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Abstract: Capillary electrophoresis (CE) has been proven to be a performant analytical method to analyze both small and macro molecules. Indeed, it is capable of separating compounds of the same nature according to differences in their charge to size ratios, particularly proteins, monoclonal antibodies and peptides. However, one of the major obstacles to reach high separation efficiency remains the adsorption of solutes on the capillary wall. Among the different coating approaches used to control and minimize solute adsorption, polyelectrolyte multilayers can be applied to CE as a versatile approach. These coatings are made up of alternating layers of polycations and polyanions, and may be used in acidic, neutral or basic conditions depending on the solutes to be analyzed. This review provides an overview of Successive Multiple Ionic-polymer Layer (SMIL) coatings used in CE, looking at how different parameters induce variations on the electro-osmotic flow (EOF), separation efficiency and coating stability, as well as their promising applications in the biopharmaceutical field.

Introduction

Since its discovery in the early 1930s by Arne Tiselius,[1] separation of charged molecules by electrophoresis has been thoroughly investigated worldwide. The capillary format used from the 1980's allows better automation, fast analysis, and facilitates solute quantification.[2] Due to its high separation efficiency,[3] capillary electrophoresis (CE) is a powerful separation technique, especially for large macromolecules and biopolymers. It may find applications in many different fields, as was shown in a recent review, for organic or inorganic compounds.[4] In the ideal case, if the peak dispersion is only controlled by axial diffusion, about one million theoretical plates should be typically obtained for protein analysis.[5] However, in practice, much lower separation efficiencies are obtained for protein analysis, with typically a factor of at least 10. This difference between theoretical and experimental values can be explained by different phenomena such as electromigration dispersion, temperature gradient, and solute adsorption onto the capillary wall.[6],[7] The prevailing cause among the previously cited sources of dispersion, may strongly depend on the experimental conditions such as the background electrolyte conductivity, the separation voltage, the sample concentration, and the difference between solute and co-ion mobility. However, it was recently demonstrated that capillary wall adsorption is a major cause of peak broadening for protein analysis, even in the case of low residual adsorption.[5] This residual adsorption propels CE into the chromatographic world[5] with a strong contribution of mass-transfer resistance to the separation efficiency. This holds true even if CE does not present any dispersive parabolic profile of the velocities as encountered in chromatography. Performing protein separation at different separation voltages in CE allows to quantify the residual adsorption by the determination of protein retention factors (k), as defined in chromatography, using basic theoretical developments derived from the theory of electrochromatography.[5] Retention factors as low as 10^{-2}-10^{-1} were determined for the CE separation of proteins on coated capillary but this small residual adsorption significantly impacts the peak broadening, especially at high separation voltages.[5] It was concluded that the determination of protein retention factors in CE is a very useful and quantitative way to estimate the performances of capillary coatings toward protein adsorption, independently of the electroosmotic mobility, the capillary length and the migration times.[5] Due to the high impact of retention factors on plate heights (H scales as k^2), it is of major interest to find new coating protocols leading to lower residual protein retention factors.

Capillary coatings are generally classified into different categories: permanent (covalently bonded), semi-permanent (physically adsorbed) and dynamic (the coating polymer or reagent is placed in the background electrolyte).[8] Permanent coatings require chemical activation and reaction onto the capillary surface with reagents and sometimes in-situ polymerization. They are time-consuming to prepare in the lab, and therefore are, most of the time, bought from commercial sources. Dynamic coatings require additives in the background electrolyte, which may result in higher capillary surface covering,[9] however they are rarely compatible with mass spectrometry (MS) coupling and may interfere with separation conditions. Semi-permanent coatings can be neutral or charged. In the latter case, Successive multiple ionic-polymer layers

(SMIL) are widely used and have the advantages of mostly resorting to commercial polymers and being easily deposited on capillary surface by automated protocol based on simple rinses. Electrostatic interactions are the main forces, but hydrogen bonding or other weak interactions can also play a role. SMIL coatings were introduced for the first time in CE by Katayama.[10] However, since their first introduction in the late 1990's, polyelectrolyte (PE) multilayers represent an important scientific field in itself, with many other applications than CE coatings, such as surface treatment,[11] cell growth,[11] selective membranes for gas separation and neurochemical monitoring,[12] and reflective displays.[12] SMIL coatings have also been applied to microchip CE, where they are particularly useful to control the EOF.[13]

Figure 1 depicts how SMIL coatings are constructed. To begin, the bare silica capillary is flushed with 1 M NaOH, water and the construction buffer. Next, the polycation is flushed through the capillary and interacts electrostatically with the negatively charged capillary surface. The capillary was then rinsed with the construction buffer to remove the free polycation molecules in excess, resulting in the first layer of the SMIL coating. The polyanion is then flushed through the capillary, followed by the construction buffer, resulting in the second layer of the SMIL coating. The process is repeated until the desired number of layers is reached, alternating between the polycation and the polyanion, and ending with a last construction buffer flush. The SMIL coating is positively charged if the polycation is the last layer, or negatively charged if the polyanion is the last layer. Finally, a wait time may be added, as well as flushing with water and BGE before proceeding with the CE analysis.

