Review

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Microchannel wall coatings for protein separations by capillary and chip electrophoresis

The necessity for microchannel wall coatings in capillary and chip-based electrophoretic analysis of biomolecules is well understood. The regulation or elimination of EOF and the prevention of analyte adsorption is essential for the rapid, efficient separation of proteins and DNA within microchannels. Microchannel wall coatings and other wall modifications are especially critical for protein separations, both in fused-silica capillaries, and in glass or polymeric microfluidic devices. In this review, we present a discussion of recent advances in microchannel wall coatings of three major classes – covalently linked polymeric coatings, physically adsorbed polymeric coatings, and small molecule additives. We also briefly review modifications useful for polymeric microfluidic devices. Within each category of wall coatings, we discuss those used to eliminate EOF, to tune EOF, to prevent analyte adsorption, or to perform multiple functions. The knowledgeable application of the most promising recent developments in this area will allow for the separation of complex protein mixtures and for the development of novel microchannel wall modifications.

Keywords: Capillary electrophoresis / Chip electrophoresis / Microchannel wall coating / Review
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Abbreviations: DEA, *N,N*-diethylacrylamide; **DMA**, *N,N*-dimethylacrylamide; **epoxy poly(AG-AA)**, poly(acrylamide-*co*-allyl-β-D-glucopyranoside-*co*-allyl glycidyl ether); **GPTMS**, 3-glycidoxypropyltrimethoxysilane; **HEC**, hydroxyethylcellulose; **HPMC**, hydroxypropylmethylcellulose; **PDMA**, poly(*N,N*-dimethylacrylamide); **PDMS**, poly(dimethylsiloxane); **PEO**, poly(ethylene oxide); **PHEA**, poly(*N*-hydroxyethylacrylamice); **PMMA**, poly (methyl methacrylate; **PS**, polystyrene; **PSS**, poly(styrene sulfonate), **PETG**, poly(ethylene terephthalate glycol); **PVA**, poly (vinyl alcohol); **Q-PzI**, (*N*-methyl-*N*-ω-iodobutyl)-*N*'-methylpiperazine; **SMIL**, successive multiple ionic layer; **TEPA**, tetraethylpentamine; **TMBD**, *N,N,N*',*N*'-tetramethyl-1,3-butanediamine

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1 Introduction

1.1 General aspects

Capillary electrophoresis has emerged as a dominant bioseparation technique, playing a leading role in the Human Genome Project and the more than 525 other genome projects currently underway worldwide [1]. The more recent development of microfluidic devices will allow for faster [2], more efficient [3], and fully integrated [4–6] biological separations. During the development of both CE and microfluidic analysis systems, advancements in hardware [7] and software [8, 9] have outpaced advancements in soft materials that are essential for the integration of multiple procedures within a single apparatus. Polymers enabling protein extraction, purification, and efficient separation [2] will be critical for the success of proteomic analyses within these devices. This review will focus on developments in microchannel wall coatings and other wall modifications.

The necessity for microchannel wall passivation is well understood. The regulation of EOF and the prevention of analyte adsorption is essential for the rapid, efficient separation of proteins within microchannels. The prevention of basic (positively charged) protein adsorption during the separation of complex protein mixtures has proved to be especially challenging [10, 11]. Ideally, microchannel walls should be modified to allow for the separation of a mixture of acidic and basic proteins, but it may also be desirable to use the microchannel wall coating to "tune" the magnitude and direction of EOF for a specific separation requirement.

One issue that complicates the area of microchannel wall coatings is the lack of comparative quantitative data. Coating characterization techniques vary widely, making comparison difficult; the quantitative data currently available does not allow for meaningful comparisons between coatings produced in different laboratories. We hope that current and future researchers will realize the importance of comparative quantitative analysis and incorporate some of the analytical techniques presented in this review. More importantly, a standard characterization protocol should be established so that meaningful comparisons may be made.

In this review, we will briefly cover aspects of protein adsorption. Microchannel wall coatings are separated into three major categories – covalently linked polymeric coatings, physically adsorbed polymeric coatings, and small molecule additives. Within each category of wall coatings, some may be used to eliminate EOF, tune EOF, prevent analyte adsorption, or to perform multiple functions. This review will focus on wall coatings and modifications that have utility for protein separations (see [12–14] for reviews of coatings for DNA separations). In addition, we will briefly cover polymeric microchannel surface modification.

1.2 Mechanism and physics of protein adsorption

There is extensive literature on the adsorption of proteins to many types of surfaces, although many of the detailed studies of the mechanisms of protein adsorption have been performed on surfaces such as gold, mica, or TiO_2, *i.e.*, many surfaces other than fused silica. Some general trends should be applicable to all surfaces, although it is not likely that all of the detailed conclusions of studies made on one surface will be applicable to all other surfaces, or be necessarily relevant in the field of capillary electrophoresis. It is the intent of this section to give a broad overview of some of the interesting phenomena observed with protein adsorption to surfaces.

The interactions of proteins with surfaces can be of two general types: biospecific and nonspecific. Biospecific interactions, such as the interaction of an antibody with an antigen-bearing surface, or binding of avidin to a biotinylated surface, are highly specific, relying on a close complementarity between the protein and the surface. Such interactions can be exploited, for example, in immunoaffinity chromatography, where an antibody bound to a solid support is used to selectively remove a specific protein from a mixture. Nonspecific interactions, on the other hand, include forces such as electrostatic attraction or repulsion, hydrophobic interactions, and Van der Waals forces. One or more of these forces is always present between a protein and a surface, although the magnitude of the interaction will depend on the surface. A single protein may exhibit both biospecific and nonspecific interactions with surfaces – immunoglobulins, for example, are known for their strong affinity for a single antigen, but also show a marked tendency to adsorb nonspecifically on many surfaces. Although biospecific interactions are important and useful, the remainder of this discussion will focus exclusively on the challenging problem of nonspecific protein adsorption.

Despite substantial research effort, the understanding of nonspecific adsorption remains incomplete. Nonspecific interactions between surfaces and proteins usually in-

volve hydrophobic interactions [15], although electrostatic interactions may be present as well with charged or polar surfaces, or in the presence of an electric potential applied to the surface [16]. The process can be thought of in terms of several discrete steps [17]: (1) diffusion of protein from the bulk solution to the vicinity of the surface; (2) reversible adsorption on the surface; and (3) irreversible denaturing of the protein on the surface. Step 2, the reversible adsorption, can be further broken down into five subprocesses, which can be considered sequentially for thermodynamic purposes. The subprocesses are [18]: (a) removal of water molecules and ions from the electrical double layer surrounding the protein; (b) removal of water molecules and ions from the vicinity of the surface; (c) hydrophobic and other interactions between the protein and surface; (d) structural rearrangement of the protein upon adsorption (not necessarily the irreversible denaturing of step 3 above); and (e) structural rearrangement of excluded water molecules or ions in the bulk solvent. Consistent with these subprocesses, it is expected that the affinity of a protein for a surface will depend not only on the strength of the interactions between the protein and the surface (process c), but also the structure of the protein adopted on the surface (process d), and the ionic strength and nature of salts present in solution (processes a, b, and e).

Using the conceptual framework of these subprocesses, the free energy change of protein adsorption to hydrophobic chromatography supports was recently studied by equilibrium binding analysis (*i.e.*, adsorption isotherms) and isothermal titration microcalorimetry [18]. Lin *et al.* studied the interaction of lysozyme, myoglobin, and RNase A with hydrophobic surfaces bearing either butyl or phenyl groups, in the presence of either Na_2SO_4 or $(NH_4)_2SO_4$. Protein affinity for the surface was enhanced in the presence of Na_2SO_4 relative to $(NH_4)_2SO_4$. It was hypothesized that Na_2SO_4 enhances hydrophobic interactions between the protein and the surface, consistent with the greater molal surface tension increment ($d\gamma/dc_{salt}$) for this salt. The maximum binding capacity of the proteins depended on the density of hydrophobic ligands on the surface and the size of the protein molecules.

Microcalorimetry measurements in this study suggested that binding of proteins to the hydrophobic surfaces are largely entropically driven, with the $T\triangle S$ term contributing at least half of the free energy of binding even for exothermic (negative $\triangle H$) processes. Enthalpy of adsorption ranged from about 0 to -10 kJ/mol, whereas entropy of binding ranged from about 30 to 80 J/mol K, Processes (a)–(c) above, dehydration of the protein and surface, and hydrophobic interactions, made the largest contribution to the overall free energy changes, with additional favorable π–π interactions between the protein and the surface contributing to the enthalpy of binding in the case of the phenyl-derived surface.

These results should be generally applicable to hydrophobic surfaces. Polar or charged surfaces should show the same overall trend, although there may be some subtle differences. For a surface such as glass or silica, which can hydrogen-bond extensively with water, there will be an unfavorable enthalpy change, and perhaps a smaller gain in entropy, associated with dehydration of the surface. In addition, the nature of the interaction between the protein and the surface will be altered. Van der Waals forces will still be present, although localized permanent dipole-permanent dipole interactions may increase. In addition, electrostatic charge-dipole and charge-charge interactions, as well as hydrogen bonding between the protein and the surface, may become important, depending on the polar or charged side chains that are present at the interface. Because proteins typically have both positive and negative charges present on their surface, the overall effect on the enthalpy of interaction will depend on a balance between electrostatic attraction and repulsion.

Once adsorbed on a surface, protein molecules have two possible fates: desorption back into solution, or denaturing on the surface, which is essentially irreversible. Unlike small molecules, which display reversible, concentration-dependent equilibrium adsorption and desorption, the desorption of proteins and other macromolecules occurs as an "exchange process" [19]. If a surface that has been covered with adsorbed protein is placed into a pure solvent with no protein present, little or no desorption is detected. However, if the protein-coated surface is placed into a solution of the same protein, molecules from solution will adsorb and displace the pre-adsorbed species (this can be shown, for example, with radiolabeled proteins). Additionally, different proteins with different affinities for the surface can displace one another. Larger proteins tend to have higher affinities for a surface, but due to their large size have lower diffusion rates, and hence take a longer time to migrate to the surface. Small proteins tend to arrive first and adsorb, and are gradually displaced as the larger proteins with higher affinities for the surface arrive. This gradual displacement is termed the Vroman effect [17, 19, 20]. In the case of blood serum proteins, for example, albumin would be the first major protein to arrive at the surface and adsorb, followed by immunoglobulins, followed by fibrinogen, followed eventually by high molar mass clotting factors kininogen and prekallikrein [17].

