It should be mentioned, however, that this set of cationic proteins represents a significant analytical challenge for this very thin coating.

For comparison, a similar separation was attempted in a clean, NaOH-rinsed uncoated capillary. As would be expected, separation of the cationic proteins was very poor to impossible, and assignment of individual peaks was not possible (data not shown).

One of the advantages of coatings that retain electroosmotic flow is that, in principle, oppositely charged analytes can be separated in a single run. This has been demonstrated for this coating by separating the same four proteins using a 25 mM Tris-Bicine buffer at pH 8.24. At this pH, lysozyme, cytochrome *c*, and ribonuclease A are still positively charged, but myoglobin has a net negative charge. Typical results for the analyses of each protein are shown in Fig. 3. Myoglobin and ribonuclease A clearly elute on either side of the neutral peak (in this case a downward peak corresponding to the zone of water introduced during sample injection). In this buffer the lysozyme and cytochrome *c* peaks tail significantly; this may be due to the increased charge of the surface at this pH leading to stronger electrostatic interactions. The same mixture of proteins was also analyzed in 25 mM Tris-Bicine buffer at pH to 8.74; in that case, similar sharp peaks for ribonuclease A and myoglobin are observed, while no peaks are observed for cytochrome *c* or lysozyme, presumably due to complete adsorption of the proteins on the capillary surface.

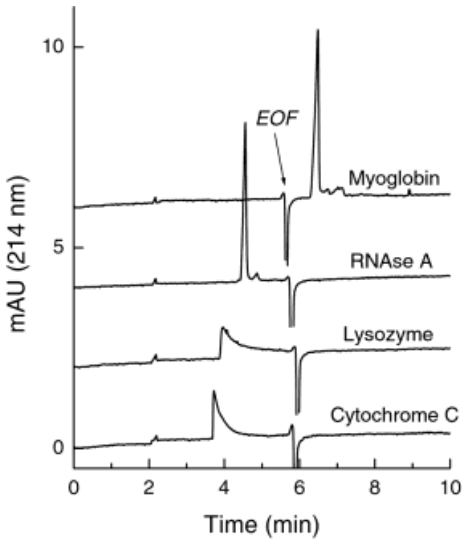


Figure 3. Analysis of individual proteins in EG_3-silane coated capillary 25 mM Tris-Bicine buffer, pH 8.24. The downward peaks, corresponding to the migration time of water in the sample, have been truncated for visual clarity. Electrophoresis conditions same as in Fig. 2.

Separations with common "acidic" (p*I* < 7) proteins including ovalbumin, α-lactalbumin, β-lactoglobulin, and trypsin inhibitor were also performed. At neutral pH, these proteins have significant electrophoretic mobility in opposition to the EOF, and thus migration times are very long, and in many cases the proteins did not elute. In this case, separation conditions, particularly buffer pH, must be chosen appropriately to give faster EOF (achieved at higher buffer pH) and reduced net negative charge on the protein (achieved at lower buffer pH). An example showing a separation of trypsin inhibitor and neutral marker at pH 6.1 in the EG3-silane coated capillary is presented in Fig. 4. The trypsin inhibitor peak in this separation has an efficiency of 17 000 plates per meter.

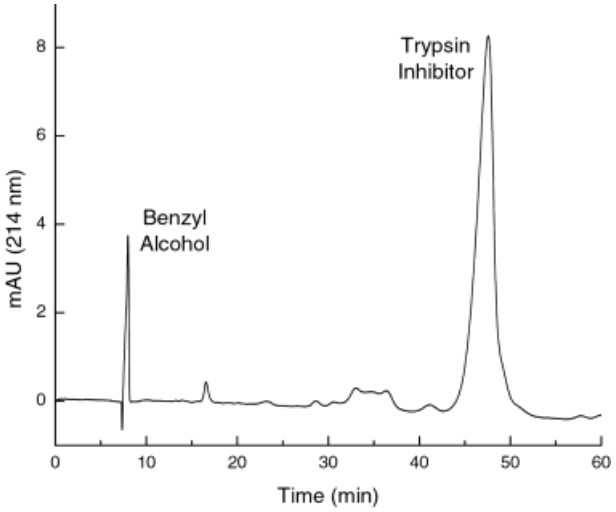


Figure 4. Separation of neutral marker (benzyl alcohol) and trypsin inhibitor EG_3-silane coated capillary. Capillary, 50 μm × 25 cm long (20.4 cm effective length); buffer, 50 mM sodium acetate, pH 6.1; field strength, 300 V/cm.