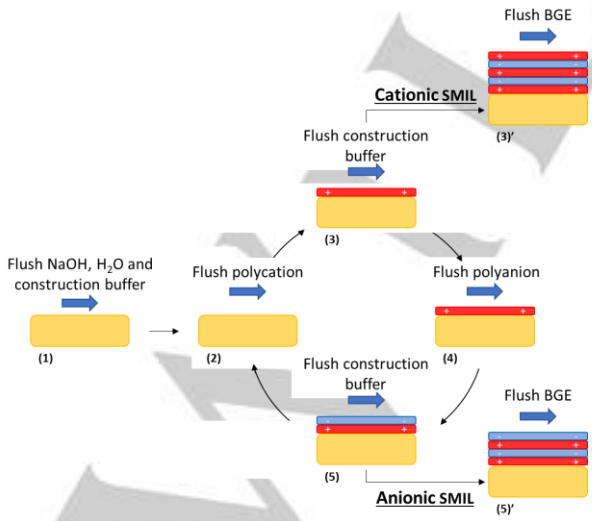

Figure 1. Schematic representation of the construction of 4- and 5-layer SMIL coatings.

The application of SMIL coatings in CE with the aim to reduce protein adsorption is a promising approach but it is challenging due to the large number of parameters controlling the final state of the capillary coating. Among these parameters, we can cite the pH and ionic strength (*I*) of the construction and separation buffers, the nature, molar mass and concentration of the polyelectrolytes, and the number of polyelectrolyte layers. Due to the high number of possible polyelectrolytes differing in their chemical nature, these parameters already represent a huge number of experimental combinations with a large variety of resulting coatings in terms of thickness, hydration, stability, surface charge and finally, in terms of protein residual adsorption onto the capillary call. Polyelectrolytes can be considered as strong or weak, the strong ones being fully ionized at all pH, and the weak ones being only partially ionized. High electro-osmotic flow mobilities (positive or negative, depending on the polyelectrolyte forming the last layer) are obtained leading to separations of proteins in counter-electroosmotic mode. The recent developments of SMIL applications in CE follow two axes: (*i*) the quantification of the residual adsorption in order to fairly rank the performances of the different SMIL coatings between them; and (*ii*) the optimization of the physicochemical conditions of SMIL coating construction to improve separation performances.

This review will provide an overview of SMIL coatings used in CE from their first use in 1998 until 2020. The effect of the different coating parameters/protocols on both separation efficiency and coating stability are discussed, as well as the applications of SMIL coatings in CE. For a better understanding of the polyelectrolyte multilayer systems and their physicochemical behavior, a selection of experimental data and conclusions obtained for applications outside of CE will be also reported. In the following, SMIL systems were noted as $(polycation/polyanion)_x$, where x is the number of bilayers.

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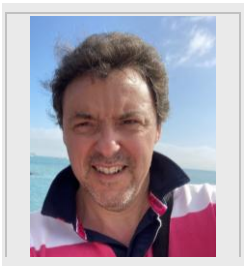
Sébastien Roca received his Master of Engineering degree from INSA Rouen Normandie, in 2020. He is currently investigating adsorption phenomenon in CE (using SMIL coatings) and Taylor dispersion analysis (TDA), as a PhD student at IBMM, Montpellier, France. He aims to stay in public research as a teacher and researcher.



Dr. Laurent Leclercq obtained his PhD in macromolecular chemistry and physical chemistry in 1997 at the University of Lille (France) under the supervision of Prof. Michel Morcellet and Prof. Bernard Martel. After a 2-year post-doc at the Max-Planck-Institute for Polymer research in Mainz (Germany), he joined the University of Montpellier in 1999, as a CNRS researcher. His research work is focused on the understanding of the interactions between (bio)polymers, with important applications in the biomedical and pharmaceutical fields.

Prof. Hervé Cottet obtained his PhD in analytical chemistry in 1999 at the ENSCP (Paris, France) under the supervision of Prof. Pierre Gareil. After a one year post-doc at the Technical University of Eindhoven (the Netherlands), he joined the University of Montpellier in 2000. In 2007, he got a full professor position. His research work concentrates at the interface between separation sciences, physical chemistry, polymers and pharmaceutical sciences. He is interested in fundamentals and practical/industrial applications of CE and TDA.

1. Coating electrolytes

Both the nature and the pH of the construction buffer used for SMIL coatings have a strong impact on coating stability, on intra-capillary repeatability and inter-capillary reproducibility, as well as peak resolution, which gives a measure of the system's ability to separate compounds based on the distance between their peaks and their widths at half height.