Very little is known of the actual mechanism of the exchange between a molecule on the surface and a molecule in solution. Experimentally, the rate of displacement from the surface appears to be a first-order kinetic pro-

cess, as demonstrated by Ball [19] for immunoglobulin G (IgG) on a titanium surface. In particular, the rate of release of radiolabeled IgG from the surface (replaced by unlabeled IgG from solution) can be written as:

$$\frac{d\Theta}{dt} = -k(C_{bulk})\Theta^{\beta} \quad (1)$$

where Θ is the difference between the surface concentration of labeled IgG at time t and the surface concentration at infinite time, or $\Theta = \Gamma_{IgG^*}(t) - \Gamma_{IgG^*}(t = \infty)$. The dependence on $\Gamma_{IgG^*}(t = \infty)$ indicates that a certain fraction of the protein molecules are irreversibly adsorbed, and do not participate in the exchange reaction. The rate "constant" k was found to increase linearly with the concentration of unlabeled protein in bulk solution, C_{bulk}. The exponent β was determined experimentally to be about 1, consistent with a first-order process. Interestingly, the surface concentration of irreversibly bound protein, $\Gamma_{IgG^*}(t = \infty)$, decreased linearly as the bulk concentration of unlabeled protein increased. This effect has not yet been satisfactorily explained, but one hypothesis is that, at higher bulk concentrations, the proteins on the surface are displaced more quickly, and thus their average residence time on the surface is shorter.

Adsorbed proteins generally undergo conformational changes at surfaces. It has been shown, using techniques such as circular dichroism, that proteins on different surfaces experience substantial loss of α-helix or β-sheet content or other major secondary and tertiary structural elements [21–23]. The phenomenon is particularly notable with proteins adsorbed on hydrophobic surfaces, and can be attributed to the entropy gain resulting from protein or surface dehydration and conformational changes. It is believed that proteins initially form a relatively small number of contacts with the surface, but after adsorption they slowly undergo conformational changes that enhance binding to the surface. This is consistent with observations that the tendency to desorb or exchange decreases with increased residence time on the surface [19, 24]. It is very difficult to show conclusively that the two processes (denaturing and irreversible binding) are identical. However, it has been shown by Fourier transform infrared (FTIR) studies of RNase A on hydrophilic surfaces that irreversible binding occurs on the same time scale as conformational changes, such as a loss of β-structure and an increase in turns and disordered structure [25]. The time scales for the conformational changes were on the order of 20 h for RNase A on a TiO_2 surface, and 10–15 h on a Germanium surface. It was later shown that the time scale for surface denaturation of two proteins, RNase A and Apo-α-lactalbumin, correlated well with the free energy of thermal denaturation, and hence with the thermodynamic stability of the folded structure [25].

Intermolecular interactions and clustering of proteins on surfaces may be responsible for some of the equilibrium adsorption properties of proteins. Proteins often exhibit ideal, Langmuir-like adsorption isotherms. The Langmuir isotherm, however, assumes no interaction between molecules on the surface. Minton [26] has presented a statistical thermodynamic model for protein adsorption that incorporates excluded volume interactions, attractive forces between molecules, and formation of clusters. Depending on the magnitudes of these effects, and the type of packing assumed for the clusters, a range of behaviors can be predicted, including positive and negative cooperativity in binding, or apparently ideal behavior resulting from competing positive and negative effects. Minton hypothesizes that real systems with apparently ideal behavior may conceal a balance between attractive and repulsive interactions between adsorbed molecules.

A variety of models have been presented for the kinetics of protein adsorption. These range from simple models assuming first-order kinetics, with the rate of adsorption proportional to bulk concentration and availability of sites [19], to more complex models including the effects of denaturing [27], diffusion from the bulk to the surface [17], or cluster formation [28]. Experimentally, a range of behavior can be observed, although a strong history dependence is often observed. That is, the kinetic behavior for a particular surface depends on "where that surface has been," and specifically whether any irreversible adsorption transitions have occurred on the surface. Multistep kinetic experiments, in which a Si(Ti)O_2 surface was exposed to a series of cytochrome *c* solutions of different concentrations, including rinse steps, show that the rate of adsorption is sensitive to the structure of any existing adsorbed layer on the protein surface [29]. This behavior seems to be consistent with a recent kinetic model proposed by Minton [28], which accounts for two pathways for protein adsorption: either directly onto the surface, or onto the upper surface of a pre-existing cluster followed be rearrangement of the cluster. Experimentally observed behavior, such as noncooperative Langmuir-like kinetics, or positive cooperativity require that the second pathway is dominant.

2 Covalently linked polymeric wall coatings

The most prevalent, and perhaps the most effective, strategy for preventing biomolecule adsorption and improving resolution in the electrophoresis of proteins has been covalent wall modification. The suppression of EOF by uncharged silane reagents and covalently bound polymers has been attributed to at least three effects in the literature: the modification of the ζ-potential by elimination of ionizable Si-OH groups on the fused-silica surface,

"shielding" of remaining charged groups on the surface by polymer or substituents on the alkylsilane reagent, and the increase in viscosity in the layer of coating near the wall. The relative contribution of all of these effects should be related to both the thickness and density of the covalently bound layer. Covalent wall coatings can be divided into two categories: covalently bound polymeric wall coatings and covalently bound small molecules, such as alkylsilane reagents. The two categories overlap somewhat, as the first step in creating a covalently bound polymer coating is often the derivatization of the surface with a bifunctional reagent to anchor the coating to the wall.

2.1 Initial derivatization of the microchannel wall

Covalently bound polymer coatings for protein analysis have received more attention from researchers over the past two decades, although the earliest reports of protein analysis by capillary electrophoresis employed a simple silane reagent. Procedures for silanization of capillaries, including the preconditioning of the silica surface, type of silane reagent (*e.g.*, methoxysilanes *versus* chlorosilanes), and silanization solvent vary widely. By comparing numerous reports of EOF velocity measured by different groups following silanization, or coating with relatively thin polymer layers bound to a silane sublayer, it can be inferred that the density of wall coverage varies substantially from study to study.

Munro *et al.* [30] recently examined the silanization reaction in detail for preparing coatings for capillaries and microchannel devices, with the aim of producing robust, dense, and reproducible surface coverage. Their conclusions regarding the silanization step should translate well polymer coatings providing reduced or eliminated protein adsorption prepared by silanization. Munro *et al.* also combined an extensive review of the sometimes contradictory silanization literature with experimental testing of different procedures.

For pretreatment of the silica surface to produce a smooth reactive surface, the authors settled upon treatment with a 5:1:1 mixture of boiling water, ammonium hydroxide, and hydrogen peroxide as the most effective treatment, while their literature review suggests that treatment with 1 M NaOH is the least effective (although very common in preparing coatings for capillary electrophoresis). It is unclear whether hydrogen peroxide, an oxidizing agent, has an effect on the fully oxidized fused-silica surface. Based on work by Fairbank and Wirth [31], it was determined that equilibration with an atmosphere at 50% humidity provides the correct hydration of the silica surface for reaction with trichlorosilanes.

The choice of silanization solvent and silanizing agent also has a significant impact on the stability of the silanized microchannel surface. Toluene was chosen as the silanization solvent because of its higher boiling point and better environmental compatibility compared to other suitable organic solvents such as carbon tetrachloride or benzene [32–34]. Coatings prepared using toluene as the silanization solvent had superior stability compared to those prepared either with 95% ethanol, or with an aqueous solvent with an acetic acid catalyst. Monochlorooctylsilanes and trichlorooctylsilanes were compared; the trichloro reagents were cured at 150°C following coating to promote cross-linking of the silanes, whereas this step was not used for the monochloro reagents. The monochloro silane was found to more effectively reduce the number of silanol groups on the surface, and hence suppress EOF to a greater extent. It is not clear from the experimental data in this report if this was due to greater surface coverage by the monochloro silane, or because incomplete cross-linking of the trichlorosilane leads to the presence of free silanols at the surface [30]. Monochlorosilanes can provide denser surface coverage, but the cross-linking of di- and tri-chlorosilanes is known to improve the stability of the silane coating.

Diethylamine was tested as a catalyst for improving the coupling of silanes to the surface; it was found to increase surface coverage slightly, as evidenced by a slight reduction of EOF in the diethylamine-treated capillary compared to one that was not treated with diethylamine, but the effect was not considered significant. Huang *et al.* [35] 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 suppresses EOF by 24%, while a C_8 alkyl chain suppresses EOF by 65%. In addition, the density of packing affects EOF suppression; a mixed monolayer of C_1 and C_8 alkyl chains is more effective than a mixed layer of C_3 and C_8 alkyl chains, which is not able to pack as densely on the surface. "Capping" of any remaining accessible surface silanol groups with trimethylsilane, following the initial silanization with chlorooctylsilane, was also found to have only a small effect. This small effect may be attributed to incomplete capping of the silanol groups by trimethylsilane. During the polymerization step, the reaction mixture was buffered at pH 8 to prevent hydrolysis of the silane coating.

Borrowing an approach from liquid chromatography, Jorgenson and Lukacs [36] used 3-glycidoxypropyltrimethoxysilane (GPTMS) to create a thin coating with hydrophilic diol groups exposed to the solution. The information presented in this reference does not specify the exact nature of the coating. Jorgenson and Lukacs [37] refer to the technique of Regnier and co-workers, which

used GPTMS as a bifunctional reagent to derivatize silica supports, using the epoxide group as a point of attachment for further derivatization. A subsequent reference, however, Shao *et al.* [38] implies that GTPMS was used alone, with no further modification. Since the epoxide group would be unstable in aqueous solution, this would result in a coating with polar diol group exposed to solution. Various acidic and basic proteins were separated successfully, although peak efficiency was relatively low, and the stability of the coating was a problem. In addition, relatively high temperature (90°C) was required to create the coating. The coating retained a significant amount of EOF, which was beneficial in allowing for the elution of positively charged, neutral, and negatively charged analytes in a single experiment.

Silanization of the wall with GPTMS has served as the starting point for covalent attachment of various polymers [37, 39–41], although this reagent has again been reported recently with no farther modification to create a thin, covalently bound diol coating [38], seemingly with reasonably good results [38]. Following recommendations for optimization of the diol-bonding reaction for silica particles for chromatography [42], the coating was deposited from aqueous solution at room temperature, representing a simplification of the procedure from previous reports. This very thin coating suppressed EOF by $\sim$80% in the pH range of 5–10, indicating substantial derivatization of the surface silanols. Peak efficiencies between 500 000 and 1 000 000 theoretical plates per meter were obtained for a variety of basic and acidic proteins, with an RSD of $\sim$1.6% in migration time, based on 10 runs. The coating was stable for over 300 runs at pH $<$ 8, although it was not indicated whether this is valid for proteins, or for small biomolecules with less tendency for nonspecific adsorption.