3.4 Stability of EOF during protein separations

EOF was monitored by inclusion of a neutral marker (benzyl alcohol) during experiments with separation of cationic proteins. Two cases, with capillaries from two different batches of coating, are illustrated in Fig. 5. Figure 5A shows EOF as measured for 237 consecutive runs described in Section 3.2, including the SDS cleaning procedure [82]. In this case, the EOF remains quite stable over the course of the first 214 runs, after a small initial decline. Some discontinuities appear, primarily at points when the instrument was left unattended overnight and restarted the next morning. Following these discontinuities, the EOF quickly returns to its original pattern. The other clear discontinuity occurs immediately following the SDS cleaning procedure at 72 h. Presumably some SDS remains bound to the capillary surface after this procedure,

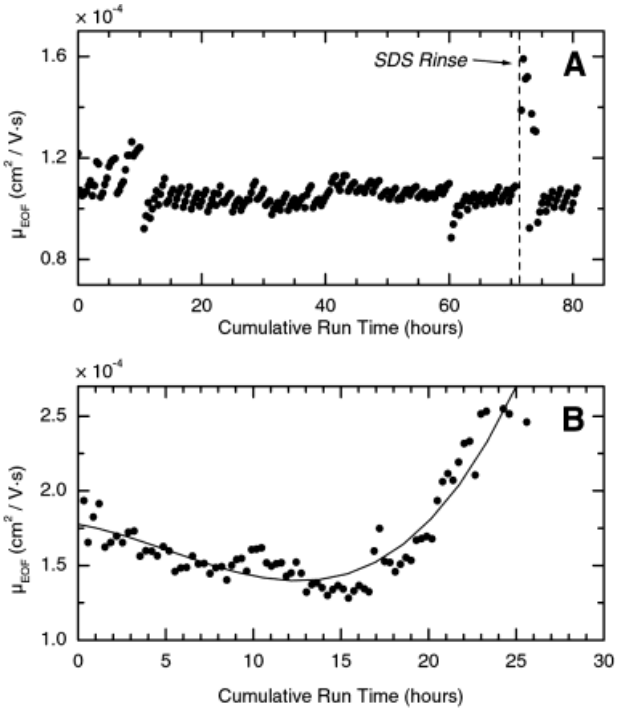


Figure 5. Stability of EOF EG_3-silane coated capillary during separations of cationic proteins lysozyme, cytochrome *c*, ribonuclease A, and myoglobin. (A) EOF during the separations illustrated in Fig. 2. The dashed line at 72 h marks the point at which the capillary was rinsed with 2% SDS. Conditions for (A) same as in Fig. 2. (B) EOF measured in another, less effective EG_3-silane coated capillary during separation of the same four proteins. The solid curve is drawn to guide the eye. Conditions for (B): 50 μm × 37.5 cm capillary (33 cm effective length); 25 mM sodium phosphate, pH 6.2; 25°C; field strength, 300 V/cm; 20.7 kPa·s (3 psi·s) injections.

leading to an increased surface charge, which diminishes over the next several runs as SDS desorbs. There is a noticeable cyclic pattern due to changing buffer vials after every fourth run. This effect is presumably due to small changes in pH due to slow hydrolysis of the running buffer, although it is unknown why the effect seems rather exaggerated in this case. As a whole, the RSD of the EOF is rather high, suggesting that absolute migration times with this coating will not be highly reproducible. Including "discontinuities", the RSD of EOF for the first 214 runs (about 3 days) was 5.1%, with 2.9% RSD for a single 24 h period of relatively stable operation from hours 16–40.

Figure 5B shows EOF measured in 86 consecutive runs with cationic proteins at pH 6.2. In this series of runs, the EOF declined slowly, presumably due to slow adsorption of protein and accumulation of positive charge at the capillary surface. Eventually, however, the EOF begins

to increase rapidly, eventually approaching that for an uncoated capillary, and meanwhile separation quality declines (data not shown). This could be due to slow hydrolysis of the covalent Si-O-Si bonds of the coating. This stretch of coated capillary exhibited a relatively short useful lifetime (about 85 runs), whereas other capillaries were stable for 150 runs or more. This indicates some variability in the capillary-to-capillary reproducibility of the coating procedure. Useful lifetime of the coated capillaries was 2–3 months when stored at 4°C. Capillaries stored for 6 months or longer displayed significantly reduced EOF and poor peak shape in protein separations.