1.1. Effect of the construction buffer ionic strength on coating performance

Adding salt to the construction buffer generally leads to thicker films.[14],[15] Indeed, building a poly(allylamine)/poly(acrylic acid)-poly(styrene sulfonate)$_8$ (PAH/PAA-PSS)$_8$ SMIL coating in the presence of 0.5 M NaCl generated a 70% increase of the film thickness compared to that in the absence of salt.[14] Graul et al. showed that layer pair thickness (t_{lp}) dependence on salt concentration (c) is approximately linear for poly(diallyldimethylammonium chloride) (PDADMAC)/PSS SMIL coatings, according to Lösche et al.'s model,[16],[17] with the following equation:

$$t_{lp}(\text{Å}) = 28 + 16c \qquad (1)$$

Generally, it can be thought that thicker films lead to more distance between the solutes and bare silica wall, thus reducing their adsorption and increasing peak separation efficiency. On the other hand, the thicker the coating, the more solute diffusion in the coating is likely to take place,[18] leading to an increase in solute adsorption.

For example, Nehmé et al. compared the (PDADMAC/PSS)$_{2.5}$ SMIL coating construction in 0.01 M and 1.5 M NaCl and found that higher stability and thicker layers were obtained using the highest ionic strength[19],[20] for the separation of 5 proteins (Ribonuclease A (RNAse A), α-lactalbumin (α-Lac), Myoglobin (Myo), Lysozyme (Lyz) and Cytochrome C (Cyt C)), in 100 mM Tris-phosphate buffer (pH 2.5). The same salt concentration was studied for the (PDADMAC/PSS)$_{11}$ system, but unrepeatable results were obtained,[20] likely because a high number of layers makes the coating more sensitive to ionic strength destabilization, showing that thicker films do not always mean higher stability. In the case of a (PAH/PAA)$_{2.5}$ SMIL coating[15] used for the separation of five proteins (RNAse A, Lac, Myo, Lyz and Trypsin inhibitor (TI)) in 0.5 M acetic acid buffer (pH 2.5), the addition of salt (0.15 M NaCl) in the construction buffer led to an unstable coating. The authors suggested that the presence of salt is likely to destabilize SMILs composed of weak polyelectrolytes. Salt ions affect interactions between weak polyelectrolytes of opposite charges by decreasing the electrostatic interaction. Figure 2 displays the influence of NaCl added to the building buffer solution (20 mM HEPES buffer, pH 7.4) on the separation of the same protein mixture in 0.5 M acetic acid.[15] It was shown that the presence of NaCl impacts both migration time (t_m) and electroosmotic mobility (μ_{EOF}) for all the SMIL coatings studied. In the case of polybrene/poly(methacrylic acid)$_{2.5}$ (PB/PMA)$_{2.5}$ and (PB/poly(L-lysine citramide) (PLC))$_{2.5}$ SMIL coatings, t_m first decreased when using 0.15 M NaCl in the construction buffer and then increased using higher NaCl concentrations. For all the other SMIL coatings studied, namely (PB/PSS)$_{2.5}$, (ε-Poly-L-lysine

(εPLL)/PLC)$_{2.5}$, (quaternized diethyl aminoethyl dextran (DEAEDq)/PSS)$_{2.5}$, (DEAEDq/PMA)$_{2.5}$, t_m increased when NaCl concentration increased, due to decreasing EOF.

Separation efficiency was impacted the same way for all the SMIL systems studied in Figure 2, the best results being obtained in the absence of NaCl in the construction buffer. The most important decrease in peak separation efficiency was observed in the case of (PB/PLC)$_{2.5}$ SMIL system, i.e. from 266 000 plates/m (without NaCl) down to 45 000 plates/m (with 1 M NaCl). The lowest impact of NaCl on peak separation efficiency was in the case of (DEAEDq/PSS)$_{2.5}$ SMIL system, a couple of strong polyelectrolytes, from 107 000 (without NaCl) to 76 000 (at 1 M NaCl) plates/m.[21]

Graul et al.[16] studied the stability of a (PDADMAC/PSS)$_{6.5}$ SMIL coating for the separation of four proteins (α-chymotrypsinogen A (α-chymo A), RNAse A, Cyt C and Lyz) in 20 mM phosphate buffer at pH 6.0, and found that the best repeatability in terms of EOF mobility was obtained when each polyelectrolyte layer was deposited at an increasing NaCl concentration (first bilayer at 0.1 M NaCl, second bilayer at 0.2 M NaCl, third bilayer at 0.5 M NaCl and final bilayers at 1.0 M NaCl). The influence of NaCl in the construction buffer on EOF mobility also depended on the nature of the SMIL coating. In the case of (PB/PLC)$_{2.5}$, (εPLL/PLC)$_{2.5}$, (DEAEDq/PSS)$_{2.5}$ and (DEAEDq/PMA)$_{2.5}$ coatings, the EOF mobility was found similar in 0 M and 1 M NaCl.[21] In contrast, the strongest effect was observed for (PB/PSS)$_{2.5}$ and (PB/PMA)$_{2.5}$ coatings. In the absence of NaCl, μ_{EOF} mobilities were -62 Tiselius Units (TU, with 1 TU = 10^{-9} $m^2s^{-1}V^{-1}$) and -55.3 TU, respectively, while in the presence of 1 M NaCl, μ_{EOF} mobilities decreased (in absolute value) to -49.7 TU and -50.1 TU, respectively.[21] Salt addition slightly affected EOF stability, by increasing the RSD(μ_{EOF}) from 1.3% (without salt) to 1.9% (1 M NaCl) for (PB/PSS)$_{2.5}$ and from 0.1% (without salt) to 0.8% (1 M NaCl) for (PB/PMA)$_{2.5}$.[21]