2.2 Polyacrylamide wall coatings

2.2.1 Covalently bound, *in situ* polymerized polyacrylamide wall coatings

Covalent attachment of a polymer to a capillary for the separation of proteins was first demonstrated by Hjertén in 1985 [43] by *in situ* polymerization of acrylamide. The capillary wall was first derivatized with the bifunctional silane reagent γ-methacryloxypropyltrimethoxysilane (MPTS), leaving an acrylic group exposed on the surface of the capillary. The capillary was then filled with acrylamide, TEMED, and potassium persulfate. The free acrylic group exposed on the capillary surface serves as an anchor for growing chains of linear polyacrylamide. The coating was successful at suppressing EOF and allowing separation of proteins. Although this coating provides a hydrophilic surface that is able to suppress EOF, *in situ* polymerization of the acrylamide monomer results in the generation of numerous unattached polymer chains, which must be forced from the capillary under high applied pressure. The formation of ester bonds during silanization using MPTS also limits the stability of the coating.

Many subsequent covalent coatings have followed essentially this protocol, using a bifunctional silanization reagent to anchor the *in situ* polymerized coating to the wall. Such coatings have been described in detail in other reviews on the subject [14, 44]. Later, 7-*oct*-1-enyltrimethoxysilane, which lacks the hydrolyzable ester bonds of MPTS, was also used [45] for attachment of polyacrylamide to the microchannel wall. The double bond of 7-*oct*-1-enyltrimethoxysilane is less reactive than the acrylic group of MPTS. The conditions used for polymerization (initiation with azobisisobutylonitrile, temperature ramp to 120°C and maintain at 120°C for 2 h) are not particularly harsh, but still appear to have resulted in a stable and reproducible coating. Unlike previous procedures, which were carried out in aqueous solution, the polymerization was carried out in methylene chloride. Since acrylamide monomer has limited solubility in methylene chloride, this procedure may afford better control over the rate of polymerization, resulting in a more reproducible coating procedure.

In addition to MPTS and 7-*oct*-1-enyltrimethoxysilane, Grignard reagents [46], or catalytic hydrosilylation of olefins on a Si-H containing substrate [47] have been used to bond carbon directly to the topmost layer of the cross-linked Si-O surface, creating direct Si-C bonds. These direct Si-C bonds should be more stable to hydrolysis than the uncross-linked Si-O-Si-C bonds formed with conventional alkylsilane reagents. The approach of catalytic hydrosilylation has been used to create γ-methacryloxypropyl-modified surfaces suitable for polymerization with acrylamide and acrylamide derivatives [48, 49], such as poly(acryloylaminoethoxyethanol) (poly(AAEE)) [50] and poly[(*N*-acryloylamino)ethoxy]ethyl-β-D-glycopyranose (poly(AEG)) [51].

2.2.2 Surface-confined living radical polymerization of acrylamide

The importance of creating a smooth, homogeneous coating was recently demonstrated by Cifuentes *et al.* [52]. A series of capillaries were coated with linear polyacrylamide, polymerized *in situ.* The capillaries were subsequently fragmented, and the topography of the inner surfaces of the different capillaries was probed by atomic force microscopy. A coated capillary with a smooth inner

surface provided excellent separation of basic proteins, while a capillary with a rough, pitted, or bumpy surface provided poor separation, with tailing peaks, indicating strong interaction with the walls. The rough surface in the second capillary was thought to be due to the presence of oxygen during the polymerization reaction, which inhibits the persulfate-driven polymerization reaction.

Surface-confined living radical polymerization has been reported by Huang *et al.* [53, 54] as an approach for creating a uniform coating of monodisperse polymer chains. In this process, a benzyl chloride monolayer was deposited on the capillary surface by silanizing with 1-trichloro-2-(*m-p*-chloromethylphenyl)ethane. The surface-bonded benzyl chloride groups form radicals by atom transfer to a Cu-$(bpy)_2Cl$ catalyst; initiation of polymerization is thus localized on the surface of the capillary. Although conventional free radicals are short-lived, reacting rapidly to form a more stable product, living radical polymerization *via* an atom transfer reaction allows for the availability of free radicals to be controlled by the equilibrium between dormant and active polymer chain ends. Linear and cross-linked polyacrylamide coatings were produced by this technique [54]. Atomic force microscopy showed that the linear polyacrylamide coating was completely smooth and uniform; the cross-linked coating was more rough, but still significantly more uniform than a coating produced by the "conventional" solution polymerization of acrylamide. Both coatings provided high-efficiency separations of proteins, and the coatings were stable for at least 150 runs (see Figs. 1 and 2).

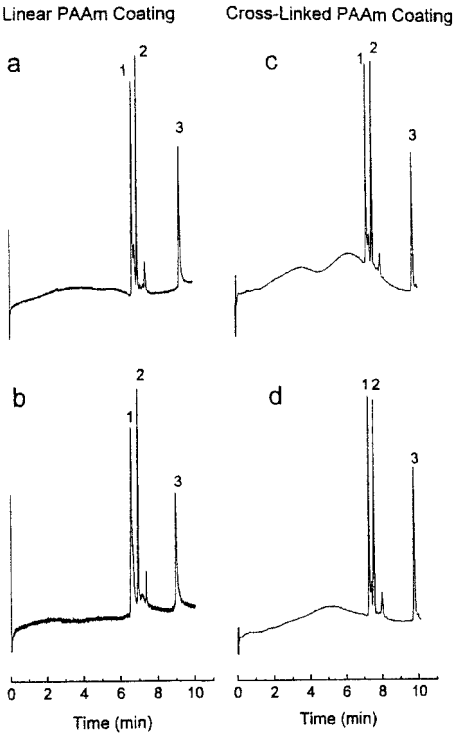


Figure 1. Reproducibility of the separation of three basic proteins performed in capillaries having surface-confined living radical polymerized polyacrylamide wall coatings: (a) LPA, fourth run, (b) LPA, 150th run, (c) cross-linked polyacrylamide, fourth run, (d) cross-linked polyacrylamide, 150th run. Sample concentration: 100 ppm each of (1) cytochrome *c*, (2) lysozyme, and (3) ribonuclease A. Separation conditions: capillary, 75 μm ID, 53 cm total length (30 cm effective); injection, 0.5 psi, 2 s; buffer, 50 mM acetate, pH 4.5; separation, 377 V/cm. Reprinted from [54], with permission.

2.2.3 Covalent attachment of preformed polymers

Other approaches to wall coatings have not relied on *in situ* polymerization, but rather on attachment of fully formed polymers to the capillary wall [55]. Covalent attachment of preformed polymers allows for *a priori* knowledge of polymer physical properties, resulting in an additional level of quality control that is not possible for *in situ* polymerized coatings.

More recently, Srinivasan *et al.* [56] created densely cross-linked layers of polyacrylamide using a simple procedure involving preformed polymers. The capillary was first silanized with (3-methacryloxyproply)trimethylsilane or chlorodimethyloctylsilane; then the capillary was filled with a polymer solution containing TEMED and ammonium persulfate or 4,4'-azobis(4-cyanopentanoic acid). Polymers studied by Srinivasan *el al.* include polyacrylamide, poly(vinylpyrrolidone) (PVP) and poly(ethylene oxide) (PEO). The capillary was sealed and baked at 80°C for 18 h. Under these conditions, the linear polymers are thought to cross-link *via* a hydrogen abstraction mechanism. The preformed polymers were simultaneously cross-linked, as evidenced by separate solution-phase experiments, and coupled to the silanized surface, and excess (unattached) polymer was flushed out. In the case of the cross-linked PVP coatings, there was not a significant difference in the separation efficiency of basic proteins analyzed in a capillary silanized with (3-methacryloxypropyl)-trimethylsilane and one silanized with chlorodimethyl-octylsilane. This is somewhat surprising due to the differences in functionality and hydrophobicity between the two silanization reagents. Microchannel wall coatings composed of preformed polymers provided high-efficiency separation of basic proteins; a similar coating based on a cationic acrylamide derivative was used to separate acidic proteins under strong anodal EOF [56].

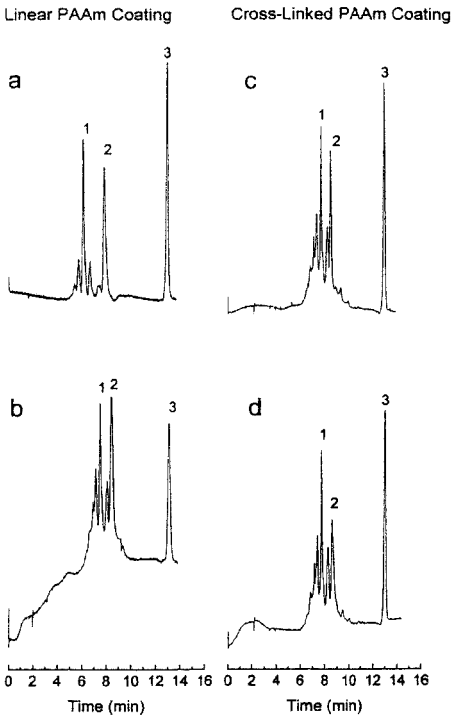


Figure 2. Reproducibility of the separation of three acidic proteins performed in capillaries having surface confined living radical polymerized polyacrylamide wall coatings: (a) LPA, first run, (b) LPA, 40th run, (c) cross-linked polyacrylamide, first run, (d) cross-linked polyacrylamide, 40th run. Sample concentration: 100 ppm each of (1) trypsin inhibitor, (2) β-lactoglobulin B, and (3) α-lactalbumin. Separation conditions: capillary, 75 μm ID, 53 cm total length (30 cm effective); injection, 0.5 psi, 2 s; buffer, 20 mM TAPS, adjusted to pH 8.8 with 2-amino-2-methyl-1,3-propanediol; separation, 283 V/cm. Reprinted from [54], with permission.

2.2.4 Covalently bound wall coatings without silanization

Additional approaches to creating covalently bound polymer coatings do not rely on an initial silanization with alkylsilanes. In an attempt to create a more stable substrate for attachment of polyacrylamide, the capillary surface was statically coated with polymethylvinylsiloxanediol, which was then cross-linked to give a more stable, highly cross-linked polyvinylsiloxanediol sublayer [57]. The exposed vinyl chains then served as points of attachment for a cross-linked polyacrylamide coating. Creating the cross-linked sublayer had the benefit of increased mechanical strength, which stabilized the coating against the high shear force necessary to flush excess, non-grafted polyacrylamide from the capillary. The coating gave excellent efficiency for protein separation and long lifetimes. The techniques that have been developed for covalent attachment of polymers to the microchannel wall may also be applied to numerous other monomers. We will briefly summarize a few of the most relevant covalent coatings used for protein separations.