4 Concluding remarks

The EG_3-silane compound created a useful coating for rapid separation of cationic proteins near neutral pH. Enough EOF was retained to separate both cationic and anionic proteins simultaneously in some cases, although the coating was not suitable for resolution of more complex mixtures of proteins with a wide range of isoelectric points. Overall separation efficiencies and stability of EOF were only moderate in light of the performance of many highly effective coatings that have been previously described. These initial results are, however, quite comparable to those of other thin, neutral coatings, and the results do show some promise that, with further optimization, a truly competitive coating may be obtained. The coating is also anticipated to be useful for other types of analytes, including protein digests and peptides.

One advantage of this coating includes simplicity of production, requiring only a silanization reaction, which is already the starting point for many other types of covalent coatings. Most coating procedures that begin with a silanization step benefit from addition of a thick layer of polymer that provides additional shielding of the silica surface, and it is possible that a silane layer on its own will not provide a sufficiently dense coating for very high performance capillary electrophoresis. It is quite impressive – but not unexpected – that the observed degree of protein resistance was obtained with an oligomer chain of only three ethylene glycol units. This is in line with previous results with EG-terminated SAMs formed by alkanethiols on gold [67–69], although it is somewhat contrary to conventional wisdom that long polymer chains are absolutely necessary to prevent adsorption in CE. Given the excellent protein resistance of the EG_3-silane coating on flat Si/SiO_2 substrates [76], perhaps better performance as a CE coating was expected, although fused-silica capillary is certainly a less controlled, more irregular surface, and thus less ideal for forming a very regular SAM coating.

Future work on this coating should focus particularly on optimizing the conditions of the silanization reaction to obtain a dense coating with extensive horizontal cross-linking. Based on the work of Wirth *et al*. [75] this may entail creating a mixed monolayer with some fraction of short (methyl or propyl) alkyltrichlorosilanes to ensure maximum surface coverage, as well as better control over moisture in the capillary. With further optimization, this coating, which requires no buffer additives, should be an excellent candidate for analysis of complex mixtures of proteins and peptides by various techniques, including CE-MS, and can also be explored as a coating for microchannels in glass microfluidic devices.

This work was supported by the DARPA-DURINT program of the Air Force Office of Scientific Research (Grant # F46920-01-1-0401), and by Northwestern University's Nanotechnology Science and Engineering Center sponsored by the National Science Foundation (Grant # NSF EEC-0118025). RJM acknowledges support from the MRSEC program of the National Science Foundation (DMR-0076097) at the Materials Research Center of Northwestern University. JS and PEL acknowledge funding from SatCon Technologies, Inc.

Received July 17, 2003

5 References

[1] Jorgenson, J. W., Lukacs, K. D., *Science* 1983, *222*, 266–272.

[2] Towns, J. K., Regnier, F. E., *Anal. Chem.* 1991, *63*, 1126–1132.

[3] Bikerman, J. J., *Surface Chemistry for Industrial Research*, Academic Press, New York 1948, pp. 382–387.

[4] Taylor, J. A., Yeung, E. S., *Anal. Chem.* 1993, *65*, 2928–2932.

[5] Figeys, D., van Oostveen, I., Ducret, A., Aebersold, R., *Anal. Chem.* 1996, *68*, 1822–1828.

[6] Kelly, J. F., Ramaley, L., Thibault, P., *Anal. Chem.* 1997, *69*, 51–60.

[7] von Brocke, A., Nicholson, G., Bayer, E., *Electrophoresis* 2001, *22*, 1251–1266.

[8] Hjertén, S., *J. Chromatogr.* 1985, *347*, 191–198.

[9] Doherty, E. A. S., Berglund, K. D., Buchholz, B. A., Kourkine, I. V., Przybycien, T. M., Tilton, R. D., Barron, A. E., *Electrophoresis* 2002, *23*, 2766–2776.

[10] Cobb, K. A., Dolník, V., Novotny, M., *Anal. Chem.* 1990, *62*, 2478–2483.

[11] Huang, M. X., Vorkink, W. P., Lee, M. L., *J. Microcol. Sep.* 1992, *4*, 233–238.

[12] Sandoval, J. E., Pesek, J. J., *Anal. Chem.* 1991, *63*, 2634–2641.

[13] Schmalzing, D., Piggee, C. A., Foret, F., Carrilho, E., Karger, B. L., *J. Chromatogr.* 1993, *652*, 149–159.

[14] Huang, X. Y., Doneski, L. J., Wirth, M. J., *Anal. Chem.* 1998, *70*, 4023–4029.

[15] Chu, C. H., Jonsson, E., Auvinen, M., Pesek, J. J., Sandoval, J. E., *Anal. Chem.* 1993, *65*, 808–816.

[16] Montes, M. C., Vanamen, C., Pesek, J. J., Sandoval, J. E., *J. Chromatogr. A* 1994, *688*, 31–45.