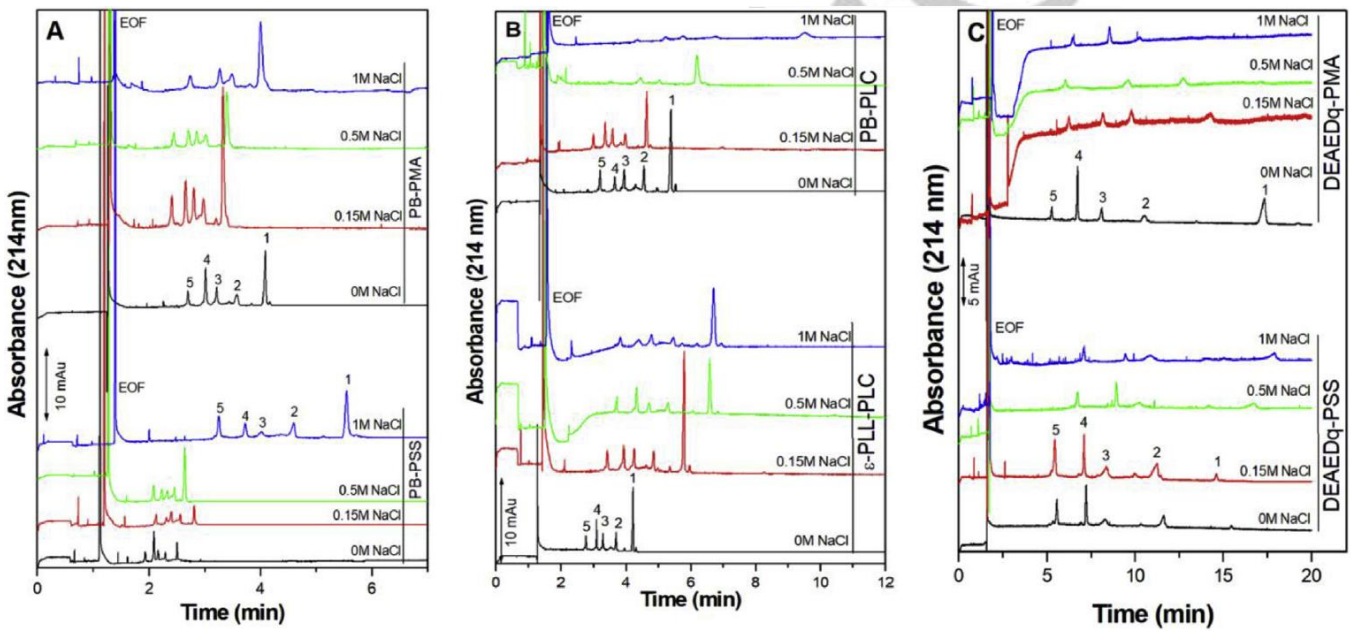


Figure 2. Electropherograms for (A) (PB/PSS)$_{2.5}$ and (PB/PMA)$_{2.5}$ SMIL, (B) (εPLL/PLC)$_{2.5}$ and (PB/PLC)$_{2.5}$ and (C) (DEAEDq/PSS)$_{2.5}$ and (DEAEDq/PMA)$_{2.5}$ SMIL. Influence of the NaCl concentration added to the construction electrolyte on the electrophoretic separation of 5 test proteins in 0.5 M acetic acid. Experimental conditions: 5-layer SMIL coatings (PB/PSS, PB/PMA, PB/PLC, εPLL/PLC, DEAEDq/PMA and DEAEDq/PLC, as indicated on the graphs), 40 cm (31.75 cm to detector) x 50 µm i.d. capillary. Electrolyte: 0.5 M acetic acid, pH 2.5. Applied voltage: -30 kV. Hydrodynamic injection: 30 mbar, 4 s (0.74% of the total capillary volume). Sample mixture: 1 g/L of each protein in water. Peak identification: Lyz (1), Lac (2), RNAse A (3), Myo (4), TI (5). Hydrodynamic co-injection of 0.01% DMF: 30 mbar, 4 s. Temperature: 25 C. Coating solutions: 3 g/L polyelectrolyte in 20 mM HEPES pH 7.4 containing NaCl at a concentration indicated on the graphs. Reprinted from Analytica Chimica Acta, Vol 1057, Laurent Leclercq, Marine Morvan, Jens Koch, Christian Neusüß, Hervé Cottet, Modulation of the electroosmotic mobility using polyelectrolyte multilayer coatings for protein analysis by capillary electrophoresis, Pages No. 152-161, Copyright (2019), with permission from Elsevier.