2.3 Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA), a highly hydrophilic polymer, is expected to be resistant to hydrophobic protein adsorption. PVA was first employed both as a dynamic buffer additive and as a physically adsorbed coating by Gilges *et al.* in 1994 [58]. PVA as a physically adsorbed coating will be discussed later; the static PVA coating was created by a simple process of thermal immobilization, in which the capillary was filled with a solution of PVA (M_w ~ 50 000 g/mol), and slowly emptied, leaving a layer of physically adsorbed PVA. The capillary was then baked, causing precipitation of insoluble crystalline PVA on the capillary wall. This permanent coating was stable and effective for separation of proteins; however, since the PVA was initially adsorbed rather covalently bound, the coating procedure was prone to errors. PVA coatings have been employed by other researchers as well; Karger *et al.* [59] have recently patented a coating based on covalently attached PVA using silanization of the capillary surface. The patented PVA coating is a significant improvement over the coating developed by Gilges *et al.* The use of an initial, *in-situ*-polymerized poly(vinyl acetate) layer, which is later hydrolyzed to form the final PVA coating, provides a more stable and higher-performance coating.

Belder *et al.* [60] have recently reported a simple new procedure for generating a highly stable, cross-linked PVA coating using glutaraldehyde as a cross-linking reagent. The capillary is first flushed with acidified glutaraldehyde, and then flushed with an acidified PVA solution. The cross-linking of PVA occurs at the interface between the glutaraldehyde and PVA solutions; the increase in viscosity and hydrophobicity of the cross-linked PVA causes it to deposit in a uniform layer on the capillary surface. The capillary is then dried by heating the capillary and flushing the lumen with nitrogen. The cross-linked coating suppressed EOF and showed excellent performance for separation of basic proteins, and excellent stability, with a lifetime of greater than 1000 runs (see Fig. 3).

This coating also provided a dramatic reduction in EOF, compared to untreated capillaries, over a wide pH range (3–10). More importantly, this coating showed little or no pH hysteresis, suggesting that silanols remaining on the capillary surface are not strongly affected by changes in buffer pH. From a practical standpoint, the lack of pH hysteresis indicates that a single capillary coated using the method suggested by Bolder *et al.* may be used for multi-

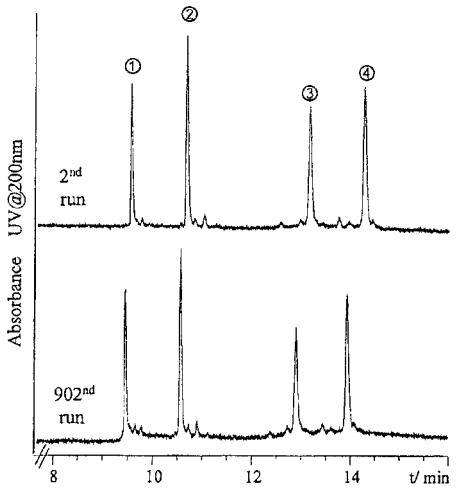


Figure 3. Second and 902nd run in long-term CE separations of basic proteins performed in capillaries coated with cross-linked, covalently bound PVA. Sample concentration: 50 ug/mL each of (1) cytochrome C, (2) lysozyme, (3) trypsinogen, (4) α-chymotrypsinogen. Separation conditions: capillary, 50 μm ID, 48.5 cm total length (40 cm effective); injection, 3 kV, 5 s; buffer, 40 mM sodium phosphate; separation, 309 V/cm. Reprinted from [60], with permission.

ple analytical protocols. It is unclear, from this work, whether the coating would have the same dramatic stability at basic pH.

2.4 Poly(ethylene oxide)

As early as 1989, Bruin *et al.* [61] attached short poly (ethylene glycol) (PEG) chains (M_w = 600 g/mol) to a capillary that had been silanized with GPTMS [61]. A similar approach was also employed by Nashabeh and El Rassi [62], 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 to create tunable EOF, or step changes in EOF in coupled capillaries [63, 64]. Huang *et al.* [65] created cross-linked layers of a copolymer of hydroxypropylcellulose (HPC) and hydroxyethylmethacrylamide (HEMA) by adsorbing fully formed HPC with HEMA monomer on a surface derivatized with 7-*oct*-1-enyltrimethoxysilane.

2.5 Covalently bound oligourethanes

In another novel approach, König and Welsch [66] have created covalently bound oligourethane coatings able to retain relatively strong EOF, which could be tuned and reversed by adjusting buffer pH. The oligourethane coating was created by first silylating the wall with urethane-terminated silane, synthesized by reacting 3-isocyanatopropyltriethoxysilane and 1,4-butanediol. The oligomeric urethanes were then built up, step by step, by flushing the capillary alternately with toluene-2,4-diisocyanate and a diol or polyol. Using a diol allowed creation of a linear oligourethane, whereas more highly branched structures could be created with polyols. Separations of some acidic proteins were improved relative to uncoated capillaries, although not dramatically, with efficiencies between 33 700 and 129 000 theoretical plates per meter. The lifetime of the coating was 160 runs at pH 9, although the authors indicated that this may be increased by modifying the initial silanization step of the coating procedure.

3 Physically adsorbed polymeric coatings

Polymers that physically adsorb to the microchannel wall to form a coating for EOF suppression or regulation and prevention of analyte adsorption have several distinct advantages over covalently linked polymer coatings. These advantages include (i) the simplicity of coating formation, (ii) the possibility of coating regeneration, (iii) access to *a priori* knowledge of coating polymer properties, and (iv) a lessened dependence of the coating process on microchannel wall chemistry. These adsorbed polymer coatings may be neutral, positively charged, or when adsorbed on the surface of a cationic polymer layer, negatively charged.

In our discussion of adsorbed coatings, we include coatings that are used with polymer in the running buffer ("dynamic" adsorbed coatings), coatings employed without polymer present in the running buffer ("static" adsorbed coatings), and coatings that are "regenerated" by acid or base washes and then reformed using another dilute polymer solution. An ideal adsorbed coating would not require polymer to be present in the run buffer and would be simple to "regenerate", that is, to remove from the microchannel wall and replace with a fresh layer of polymer.

Indeed, as mass spectrometry (MS) becomes the dominant technique with which protein mixtures are studied, the use of dynamic adsorbed coatings could be problematic. Polymeric additives, small-molecule additives, or additional salt in the running buffer may complicate on-line MS analysis of proteins separated by CE, since these would act as contaminants for the MS analysis. The possible benefits of these types of buffer additives must be weighed against the drawbacks of sample contamination and resulting ionization problems.

Polymers are frequently used for the adsorptive coating of microchannels employed in biomolecule analysis. Typically, the capillary is first flushed with various concentrations of NaOH in an attempt to remove surface contaminants [67], and then flushed with a dilute aqueous solution containing the polymer. A small amount of the polymer may or may not be used in the running buffer to maintain the coating. The adsorbed polymer blocks most surface adsorption sites; ideally, the protein does not displace the adsorbed polymer or adsorb directly on top of it.

Since it has been hypothesized by many authors that microchannel wall coatings suppress or regulate EOF as well as prevent protein adsorption, it is important that both of these effects are quantifiable. The mobility of EOF (μ_{EOF}) may be quantified in capillaries by the method of Williams and Vigh [68] or in microchannels by the method of Huang *et al.* [69], but the quantification of protein adsorption on surfaces is more complex.

3.1 Quantitation of protein adsorption on microchannel wall coatings

In a comprehensive study, Verzola *et al.* [70] used a novel procedure involving the use of SDS to desorb myoglobin adsorbed on a microchannel wall coating to test the effectiveness of four polymers as microchannel wall coatings: hydroxypropylmethylcellulose (HPMC, $M_r = 1 \times 10^6$ g/mol), hydroxyethylcellulose (HEC, M_r = 27 000 g/mol), PVA (M_r = 49 000 g/mol), and poly(*N,N*-dimethylacrylamide) (PDMA, $M_r = 1.5 \times 10^5$ g/mol) (see also Section 4.2). The HPMC, PDMA, and PVA were effective at 0.005 to 0.02%, whereas HEC was most effective at 0.1–0.8%. Relative to some of the other additives this group has examined, *e.g.*, oligoamines, none of the polymers studied were fully effective, inhibiting protein adsorption by 50% at most. This quantitation method may also be used to determine the driving forces that govern protein adsorption. Trace analysis, which may be critical for the study of extremely dilute samples, should be possible using this technique. Despite the finding that adsorbed polymers are relatively ineffective at eliminating protein adsorption on the microchannel surface, numerous researchers have found that adsorbed polymers do improve resolution in capillary electrophoresis of proteins. It seems that the primary function of the adsorbed coatings is to suppress EOF, which also serves to improve resolution.

3.2 Uncharged adsorbed coatings

Neutral polymers have been widely used for suppression of EOF for analysis of DNA and other biomolecules. They are, however, a bit more problematic for protein separations. Hydrophilic polymers, such as methylcellulose or polysaccharides, do not adsorb strongly to the capillary surface, resulting in a coating that is easily rinsed away [71]. More hydrophobic polymers such as PVP or poly(*N,N*-dimethylacrylamide) (PDMA) form more stable adsorbed coatings, but are more likely to interact with hydrophobic patches on the surfaces of folded proteins, reducing separation efficiency [10, 13, 51].

3.2.1 Poly(vinyl alcohol)

PVA has been demonstrated as a dynamic adsorbed coating as well as a covalent coating [58]. In one approach, PVA, $M_w \sim 50\,000$ g/mol, is added to the running buffer at low concentrations (0.05 w/w%). A few capillary volumes of the PVA-containing buffer are flushed through the capillary prior to analysis, forming an initial adsorbed layer of PVA. The dynamic PVA coating effectively suppresses EOF below pH 8. Above pH 8, the EOF begins to increase, although it is still diminished with respect to uncoated capillaries. The dynamic PVA coating performed as well as or better than the thermally immobilized, covalently bound PVA developed by Gilges *et al.* in 1994 [58], with significantly less effort to generate the coating, but this adsorbed PVA wall coating is effective at low pH only (see Fig. 4). Again, the addition of PVA to the running buffer is likely to complicate downstream MS detection of proteins separated by CE.