[17] Chiari, M., Nesi, M., Sandoval, J. E., Pesek, J. J., *J. Chromatogr. A* 1995, *717*, 1–13.

[18] Chiari, M., Dell'Orto, N., Gelain, A., *Anal. Chem.* 1996, *68*, 2731–2736.

[19] Herren, B. J., Shafer, S. G., Vanalstine, J., Harris, J. M., Snyder, R. S., *J. Coll. Int. Sci.* 1987, *115*, 46–55.

[20] Huang, M. X., Plocek, J., Novotny, M. V., *Electrophoresis* 1995, *16*, 396–401.

[21] Srinivasan, K., Pohl, C., Avdalovic, N., *Anal. Chem.* 1997, *69*, 2798–2805.

[22] Zhang, J., Horváth, C., *Electrophoresis* 2003, *24*, 115–120.

[23] Belder, D., Schomburg, G., *HRC J. High Resolut. Chromatogr.* 1992, *15*, 686–693.

[24] Lindner, H., Helliger, W., Dirschlmayer, A., Jaquemar, M., Puschendorf, B., *Biochem. J.* 1992, *283*, 467–471.

[25] Gilges, M., Kleemiss, M. H., Schomburg, G., *Anal. Chem.* 1994, *66*, 2038–2046.

[26] Iki, N., Yeung, E. S., *J. Chromatogr. A* 1996, *731*, 273–282.

[27] Preisler, J., Yeung, E. S., *Anal. Chem.* 1996, *68*, 2885–2889.

[28] Chiari, M., Cretich, M., Damin, F., Ceriotti, L., Consonni, R., *Electrophoresis* 2000, *21*, 909–916.

[29] Chiari, M., Cretich, M., Stastna, M., Radko, S. P., Chrambach, A., *Electrophoresis* 2001, *22*, 656–659.

[30] Albarghouthi, M. N., Stein, T. M., Barron, A. E., *Electrophoresis* 2003, *24*, 1166–1175.

[31] Doherty, E. A. S., Meagher, R. J., Albarghouthi, M. N., Barron, A. E., *Electrophoresis* 2003, *24*, 34–54.

[32] Horvath, J., Dolník, V., *Electrophoresis* 2001, *22*, 644–655.

[33] Rodriguez, I., Li, S. F. Y., *Anal. Chim. Acta* 1999, *383*, 1–26.

[34] Chiari, M., Nesi, M., Righetti, P. G., in: Righetti, P. G. (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, FL 1996, pp. 1–36.

[35] Wiktorowicz, J. E., United States Patent #50153250, 1991.

[36] Masselter, S. M., Zemann, A. J., *Anal. Chem.* 1995, *67*, 1047–1053.

[37] Masselter, S. M., Zemann, A. J., Bobleter, O., *Electrophoresis* 1993, *14*, 36–39.

[38] Chiu, R. W., Jimenez, J. C., Monnig, C. A., *Anal. Chim. Acta* 1995, *307*, 193.

[39] Erim, F. B., Cifuentes, A., Poppe, H., Kraak, J. C., *J. Chromatogr. A* 1995, *708*, 356–361.

[40] Cifuentes, A., Rodriguez, M. A., GarciaMontelongo, F. J., *J. Chromatogr. A* 1996, *742*, 257–266.

[41] Yao, Y. J., Li, S. F. Y., *J. Chromatogr. A* 1994, *663*, 97–104.

[42] Morand, M., Blass, D., Kenndler, E., *J. Chromatogr. B* 1997, *691*, 192–196.

[43] Katayama, H., Ishihama, Y., Asakawa, N., *Anal. Sci.* 1998, *14*, 407.

[44] Katayama, H., Ishihama, Y., Asakawa, N., *Anal. Chem.* 1998, *70*, 5272.

[45] Graul, T. W., Schlenoff, J. B., *Anal. Chem.* 1999, *71*, 4007–4013.

[46] Gelfi, C., Vigano, A., Ripamonti, M., Righetti, P. G., Sebastiano, R., Citterio, A., *Anal. Chem.* 2001, *73*, 3862–3868.

[47] Corradini, D., Cannarsa, G., *Electrophoresis* 1995, *16*, 630–635.

[48] Corradini, D., Cannarsa, G., Fabbri, E., Corradini, C., *J. Chromatogr. A* 1995, *709*, 127–134.

[49] Verzola, B., Gelfi, C., Righetti, P. G., *J. Chromatogr. A* 2000, *868*, 85–99.

[50] Oda, R. P., Madden, B. J., Spelsberg, T. C., Landers, J. P., *J. Chromatogr. A* 1994, *680*, 85–92.