These findings show that weak and strong polyelectrolytes react differently to the addition of salt to the construction buffer. Indeed, the coatings built from PDADMAC and PSS, which are two strong polyelectrolytes, always showed good stability when used with high ionic strength construction buffers, while the polymer couples containing at least one weak polyelectrolyte showed a decrease in their performance and stability at high ionic strength.[21]

In addition to the construction buffer, the influence of the dissolution medium may be considered, as it has an impact on film formation and stability, even outside of CE field. Indeed, Pallotta et al.[22] studied polyelectrolyte films made of PAH or PEI and PAA incorporated with gold nanoparticles (AuNP), which have promising biomedical applications, comparing PBS (148 mM), Tris (10 mM) and Tris-NaCl (150 mM) buffers at pH 7.4 as polycation dissolution media. Through successive deposition cycles, the PEI/PBS film showed no increase in absorbance, which is correlated to poor AuNP loading, while the other coatings showed linear increase. Film construction was also monitored by quartz crystal microbalance, which revealed that the films in PBS buffer were unstable and those in Tris-NaCl had the best linearity, confirming the importance of counter ions in the building medium.

1.2. Effect of the construction buffer pH on coating performance

Modifying construction buffer pH can affect the multilayers' construction if weak polyelectrolytes are used.[15] Deposition of poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA) polycation at pH 5 and 7 was investigated on a silica wafer in combination with sodium alginate polyanion (SA).[23] It was shown by atomic force microscopy (AFM) that stretched PDMAEMA chains are formed at pH 5, leading to smooth and homogeneous films. In contrast, thicker and more heterogeneous films were obtained at pH 7, where PDMAEMA chains are coiled. This is due to the polymers' points of zero charge (*pzc*), i.e. the pH at which the polyelectrolyte has a neutral net electrical charge. Indeed, the *pzc* of SA being pH 3.4, the polyanion was thus fully charged from pH 4 to 10. However, PDMAEMA behaves as a weak polyelectrolyte: it is fully charged between pH 3 and 5.5, and gradually decreases in charge until the *pzc* is reached at pH 8.1. This enabled the precise modulation of the film thickness through construction buffer pH.

On the other hand, acetic acid (0.5 M, pH 2.5) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) were used as building buffers in the case of $(PAH/PAA)_{2.5}$ SMIL coatings for the separation of 5 proteins (RNAse A, Lac, Myo, Lyz and TI) in 0.5 M acetic acid pH 2.5 background electrolyte (BGE). With the acidic buffer, separation efficiency was only around 5000 plates/m with RSD(t_m) of 2%. This is likely due to the fact that the polyanion was not fully ionized at pH 2.5. In contrast, using a neutral buffer ensured full ionization of the two polyelectrolytes (pK_a of PAH = 8.5 and pK_a of PAA = 4.2) and led to a SMIL coating with separation efficiency of around 55 000 plates/m and RSD(t_m) of about 2%.[15]

2. Impact of the BGE

The running electrolyte, or BGE, used for CE analysis should also be considered, as its pH and ionic strength may have an impact on EOF mobility and separation efficiency.

Better separation efficiencies were generally found when increasing the ionic strength of the BGE. For example, Haselberg et al.[24] studied the impact of BGE ionic strength on a $(PB/DS)_{1.5}$ cationic SMIL, by changing BGE concentration, and therefore ionic strength (from 50 mM up to 1000 mM acetic acid at pH 3), for the analysis of oxytocin. The lowest ionic strength led to approximately 143 000 plates/m and the optimal separation efficiency was found at 429 000 plates/m at 525 mM BGE. Intra-capillary repeatability with RSD(t_m) less than 1.4% was reported. Over 525 mM, neither the separation efficiency nor the peak resolution were improved. Changing the pH of the 525 mM acetic acid BGE (between 3 and 5) did not affect these findings.

Similarly, separation efficiency and stability were studied on a $(PB/PVS)_1$ SMIL system by Catai et al.[25] Insulin (In), α-lac, β-lactoglobulin B (β-lac B) and β-lactoglobulin A (β-lac A) were analyzed in sodium phosphate buffer at three different concentrations: 25, 50 and 100 mM (pH 7 constant). Better separation efficiency was found with the higher ionic strength BGE. When comparing 25 mM with 100 mM BGE, separation efficiency improved from 240 000 plates/m to 340 000 plates/m, for In, from 140 000 plates/m to 200 000 plates/m for α-lac, from 80 000 plates/m to 324 000 plates/m for β-lac B, and from 50 000 plates/m to 296 000 plates/m for β-lac A, respectively. Tris-phosphate buffers at 200 mM, 300 mM and 400 mM (pH 7) were also tested as BGE. It was shown that the separation efficiency increased with BGE ionic strength: 320 000 plates/m were achieved with the 200 mM buffer, 500 000 plates/m with the 300 mM BGE and 680 000 plates/m with the 400 mM BGE for In. As a general trend, increasing BGE ionic strength can decrease the solute adsorption by an effect of competition of interaction on the capillary wall, and thus increase the separation efficiency.