Gilges *et al.* [58] also compared PVA to three other hydrophilic, hydroxyl-bearing polymers, including dextran, HEC, and HPMC. The dynamic PVA coating provided the

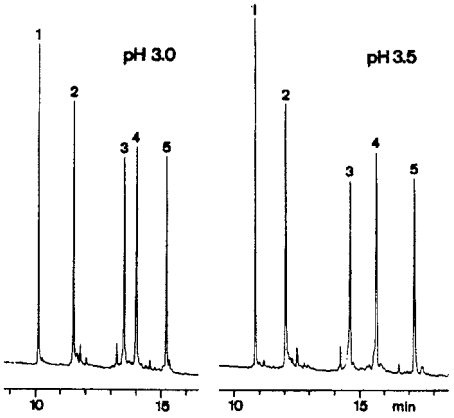


Figure 4. Separations of basic proteins in a capillary adsorptively coated with PVA. Sample concentration: 0.2 mg/mL each of (1) cytochrome *c*, (2) lysozyme, (3) trypsin, (4) trypsinogen, (5) α-chymotrypsinogen. Separation conditions: capillary, 50 μm ID, 70 cm total length (57 cm effective); injection, 10 kV, 5 s; buffer, 70 mM sodium phosphate; separation, 429 V/cm; temperature, 20°C. Reprinted from [58], with permission.

poorest peak shape and efficiency when separating a mixture of basic proteins, while HPMC gave the best performance, with peak efficiencies of 1.5 million theoretical plates per meter. The dextran was hypothesized to be too highly water-soluble, and thus less strongly adsorbed to the surface than HPMC. All of the hydroxylic polymers were ineffective as dynamic coatings above pH 5 due to insufficient adsorption of the polymer on the capillary surface. Interestingly, the dynamically adsorbed polymers performed differently in capillary tubing from different suppliers. HEC performed reasonably well in capillary tubing from Polymicro Technologies (Phoenix, AZ, USA), whereas it did not perform well in capillary tubing from MicroQuartz (Munich, Germany). PVA, in contrast, displayed the opposite trend with respect to the capillary source. This dependence on surface properties from different suppliers was not an issue for covalently bound PVA coatings.

3.2.2 Poly(ethylene oxide)

Polyethylene oxide (PEO) has been studied as a microchannel wall coating for the suppression of EOF and the separation of basic proteins at pH 3–7 [72, 73]. PEO is believed to coat the surface by hydrogen bonding with the surface silanol groups. Microchannel wall coatings composed of PEO have been shown to provide better protein separation efficiency than capillaries coated with HEC and HPC. The coating was accomplished by first rinsing the capillary with 1 M HCl, followed by flushing with a 0.2 w/v% solution of PEO with $M_r = 8 \times 10^6$ g/mol in 0.1 M HCl, followed by a rinse with buffer. This technique of coating by adsorption prior to analysis proved to be more effective than dynamically coating with PEO in the running buffer, and the coating could be regenerated by repeating this procedure. PEO was modestly effective at suppressing EOF, reducing μ_{EOF} by one order of magnitude at pH 7. The coating is unstable at higher pH, and could be slowly degraded over the course of a long run by the increase in pH at the cathode due to electrolysis of the buffer.

3.2.3 Pluronic (PEO-PPO-PEO triblock copolymers)

Pluronic polymers (PEO-PPO-PEO triblock copolymers) have been effective at suppressing EOF and improving the separation efficiency of proteins [74]. A variety of different copolymers, with varying ratios of the sizes of the PEO and poly(propylene oxide) (PPO) blocks, and varying overall molecular weight, were tested. The most effective triblock tested, F-108, had the average structure $PEO_{129}PPO_{56}PEO_{129}$, and $M_r \sim 14\,000$ g/mol. The coating procedure required first silylating the capillary with alkyl di- or trichlorosilanes. This provides a hydrophobic surface to which the PPO segment of the polymer adsorbs, leaving the more hydrophilic PEO segments exposed to the solution [75]. It is critical that coverage of the alkyi di- or trichlorosilane layer is maximized in order to prevent irreversible protein adsorption to the silane layer *via* hydrophobic interactions. The F-108 Pluronic provided a 60-fold reduction in EOF at pH 6, and plate efficiencies in excess of 600 000 theoretical plates per meter for separation of cytochrome *c*. Compared to some other adsorbed coatings, the procedure is fairly slow, requiring 3 h for silylation of the capillary, followed by 2 h for adsorption of the polymer. No mention was made of the stability or the possibility of regeneration of the Pluronic coating.

3.2.4 Epoxy-bearing, acrylamide-based copolymers

Chiari *et al.* [13] describe two novel epoxy-bearing, acrylamide-based copolymers for use as adsorbed microchannel wall coatings, poly(acrylamide-*co*-allyl-β-D-glucopyranoside-*co*-allyl glycidyl ether) (epoxy poly(AG-AA)) [13] and epoxy poly(dimethylacrylamide) (EPDMA) [11]. These polymers were synthesized with small amounts of allyl glycidyl ether, representing approximately 2 mol% of the total monomer used, leading to one epoxy group per 108 carbonyl groups (0.9% epoxy-bearing monomer units) in the polymers, Epoxide groups are unstable, and ring-opening reactions are rapidly catalyzed under either acidic or basic conditions [76]. It is unlikely that epoxide groups would persist long in aqueous solution, being rapidly converted to diol groups, or perhaps reacting with unprotonated silanol groups on the capillary wall. Epoxypoly(AG-AA) was used for efficient separations of acidic and basic proteins (see Fig. 5). The epoxy groups, pendant from the polymer backbone, dramatically improve adsorption to the capillary surface, relative to poly(AG-AA) with no epoxy groups. The adsorption mechanism of these epoxy-type polymers is not well understood, although the authors hypothesize that both strong hydrogen bonds between wall silanols and the epoxide groups on the polymer, and cross-linking of chains bearing sugar hydroxyls in the case of epoxy poly(AG-AA), play a role.

The epoxy-type polymers are adsorbed using a simple and quick procedure, requiring prerinsing with 0.1 M NaOH and water, followed by flushing with the polymer solution for 10 min. The adsorption of epoxy poly(AG-AA) is strong enough that no additional polymer is necessary in the running buffer to maintain the coating, and the coating could be regenerated with relative ease. In the case of EPDMA, adsorption is apparently as strong as regular

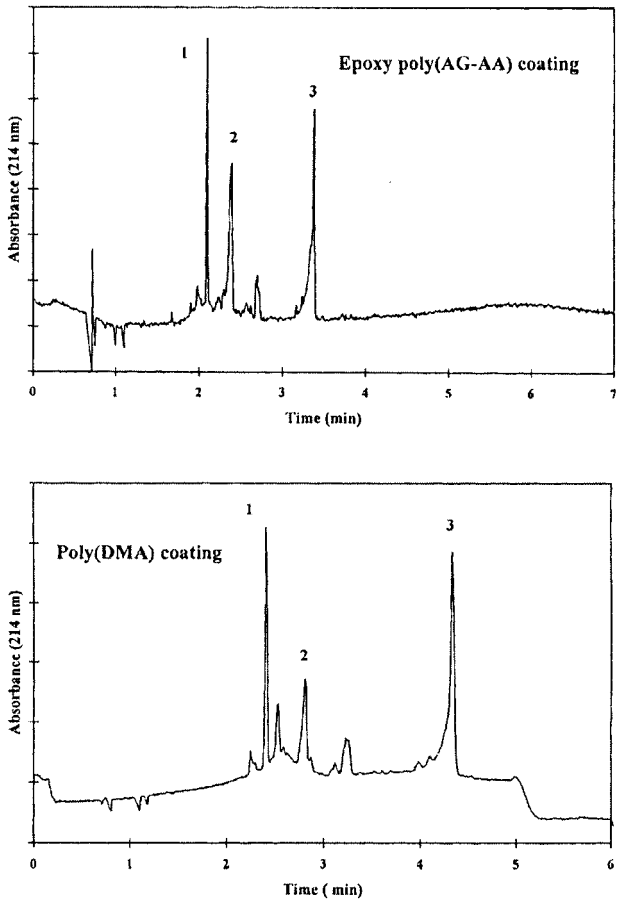


Figure 5. Electropherogram of acidic proteins obtained in PDMA (top) and epoxy poly(AG-AA) coatings. Sample concentration: 0.1 mg/mL each of (1) trypsin inhibitor, (2) β-lactoglobulin A, (3) α-lactalbumin. Separation conditions: capillary, 50 μm ID, 38 cm total length (30 cm effective); injection, 21 kV, 1 s; buffer, 25 mM bicine adjusted to pH 8.5 with Tris; separation, 500 V/cm. Reprinted from [13], with permission.

PDMA, and both epoxide-bearing coatings were as effective as PDMA in suppressing EOF. Performance of EPDMA in analysis of *R*-phycoerythrin (PHYCO) was equivalent to covalently bound polyacrylamide; however, the EPDMA was unstable at certain conditions (low ionic strength, presence of SDS, or presence of polystyrene carboxylate), at which covalently bound polyacrylamide wall coatings were stable.