[51] Janini, G. M., Chan, K. C., Barnes, J. A., Muschik, G. M., Issaq, H. J., *J. Chromatogr.* 1993, *653*, 321–327.

[52] Varghese, J., Cole, R. B., *J. Chromatogr.* 1993, *652*, 369–376.

[53] Yeung, K. K. C., Lucy, C. A., *Anal. Chem.* 1997, *69*, 3435–3441.

[54] Castelletti, L., Verzola, B., Gelfi, C., Stoyanov, A., Righetti, P. G., *J. Chromatogr. A* 2000, *894*, 281–289.

[55] Baryla, N. E., Lucy, C. A., *Anal. Chem.* 2000, *72*, 2280–2284.

[56] Emmer, A., Roeraade, J., *Electrophoresis* 2001, *22*, 660–665.

[57] Melanson, J. E., Baryla, N. E., Lucy, C. A., *Anal. Chem.* 2000, *72*, 4110–4114.

[58] Cunliffe, J. M., Baryla, N. E., Lucy, C. A., *Anal. Chem.* 2002, *74*, 776–783.

[59] Bruin, G. J. M., Chang, J. P., Kuhlman, R. H., Zegers, K., Kraak, J. C., Poppe, H., *J. Chromatogr.* 1989, *471*, 429–436.

[60] Nashabeh, W., El Rassi, Z., *J. Chromatogr.* 1991, *559*, 367–383.

[61] Nashabeh, W., El Rassi, Z., *J. Chromatogr.* 1993, *632*, 157–164.

[62] Nashabeh, W., Smith, J. T., El Rassi, Z., *Electrophoresis* 1993, *14*, 407–416.

[63] König, S., Welsch, T., *J. Chromatogr. A* 2000, *894*, 79–88.

[64] Ng, C. L., Lee, H. K., Li, S. F. Y., *J. Chromatogr. A* 1994, *659*, 427–434.

[65] Shao, X. W., Shen, Y. F., O'Neill, K., Lee, M. L., *J. Chromatogr. A* 1999, *830*, 415–422.

[66] Thorsteinsdottir, M., Isaksson, R., Westerlund, D., *Electrophoresis* 1995, *16*, 557–563.

[67] Pale-Grosdemange, C., Simon, E. S., Prime, K. L., Whitesides, G. M., *J. Am. Chem. Soc.* 1991, *113*, 12–20.

[68] Porsch, B., *J. Chromatogr. A* 1993, *653*, 1–7.

[69] Prime, K. L., Whitesides, G. M., *Science* 1991, *252*, 1164–1167.

[70] Ostuni, E., Chapman, R. G., Holmlin, R. E., Takayama, S., Whitesides, G. M., *Langmuir* 2001, *17*, 5605–5620.

[71] Chapman, R. G., Ostuni, E., Yan, L., Whitesides, G. M., *Langmuir* 2000, *16*, 6927–6936.

[72] Sagiv, J., *J. Am. Chem. Soc.* 1980, *102*, 92–98.

[73] Silberzan, P., Leger, L., Ausserre, D., Benattar, J. J., *Langmuir* 1991, *7*, 1647–1651.

[74] Fatunmbi, H. O., Bruch, M. D., Wirth, M. J., *Anal. Chem.* 1993, *65*, 2048–2054.

[75] Wirth, M. J., Fairbank, R. W. P., Fatunmbi, H. O., *Science* 1997, *275*, 44–47.

[76] Lee, S. W., Laibinis, P. E., *Biomaterials* 1998, *19*, 1669–1675.

[77] Cras, J. J., Rowe-Taitt, C. A., Nivens, D. A., Ligler, F. S., *Biosens. Bioelectron.* 1999, *14*, 683–688.

[78] Munro, N. J., Huhmer, A. F. R., Landers, J. P., *Anal. Chem.* 2001, *73*, 1784–1794.

[79] Cifuentes, A., Canalejas, P., Ortega, A., Diez-Masa, J. C., *J. Chromatogr. A* 1998, *823*, 561–571.

[80] Fairbank, R. W. P., Wirth, M. J., *J. Chromatogr. A* 1999, *830*, 285–291.

[81] Huang, M. X., Dubrovcakova-Schneiderman, E., Novotny, M. V., Fatunmbi, H. O., Wirth, M. J., *J. Microcol. Sep.* 1994, *6*, 571–576.

[82] Olivieri, E., Sebastiano, R., Citterio, A., Gelfi, C., Righetti, P. G., *J. Chromatogr. A* 2000, *894*, 273–280.