BGE ionic strength impact over resolution and efficiency was also investigated by Currie et. al, with a $(PDADMAC/PSS)_6$ coating.[26] Increasing ionic strength of the BGE was found to lead to better chiral separation of pheniramine. Indeed, by increasing the ionic strength of phosphate buffer from 10 mM to

100 mM (pH 4.5), peak resolution increased from 0.68 to 0.81 and separation efficiency increased from 65 600 plates/m to 111 600 plates/m.

The ionic strength of the BGE also has a strong effect on the EOF, as shown by Pattky et al.[27] In their experiments, the BGE was a mixture of acetic acid and formic acid (with ratio of 3:1, v/v). On a PB coated capillary, EOF was around -62 TU with 1 M BGE, and -50 TU with 2 M BGE. For the separation of a tryptic BSA digest, t_m increased from 10.5 min to 22 min when using 1 M BGE and 2 M BGE, respectively.

The pH of the BGE also has a strong effect on EOF, depending on polyelectrolyte nature. When using a strong PE, such as PB or DS, no change in EOF was observed at different pH,[28] as long as the BGE ionic strength was the same. As it can be seen in Figure 3, keeping the ionic strength constant at different pH did not affect both positively (PB monolayer, μ_{EOF} = -38 TU) and negatively ($(PB/DS)_1$, μ_{EOF} = +38 TU) charged SMIL. By comparison, the uncoated capillary showed changes since EOF increased when pH increased due to higher ionization of silanol at higher pH.

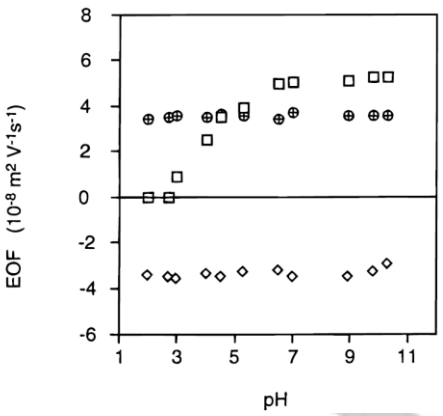


Figure 3. EOF of uncoated (□), SMIL-PB (⌖) and SMIL-DS (◇) capillaries (n=5). Conditions: detection, 214 nm; applied voltage, 7 kV; buffers, phosphate buffer at pH 2-3 (I = 0.05), acetate buffer at pH 4-5 (I = 0.05), phosphate buffer at pH 6-7 (I = 0.05) and borate buffer at pH 8-11 (I = 0.05); capillary, 75 µm i.d. x 27 cm (20 cm effective length). Reprinted with permission from H. Katayama, Y. Ishihama, N. Asakawa, *Anal. Chem.* **1998**, *70*, 2254–2260. Copyright 1998 American Chemical Society.

In the case of a $(PDADMAC/PSS)_6$ SMIL coating with two different BGE, i.e. 100 mM phosphate (pH 4.2, 5.3 and 6.0) and 100 mM sodium borate at pH 9.5, better separation efficiency was found with 100 mM phosphate buffer BGE at pH 4.2 (111 600 plates/m) than at pH 6 (35 000 plates/m),[26] but EOF was similar (29.6 TU at pH 4.2 and 29.3 TU at pH 6.0). At pH 9.5, EOF mobility remained similar (31.9 TU).

The influence of the pH of the BGE was also studied over a wide pH range (2.5-9.3) for a 25 µm i.d. $(PDADMAC/PSS)_{2.5}$ coated capillary.[29] Three buffers were tested, namely 100 mM Tris phosphate (pH 2.5), 100 mM sodium phosphate buffer (pH 7.0), and 100 mM borate buffer (pH 9.3). Similar results were found for the RSD(t_m) in the case of α-Lac, Cyt C, Lyz, Myo, RNAse A and CA proteins (< 1%). Highest separation efficiency was obtained in the following conditions: 715 000 plates, 700 000 plates and 400 000 plates with the borate buffer for Myo, RNAse A and CA proteins, respectively.

Graul et al. measured EOF modification on SMIL coatings when changing pH of a 20 mM phosphate buffer BGE,[16] but ionic strength was not kept constant which may explain this variation. Two different SMILs were tested, i.e. a cationic $(PDADMAC/PSS)_{6.5}$ and an anionic $(PDADMAC/PSS)_7$, at pH 4, 6 and 8. In terms of stability, both SMIL coatings were robust with very low RSD(μ_{EOF}) (with pH in the 4-8 range).