3.2.5 Poly(*N*-hydroxyethylacrylamide)

The novel, acrylamide-based polymer poly(*N*-hydroxyethylacrylamide) (PHEA) (polyDuramide™) has been shown to combine high hydrophilicity and the ability to physically adsorb to the microchannel wall, properties that are

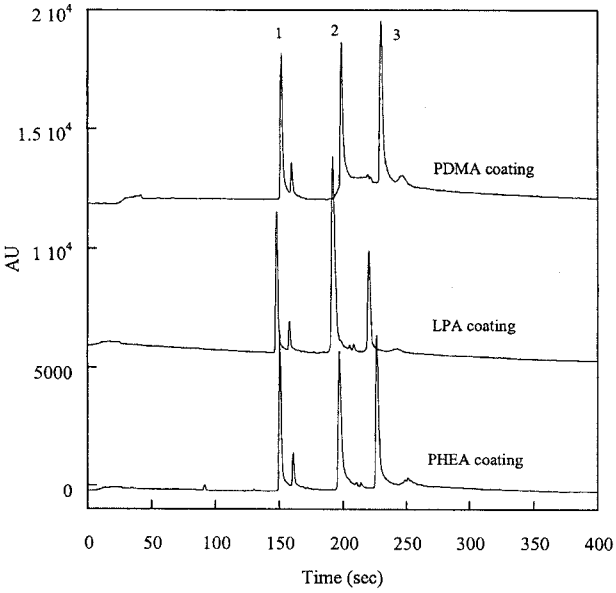


Figure 6. Basic protein separation in adsorbed PHEA-, adsorbed PDMA-, and covalent LPA-coated capillaries. Sample concentration: 0.1 mg/mL each of (1) cytochrome *c*, (2) ribonuclease A, (3) myoglobin. Separation conditions: capillary, 50 μm ID, 25 cm total length (20 cm effective); injection, 0.5 psi, 2 s; buffer, 25 mM acetate buffer, pH 4.4; separation, 500 V/cm; temperature, 25°C. Reprinted from [77], with permission.

ideal for wall coatings for protein separations. Using 1 M HCl to protonate the silanol surface prior to the adsorption step, Albarghouthi *et al.* [77] report that the EOF can be maintained at 5.3×10^{-10} m^2/V s for 50 runs at pH 4.4. The separation of basic proteins in a capillary adsorptively coated with PHEA is excellent when compared with separations performed in a capillary covalently coated with linear polyacrylamide (LPA) or adsorptively coated with PDMA (see Fig. 6). The migration times and the relative standard deviations listed in Table 1 indicate that physically adsorbed PHEA is as effective as covalently

Table 1. Migration time and migration time reproducibility (n = 50) of basic proteins separated in capillaries coated with PHEA (adsorbed), PDMA (adsorbed), and LPA (covalently bound)

Protein	PHEA (adsorbed)		PDMA (adsorbed)		LPA (covalent)	
	t (min)	RSD (%)	*t* (min)	RSD (%)	*t* (min)	RSD (%)
Cytochrome *c*	2.49	0.33	2.52	2.13	2.47	0.31
Ribonuclease A	3.27	0.37	3.28	3.66	3.19	0.43
Myoglobin	3.76	0.62	3.81	4.27	3.66	0.41

Separation conditions as shown in Fig. 6. Adapted from [77]

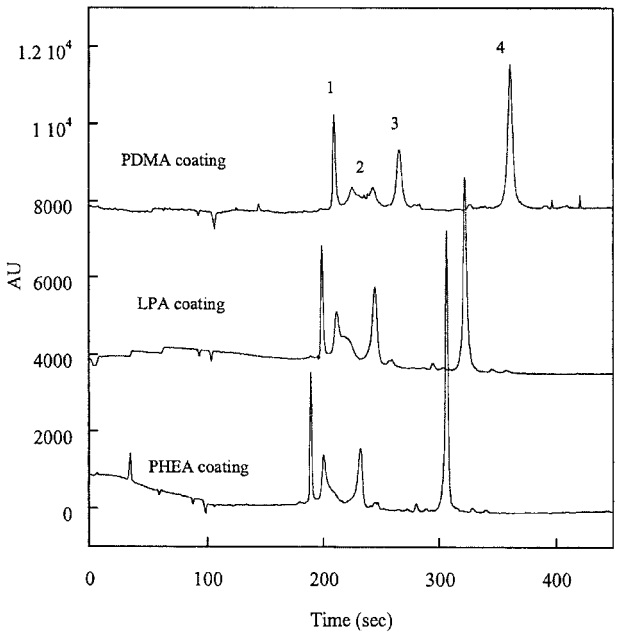


Figure 7. Acidic protein separation in adsorbed PHEA-, adsorbed PDMA- and covalent LPA-coated capillaries. Sample concentration: 0.1 mg/mL each of (1) trypsin inhibitor, (2) β-lactoglobulin A, (3) β-lactoglobulin B, (4) α-lactalbumin. Separation conditions: capillary, 75 μm ID, 53 cm total length (30 cm effective); injection, 0.5 psi, 2 s; Buffer, 25 mM Tris-Bicine buffer, pH 8.4; separation, 500 V/cm; temperature, 25°C. Reprinted from [77], with permission.

bound LPA in providing reproducible protein separations. The separation of acidic proteins also indicates that the performance of physically adsorbed PHEA wall coatings may be favorably compared to the performance of covalently bound LPA coatings. As shown in Fig. 7, the β-lactoglobulin A peak was split into two small peaks in PDMA-coated capillary and tailed severely in the LPA-coated capillaries. In the case of β-lactoglobulin A, as well as the other acidic proteins studied by Albarghouthi *et al.*, the best peak shapes were obtained using capillaries coated with an adsorbed PHEA coating. Although the use of neutral polymers as adsorbed coatings has resulted in excellent separations of both acidic and basic proteins, the use of a charged polymer as an adsorbed coating may also be used to tune EOF to a desired value or to improve the separation efficiency of a protein mixture by electrostatically repelling the proteins from the microchannel wall.

3.3 Charged adsorbed coatings

Charged polymers have also been used as adsorbed coatings, both to tune EOF and to improve protein separation. Most of the charged polymeric capillary coatings that have been reported are cationic in nature, probably because a positively charged surface enables analysis of basic proteins and other positively charged analytes that could not be analyzed in an uncoated capillary with a negatively charged surface. Because the fused-silica capillary is itself negatively charged, a layer of positively charged polymer can be adsorbed quite strongly. If a more "well-controlled" negatively charged surface is desired, a base layer of a polycation can be deposited first on the negatively charged silica surface; the polyanion can then be added on top of the polycation layer. Charged polymers can affect both the magnitude and the sign of the ζ-potential at the interface between the polymer layer and the bulk solution, and thus influence the magnitude and direction of EOF. In particular, cationic polymer coatings can provide a strong reversed EOF, from cathode to anode.

The use of charged polymer layers, while simple to produce and excellent for the analysis of a protein mixture of a single type (acidic or basic), is not practical for the separation of highly complex or unknown protein mixtures. Proteins or other biomolecules have the potential to irreversibly adsorb to the oppositely charged microchannel wall. This may be prevented by varying the ionic strength of the running buffer. In order to screen the interactions of analytes with the wall, buffers having high ionic strength must be used, which are generally undesirable for use in CE.

3.3.1 Cationic adsorbed coatings

A variety of positively charged polymers have been used both as statically and dynamically adsorbed wall coatings for analysis of basic proteins. Among the most popular is Polybrene, which is sold commercially as a capillary treatment. Polyarginine used as a dynamic coating was shown to perform similarly to Polybrene, giving high efficiency separations for a variety of basic proteins, with plate efficiencies in excess of 2 million theoretical plates per meter at pH 5; however, the polyarginine coating was significantly less stable than Polybrene at low and high pH (less than 4 or greater than 9) [78].

Polyethyleneimine (PEI) forms a very stable cationic static adsorbed coating, and can be used to analyze basic proteins in the pH range of 3–11 [79]. High molar mass PEI ($M_r = 6 \times 10^5$ to 1×10^6 g/mol) was found to adsorb irreversibly to the fused-silica surface, even after flushing with strong acid or strong base. Peak efficiencies of 300 000–500 000 theoretical plates per meter were found for several basic proteins at pH 5.5. Cifuentes *et al.* [80], however, report significantly lower peak efficiency and poor reproducibility using PEI as a buffer additive (dynamic

coating). Because of its tendency to strongly adsorb to fused-silica, PEI has been used to coat only the first few centimeters of a capillary, creating a capillary with essentially a step change in ζ-potential in the axial direction, and allowing analysis of nonuniform ζ-potential on EO flow profile [81, 82].

Chitosan, or (1→4)-2-amino-2-deoxy-β-D-glucan, has been employed as both a dynamic and a static cationic adsorbed coating for the analysis of basic proteins [83]. Chitosan is a hydrophilic polycation obtained by deacetylation of the carbohydrate chitin. The presence of 0.002% chitosan in the buffer was sufficient to reverse the direction of EOF; complete coverage of the surface was inferred by constant EOF above 0.05% chitosan in the buffer. Prolonged washing of the chitosan-coated surface with aqueous acetic acid did not reverse the direction of the EOF, indicating to the authors that the chitosan coating is fairly stable. Peak efficiencies were generally better when chitosan was used dynamically, as a buffer additive, than as a static coating deposited prior to analysis. The coating was most effective in the pH range of 3.0–5.5. A base wash, which would serve to reduce repulsive ionic interactions between the wall coating and the analyte, would be a more rigorous test of the chitosan coating.

Poly(diallyldimethyl ammonium chloride) (PDADMAC) has been used as a dynamic coating to analyze a basic single-chain antibody fragment at pH of ~7 [84]. This particular protein had proven difficult to analyze at neutral pH with neutral polymers such as HPMC, HEC, or PEO. Including PDADMAC in the running buffer improved resolution at neutral pH, revealing microheterogeneities in the protein. The ability to separate proteins at neutral, or near-neutral, pH may allow for the resolution of other important proteins.

3.3.2 Successive multiple ionic layer (SMIL) adsorbed coatings

The approach of polyelectrolyte multilayers has been used to generate negatively charged surfaces with reduced tendency for protein adsorption. In a technique called successive multiple ionic layer (SMIL) coating, Katayama *et al.* [85, 86] used alternating rinses with oppositely charged polymers to create layers of alternating charge. A layer of the cationic polymer Polybrene is first deposited on the bare silica surface. The anionic polymer dextran sulfate (DS) was then deposited to create a negatively charged polymer surface. This SMIL with a DS surface displayed EOF from anode to cathode (the same as an uncoated capillary) that was independent of pH in the range of 2–11, permitting separations in the presence of EOF at acidic pH that would be impossible in an uncoated capillary. The SMIL with a DS surface also showed good efficiency for the separation of acidic proteins at pH 7.4.

The SMIL approach was extended to the addition of another layer of Polybrene on top of the anionic polymer layer. This three-layer Polybrene surface was significantly more stable than a capillary coated with a single layer of Polybrene, perhaps due to stronger interaction between Polybrene and the anionic polymer, compared to the interaction between Polybrene and the silica surface. Peak efficiencies were also improved in the three-layer Polybrene capillary, relative to a single Polybrene layer.

In a similar approach, a SMIL coating was generated with alternating layers of PDADMAC and poly(styrene sulfonate) (PSS) [87]. Up to six alternating layer pairs of PDADMAC and PSS were deposited onto capillary surfaces, and variables such as polymer and salt concentration during the deposition process were examined. As expected, the direction of EOF depends on the charge of the outermost layer. Beyond the first layer pair (*i.e.*, one layer of PDADMAC and one layer of PSS), the velocity of EOF was independent of the number of layer pairs. Separation efficiency for basic proteins was improved in a capillary with 6.5 layer pairs of PDADMAC/PSS, with PDADMAC as the outermost layer, compared to a capillary with only a single layer of PDADMAC.

Currently, there is little quantitative data on protein recovery in on-chip microchannel electrophoresis. It is possible that increasing the number of charged layer pairs of any type reduces protein adsorption, but to our knowledge, this has not been investigated. The use of polymeric additives as microchannel wall coatings has been simplified to the point that it requires only a short time (on the order of an hour) and the use of dilute polymer solutions and dilute acidic or basic pretreatment solutions. In an attempt to further simplify the modification of the microchannel wall, the use of small molecules, which can be added to the running buffer or used as a static coating, have been investigated.

4 Small molecule additives

A wide variety of small molecules have been employed as buffer additives to reduce protein adsorption. These range from simple inorganic salts to various types of amines, surfactants, and multifunctional molecules. Additives may be charged or uncharged, and often display some affinity for the silica surface. Due to the nonpolymeric nature of these additives, any physically adsorbed layer on the silica surface is likely to be quite thin. The mechanism of action of small molecule additives in coat-

ing formation may simply be to block the silanol groups on the capillary surface to prevent protein adsorption. Some additives are also able to "tune", modify, or reverse EOF, depending on the charge and surface activity of the molecules.

4.1 Salts

Salts play an important role in the extent of protein adsorption. The nature and concentration of salts in solution significantly affect the thermodynamics of protein adsorption, with the molal surface tension increment (a measure of the strength of the salt-water interaction) being an important parameter [18]. The addition of salt, as suggested originally by Green *et al.* [88] is not necessarily an effective way to eliminate protein adsorption in capillary electrophoresis: addition of a strong electrolyte such as potassium chloride may improve separation slightly (relative to no additives), but adsorption is not eliminated, and Joule heating becomes a problem due to the high conductivity of the salt solution [80]. In fact, the addition of zwitterionic salt, such as trimethylammoniumpropane sulfonate, improves protein separation efficiency, not by preventing protein adsorption, but by increasing the ionic strength as well as the dielectric constant of the buffer solution, which serves to increase the mobility of the analyte without an increase in field strength [89].

4.2 Amines

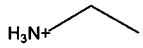
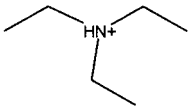
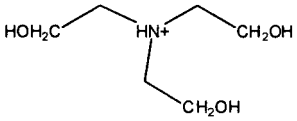
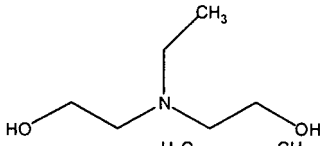
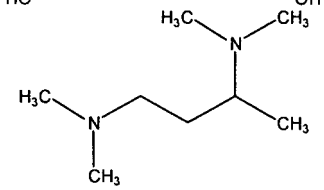
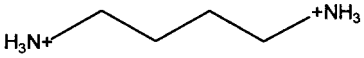
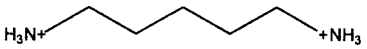
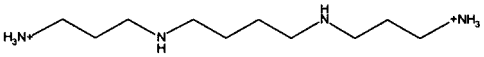
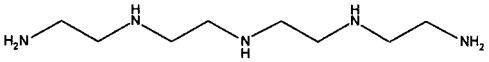
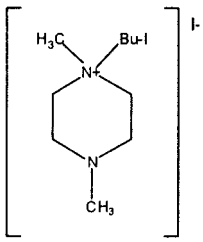
Amines have been used as buffer additives in capillary electrophoresis, both to reduce protein adsorption and to reduce or reverse EOF [90–93]. Tertiary amines such as triethanolamine [93], triethylamine or *N*-ethyldiethanolamine [91], diamines such as *N,N,N',N'*-tetramethyl-1,3-butanediamine (TMBD) [92], putrescine, cadaverine [93], and α, ω-bis-quaternary ammonium alkanes [94], primary amines, such as ethylamine [93], and oligoamines such as tetraethylenepentamine (TEPA) and spermine [93] have all been employed as buffer additives (see Table 2). Alkylamines are believed to interact strongly with silanol groups at the capillary surface, modifying the charge at the surface. At high enough concentrations of alkylamines, the sign of the ζ-potential can be reversed, reversing the direction of EOF. Such additives are most effective at preventing adsorption with basic (positively charged) proteins.

Verzola *et al.* [93] have studied quantitatively a wide variety of amine additives, ranging from monoamines to oligoamines, using an innovative method for on-line quantification of protein adsorption by saturating the capillary surface with protein and then desorbing with SDS (see also Section 3.1). The different amines were "rated" using two different concentrations of interest: the concentrations of amine in solution required for 50% and 90% inhibition of protein binding. By this standard, mono- and diamines were less effective, while the oligoamines, especially spermine and TEPA, are most effective, requiring $\sim$1 mM concentrations to achieve 90% inhibition of protein adsorption. Interestingly, this study also establishes that washing with 1 M NaOH or 1 M HCl still leaves traces of protein adsorbed on the capillary, whereas washing with a 60 mM SDS solution seems to entirely desorb all proteins.

Another novel use of amines in protein separation has been reported by Gelfi *et al.* [90]. In this study, a trifunctional diamine is used to treat the surface of the capillary at alkaline pH prior to protein analysis. The diamine is a quaternarized piperazine derivative: (*N*-methyl-*N*-ω-iodobutyl)-*N'*-methylpiperazine (Q-PzI). This compound is able to form a covalent link with the silica surface, as well as a salt bridge and multiple hydrogen bonding interactions. Again, using the approach of desorption of adsorbed myoglobin with SDS [93], Verzola *et al.* have studied the efficacy of Q-PzI in inhibiting protein adsorption, alone or in combination with other additives. Mixing different additives was shown to significantly degrade performance – a ternary mixture of HEC, TEPA, and Q-PzI was significantly less effective in preventing protein adsorption than TEPA or Q-PzI alone [95]. The covalent link, a Si-O-C bond, is unlikely to be stable under aqueous conditions which would limit the stability of the Q-PzI coating. The use of a ternary mixture may be less effective due to competition among the three components for hydrogen bonding sites on the fused-silica surface.

Yang and El Rassi [96] have used alkylsilanes incorporating quaternary amines to create a covalently bound, cationic sublayer, to which neutral polymers such as epoxybutane-HPC (EBHPC), or nonionic surfactants such as Brij 35 can be dynamically adsorbed during the run. This cationic coating could be used for separation of basic proteins; if instead of a neutral polymer, the anionic polymer hyaluronic acid was adsorbed to the surface, acidic proteins could be separated. Cifuentes *et al.* [97, 98] have also demonstrated that including cationic buffer additives such as morpholine, and especially tetraazamacrocycles, in the running buffer improved separation of basic proteins in capillaries that had been covalently modified with "standard" cross-linked polyacrylamide coatings. It was hypothesized that the buffer additives were able to dynamically coat any bare patches on the capillary surface that were not adequately shielded by the covalent coating.

Table 2. Selected amines utilized for dynamic coatings

Amine	Structure	50% Inhibition (mM)	90% Inhibition (mM)
Ethylamine		76	230
Triethylamine		38	193
Triethanolamine		160	560
N-Ethyldiethanol amine		n/a	n/a
TMBD		n/a	n/a
Putrescine		47	170
Cadaverine		31	88
Spermine		0.01	1
TEPA		0.3	1.3
Q-PzI		n/a	n/a

Adapted from [90–93]
n/a, not available

4.3 Surfactants

Various classes of surfactants, including anionic, cationic, zwitterionic, and nonionic surfactants, have also been successfully employed as additives for reducing protein adsorption in capillary electrophoresis. These surfactants have different mechanisms of action. Cationic surfactants such as CTAB, didodecyldimethylammonium bromide (DDAB), and fluorosurfactants reverse the charge at the surface of the capillary, and thus can cause strong reversed EOF [80, 99, 100]. CTAB, for example, aggregates at the capillary surface, giving the surface a net positive charge, repelling basic proteins. Unlike SDS, CTAB interacts to a different extent with different proteins, imparting each with a different charge-to-mass ratio, which can sometimes enlarge the difference in protein migration times [80]. Both anionic and cationic surfactants tend to be strongly denaturing, limiting their effectiveness when maintaining the native conformation of proteins is required.

The structure of cationic surfactant aggregates at the fused-silica surface was recently characterized by atomic force microscopy (AFM) [101]. Consistent with geometrical arguments for optimal packing of surfactant molecules, CTAB was shown to form spherical aggregates on the silica surface, changing to cylindrical or rod-like structures as ionic strength of the buffer increased. The double-chained surfactant DDAB, on the other hand, formed a smooth bilayer. Since DDAB forms a uniform bilayer covering the entire surface, it is expected to be more effective than CTAB at inhibiting adsorption, although its cationic nature interferes with separation of anionic proteins. Since EOF is not always desirable, zwitterionic surfactants have been used to effectively neutralize charge at the capillary surface, suppressing EOF by up to 90%, while providing high efficiency separations for basic proteins [102].

The double-chained zwitterionic phospholipid 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC) was recently employed for protein separations. DLPC adsorbs strongly to the capillary wall, thus it can be used as a statically adsorbed coating, without being present in the running buffer. Like DDAB, DLPC is expected to form a smooth bilayer, but since it is zwitterionic, it does not interact electrostatically with either acidic or basic proteins. High-efficiency separation of a variety of proteins was demonstrated, with peak efficiencies ranging from 15 000 to 1.4 million theoretical plates per meter. The coating was refreshed between each run by simply flushing with a solution of DLPC for 5 min, followed by flushing with buffer to clear excess surfactant from the capillary. Protein recovery was 93%, measured using a variation on the method of Towns and Regnier [81], wherein the areas of protein peaks are compared after migrating different lengths in the capillary. Several studies have combined the processes of covalent modification with dynamic coating. Towns and Regnier [103] covalently modified capillaries with a hydrophobic octadecylsilane reagent, followed by dynamic coating with nonionic surfactants. This approach reduced EOF 5- to 8-fold, while improving resolution of proteins, it had the drawback of requiring the addition of surfactant to the running buffer.

Again, using their protocol for on-line measurement of protein adsorption, the Righetti research group [104] has quantified the effectiveness of adsorbed nonionic and zwitterionic surfactants at preventing adsorption of proteins. Overall, the nonionic surfactants (detergents like Triton X-100 and Tween 20) were much less effective than zwitterionic surfactants such as palmityl sulfobetaine (SB-16). As an illustrative example, a solution of $>$10% nonionic Triton X-100 was required to reduce protein adsorption by 90%, *versus* only 0.3% of the zwitterionic SB-16. For zwitterionic surfactants, the inhibition of protein adsorption increased as the length of the carbon chain increased and the CMC decreased. For amines, those with the highest CH_2/N ratio were the most effective. And for adsorbed polymers, PDMA, the most hydrophobic of the four studied, was the most effective. This result is counterintuitive, as hydrophilic coatings or additives might be expected to resist protein adsorption most effectively. Interestingly, following their extensive study of surfactants, amines, and adsorbed polymers, the Righetti group concludes that (i) some protein adsorption is inevitable, and that even 90% inhibition of adsorption is difficult to achieve, and (ii) the efficacy of all additives is closely related to their hydrophobicity.