A similar study was performed on a $(PB/DS)_{1.5}$ coating in phosphate buffer (without maintaining ionic strength constant) for the analysis of 25 different acids.[30] Better separation efficiency was obtained at pH 3.1 (24 peaks were observed among the 25 acids analyzed). EOF remained stable at higher pH until 10, showing good robustness for this coating.[31]

In the case of a $(PB/PSS)_2$ coated capillary[25] and 300 mM Tris buffer BGE at pH 7.0 and 8.2 used for the analysis of 4 proteins (In, α-lac, β-lac A and β-lac B), the neutral BGE led to a similar analysis time (10 min) compared to the basic one (11 min), with low RSD(t_m) in both cases (≈ 0.5%), but lower separation efficiencies were obtained for the neutral BGE (470 000 plates/m versus 560 000 plates/m for the basic one).

Haselberg et al. studied the impact of nature and pH of BGE on the same $(PB/DS)_{1.5}$ SMIL system.[32] Two different BGEs (175 mM acetic acid pH 2.7 and 100 mM Tris-phosphate pH 8) were tested for the separation of 4 proteins (α-Chymo A, RNAse A, Lyz and Cyt C). EOF decreased from -57.9 TU using the acetic acid buffer at pH 2.7 to -13.4 TU using the Tris-phosphate buffer at pH 8, mainly due to higher ionic strength in the TRIS-phosphate buffer. RSD(μ_{EOF}) was very low (intra-capillary RSD around 0.2% on 3 runs and inter-capillary RSD = 0.63-0.89% on 5 capillaries). Coating stability was investigated in 100 mM Tris-phosphate buffer at different pH (3, 4, 5, 6, 7 and 8). All RSD values were below 0.81% regardless of pH, showing great repeatability and stability.

Likewise, Villemet et al.[33] investigated the effect of BGE pH on the separation on peanut allergens with a $(PB/DS)_{1.5}$ coated capillary and a BGE composed of 100 mM phosphoric acid, 70 mM Tris, and 3 g/L polysorbate 80 (PS) at pH 2.5. They observed that lowering the pH led to increased electrophoretic mobilities of the proteins and shorter migration times, but peak resolution was lost at pH 2.2. BGE composition was then modified, keeping pH and ionic strength constant and varying the concentrations of Tris and glycine in phosphoric acid, and it was shown that glycine improves the separation of the early migrating proteins and Tris the later ones, highlighting the importance of BGE co- and counter-ions.

Finally, a similar study was carried out on $(PAH/PSS)_x$ coatings,[34] in a BGE composed of 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 7.1 until EOF stabilization, followed by similar MES buffer at different pH (5.5, 6.1 or 6.6) but constant ionic strength (by adding NaCl). The authors showed that both the number of layers and the nature of the last layer have a strong influence on EOF. Finishing SMIL coatings with a polycation layer led to increased EOF. For example, with $(PAH/PSS)_{1.5}$, EOF increased from -37 TU (at pH 7.1) to -45 TU (at pH 5.5). With $(PAH/PSS)_{3.5}$, EOF increased from -40 TU (at pH 7.1) to -49 TU (at pH 5.5). With $(PAH/PSS)_{7.5}$, EOF increased from -35 TU (at pH 7.1) to -45 TU (at pH 5.5). In contrast, with a polyanionic last layer, such as with the $(PAH/PSS)_4$ coating, EOF remained stable (≈ 40 TU) at 3 different pHs (6.6, 6.1 and 5.5). In the case of the $(PAH/PSS)_8$ coating, EOF decreased from 45 TU (at pH 7.1) to 30 TU (at pH 5.5). Other SMIL coatings were also tested by the same authors, namely $(PDADMAC/PSS)_x$ and $(PDADMAC/PMA)_x$. PDADMAC and PSS are considered as strong polyelectrolytes which are fully ionized at all pH, as opposed to weak ones such as PMA and PAH, which are only partially ionized depending on the pH. The authors found that coatings built from strong polyelectrolytes (like $(PDADMAC/PSS)_2$) did not change in EOF (EOF changes <1%), while coatings built with one or two weak polyelectrolytes (like $(PAH/PSS)_x$ or $(PDADMAC/PMA)_x$) had dramatically different EOF mobilities (changes >10%), when pH varied from 5.5 to 6.6.

3. Number of layers

When looking at SMIL coatings, one major factor to consider is the number of layers. As the number of layers increases, the coating becomes thicker, which may lead to an increase in solute adsorption as was mentioned earlier.[18] The impact of the number of layers also strongly depends on the hydration of the SMIL layers, and therefore differs for each polymer couple.[35]