The relationship between adsorbed polymer coating hydrophobicity, EOF suppression, and analyte adsorption was also investigated by Doherty *et al.* [105]. In this study, it was found that a critical hydrophobicity and polymer chain length is required for *N,N*-dialkylacrylamide polymers that physically adsorb to the microchannel surface. Through the use of streaming current measurements and scanning angle reflectometry, the conformation of the adsorbed polymer layer could be deduced. PDMA was found to adsorb in a thick, "loopy" conformation ($\sim$120 nm) that was able to suppress EOF. As the fraction of *N,N*-diethylacrylamide (DEA) in a random copolymer of DEA and *N,N*-dimethylacrylamide (DMA) was increased, the adsorbed polymer layer became "flatter" and less able to suppress EOF. The separation efficiency of analytes, in this case dsDNA, was shown to be dramatically reduced by a small change in the hydrophobicity of the coating, even when the adsorbed polymer chain length was adjusted so that the EOF suppression ability of the coatings were effectively identical (see Fig. 8). We predict that the hydrophobicity of the adsorbed polymer layer will have an even more detrimental effect of the efficiency of protein separations. In comparison to the work of Verzola *et al.* [70], PDMA may have been more effective than HEC, HPMC, and PVA because of the stability or conformation of the adsorbed polymer layer.

5 Polymeric microchannel modifications

Although fused-silica microchannels have allowed for rapid, efficient biomolecular separations, microfluidic devices have been investigated in an attempt to more fully realize the benefits of a truly integrated, miniaturized bioseparation system. Some of the advantages of microfluidic analysis systems include the further reduction of reagent and sample consumption, the integration of several functions on a single device, the elimination of auxiliary equipment, which would facilitate bedside, or on-site,

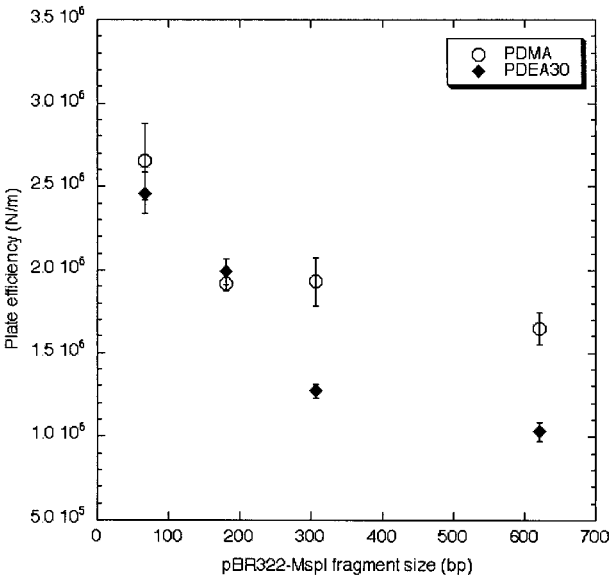


Figure 8. Effect of coating polymer hydrophobicity on pBR322-*Msp*I dsDNA separation. Adsorbed polymeric wall coatings were matched for their electroosmotic mobility. Error bars denote the standard deviation ($n = 3$). PDEA30 is a random, linear copolymer having the composition 30 wt% DEA/70 wt% DMA. Separation conditions: capillary, 50 μm ID, 44.5 cm total length (39.5 cm effective); separation matrix, 3.0 w/v% 1.2 MDa LPA dissolved in 1 × TTE (50 mM Tris, 50 mM TAPS, 2 mM EDTA); prerun electrophoresis, 169 V/cm for 5 min; injection, 15 kV, 3 s; separation, 169 V/cm; temperature, 20°C. Reprinted from [105], with permission.

analysis, and the reduction of time required for sample analysis. Early microfluidics research focused on glass microchannels, which could be fabricated by well-known wet lithography techniques [106–108]. In addition, a variety of glass substrates have well-characterized surface and optical properties that could be related to the properties of fused-silica. Unfortunately, glass microfluidic devices must be serially produced using hazardous chemicals, elevating the cost per device. Since disposable devices are desirable for many potential applications of microfluidic devices, alternate materials must be considered [109].

Polymers have numerous advantages over glass as a substrate for microfluidic devices. Most importantly, the mass production of polymeric devices is currently far less expensive than the mass production of glass devices. In addition, there are multiple methods of polymeric microchannel production, including imprinting [110], injection molding [111], X-ray lithography [112], and laser ablation [113, 114], which provides additional manufacturing flexibility. Although many polymers have inferior thermal and optical properties when compared to glass, there are several polymeric materials that have been investigated as microfluidic substrates, including poly(dimethylsiloxane) (PDMS) [115], poly(methyl methacrylate) (PMMA) [116–118], polycarbonate [113, 119–121], polystyrene (PS) [113, 122, 123], cellulose acetate [113], copolyester [124], poly(ethylene terephthalate) (PET) [121], and poly(ethylene terephthalate glycol) (PETG) [114]. Since more native microchannel surface chemistries are possible with this diverse group of polymers, EOF within polymeric microchannels can vary widely. Currently, electroosmotic pumping is the transport method of choice within microchannels; it is preferable to make modifications to the microchannel surface to regulate EOF, or to isolate EOF to certain areas of the chip, but not to eliminate EOF entirely.

The surface chemistry of most polymeric microfluidic substrates is not highly charged, sometimes making a wall treatment or coating unnecessary for rapid, efficient biomolecule separations [111]. However, these polymers are also slightly hydrophobic, leading to poor wettability and deleterious analyte-wall interactions. Thus, modification of the wall to increase hydrophilicity and to provide EOF for fluid transport is still required.

5.1 Charged adsorbed coatings for chips

The Locascio research group has investigated the use of polyelectrolyte multilayers, which is a promising approach to create uniform surfaces with consistent EOF properties on polymeric substrates. Barker *et al.* [122, 123] have successfully applied polyelectrolyte multilayers composed of PSS and poly(allylamine hydrochloride) for EOF control in PS and PETG microfluidic devices [122, 123]. Deposition of 7 pairs of electrolyte layers resulted in similar EOF mobilities on the two different substrates, despite differences in mobilities of the native plastics. The charged surfaces also facilitated easy filling of microchannels with aqueous buffers, as compared to the less hydrophilic untreated plastic channels. Polyelectrolyte multilayers are sufficiently robust to withstand long-term storage and may be regenerated by flushing with a dilute polyelectrolyte solution of interest [122]. The multilayer devices were not tested for protein separations; however, some peak tailing was observed with injections of carboxyfluorescein, perhaps due to hydrophobic interactions with the polyelectrolyte multilayers.

5.2 Chemical modification of the polymeric microchannel surface

Alkaline hydrolysis of ionizable groups to provide a more hydrophilic surface has been shown to produce reproducible EOF in PETG [114] and copolyesters [124]; ami-

nolysis of PMMA surface esters yields an amine-terminated surface that may undergo further reactions [116]. While the use of chemical treatments and adsorbed polymer layers is a simple and effective method of microchannel wall modification, the use of laser treatment of polymeric substrates, the use of a laser to ablate μm-sized features in the polymeric substrate or to modify preformed channels has emerged as the dominant wall modification technique to produce a hydrophilic surface and to regulate EOF.

5.3 Laser modification to the polymeric microchannel surface

Briefly, laser ablation involves the use of short, UV-wavelength laser pulses that when absorbed by the polymeric substrate, lead to polymer chain scission, either by thermal degradation or photodegradation [125], and finally, to vaporization. Vaporization by-products, including gases, polymer molecules, and small polymer particles, may be cleaned by successive laser pulses or swept away using an inert gas [126, 127]. Thus, ablation may be used to produce channels. At laser power levels below the ablation threshold, the laser power may also be used to modify the chemical nature of the microchannel surface.

The use of UV lasers to modify the surface of a preformed polymeric microchannel allows for very fine control of surface chemistry, permitting control of the EOF and of the flow profile within the microchannel. Johnson *et al.* [117, 118] demonstrated the usefulness of a 248 nm KrF laser at low power to produce carboxylate groups on the surface of a PMMA microfluidic device. The effects of the type of sweep gas and sonication of the ablated substrate on the final charge density of the PMMA surface was also investigated [117]. This work was later expanded by Pugmire *et al.* to include PETG, poly(vinyl chloride) (PVC), and polycarbonate [120]. Roberts *et al.* [113] also used a UV laser (193 nm) to modify the charge density of PS, polycarbonate, and PET surfaces. The ability to modify a microchannel in this manner eliminates the need for wall coatings or complicated microchannel geometries, which will facilitate the development of these polymeric materials as inexpensive, disposable diagnostic devices.

6 Conclusions and future directions

In conclusion, promising microchannel wall modifications for the facile, rapid coating of microchannel walls include polymers that physically adsorb to the microchannel wall and do not require additional polymer in the running buffer, including PVA, epoxy poly(AG-AA), and PHEA, and a variety of small molecule additives, including zwitterionic surfactants and oligoamines. If a covalently bound wall coating is used, extreme care must be taken when choosing the silane reagent as well as the pretreatment and reaction conditions. At present, there is no universal wall coating that will function well for all types of protein analysis. The separation of complex samples (as might result from direct cell lysis) will present an especially difficult challenge [128].

The results of studies carried out by Verzola *et al.* [70] indicate that the complete inhibition of protein adsorption by microchannel wall modification is currently not possible. This general result has several interesting implications. Most importantly, the loss of a small percentage of protein to irreversible adsorption makes trace analysis of an unknown protein mixture highly unlikely to be successful. In fact, certain proteins, especially those with active sites able to bind with the modified microchannel wall or those with limited stability in the running buffer, may not be detectable in trace amounts by CE.

Since polymeric microfluidic devices have tremendous potential as disposable bed-side, or on-site, devices, the development of simple modifications of these polymer microchannel surfaces to regulate EOF and to prevent the adsorption of proteins and other complex biomolecules that would be present in a raw biological sample will be critical. To date, chemical treatments, including aminolysis and hydrolysis, SMIL coatings, and UV laser ablation have been developed in an attempt to regulate EOF within a wide variety of polymeric microchannels. Further research into the regulation of EOF within microchannels, fabricated from glass or plastic, is currently underway in our laboratory.

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