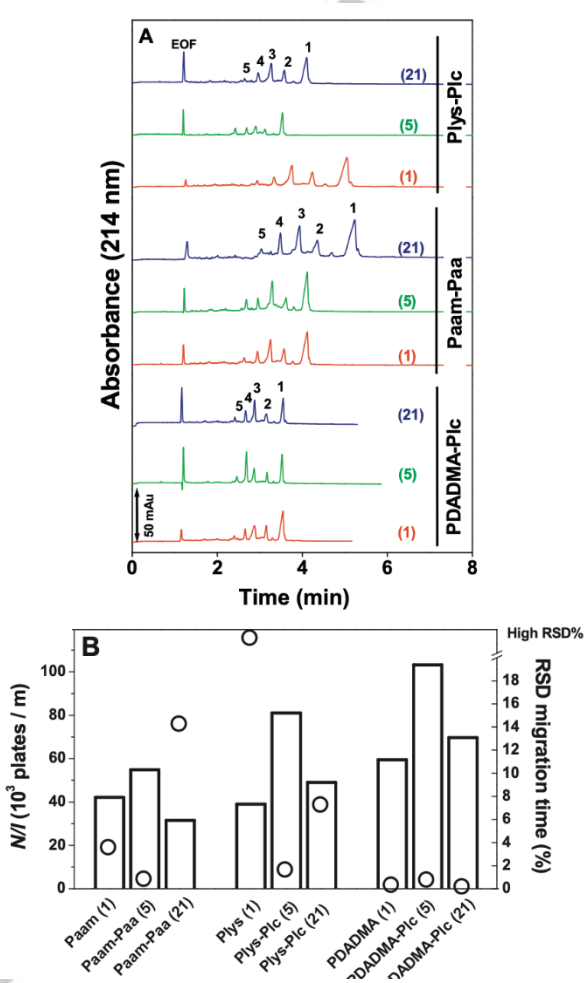


Figure 4. Influence of the number of polyelectrolyte layers on the separation of five test proteins (A) and the corresponding figures of merit on separation efficiency and migration time repeatability (B) for 3 polyelectrolyte systems. Experimental conditions: 5-polyelectrolyte layers coated capillary terminating with the polycation, 40cm total length (29.6 cm to the detector) × 50 µm i.d. Electrolyte: 0.5M acetic acid, pH 2.5. Applied voltage: −30 kV. Hydrodynamic injection: 30 mbar, 4 s. Sample mixture and peak identification: Lyz (1), Lac A (2), RNAse A (3), Myo (4), TI (5) at 1 g/L each in water. Temperature: 25°C. Reprinted from Journal of Chromatography A, Vol 1399, Samya Bekri, Laurent Leclercq, Hervé Cottet, Polyelectrolyte multilayer coatings for the separation of proteins by capillary electrophoresis: Influence of polyelectrolyte nature and multilayer crosslinking, Pages No. 80-87, Copyright (2015), with permission from Elsevier.

Bekri et al. tested three different SMIL systems with 1, 5 and 21 layers, namely PDADMAC/PLC, poly(L-lysine) (Plys)/PLC and PAH/PAA, for the separation of five intact proteins in a 0.5 M acetic acid BGE (pH 2.5).[15] All the SMIL systems performed best with 5 layers. As displayed in Figure 4, the separation efficiencies were systematically better on 5-layer coatings, achieving the highest values of N (up to 100 000 plates/m for

$(PDADMAC/PLC)_{2.5}$) and the lowest RSDs of migration times (<2%).

Nehmé et al. also studied the influence of the number of coating layers ($(PDADMAC/PSS)_{0.5}$, $(PDADMAC/PSS)_{2.5}$ and $(PDADMAC/PSS)_{5.5}$) for different ionic strengths of the coating solutions.[20] At low ionic strength, the $(PDADMAC/PSS)_{5.5}$ coating led to the most efficient separations of the model proteins (Cyt C, α-Lac, Lyz, Myo, and RNAse) in a 100 mM Tris-phosphate BGE (pH 2.5). At 1.5 M ionic strength, separation efficiency slightly increased from the monolayer to the $(PDADMAC/PSS)_{2.5}$ coating, but decreased or was unstable with the $(PDADMAC/PSS)_{5.5}$ coating. Similarly, Graul et al. tested a $(PDADMAC/PSS)_{6.5}$ coating but with lower ionic strength (0.5 M) in the last seven layers, resulting in a thinner deposit overall, and which managed to successfully separate four basic proteins0[16] This seems to indicate that coatings that are too thick, with a high number of layers as well as a high amount of salt, are not the best for protein analysis.

Further studies were done by Swords et al., who examined three polymer couples, PAH/PSS, PDADMAC/PMA, PDADMAC/PSS, with varying numbers of layers (3, 4, 7, 8, 15, 16).[34] When subjected to pH changes above 1.5 units (pH 5.1-7.1), the EOF varied very few for the coatings with 3 and 4 layers, and varied more and more as the number of layers increased. The coatings containing a weak polyelectrolyte, PAH or PMA, were unstable for the highest number of layers (15 and 16).

Moreover, Sui et al. investigated the impact of SMIL layer number using AFM, and found that $(PDADMAC\text{-}co\text{-}PAA/PSS)_{10.5}$ coatings (with PEI as the first layer) deposited on a silicon wafer showed some irregularities as the pH was changed, whereas $(PDADMAC\text{-}co\text{-}PAA/PSS)_{5.5}$ coatings were better, an effect which was amplified when PSS came as the last layer.[36] Similar results were obtained for quaternized poly(vinyl imidazole) (QPVI) and PSS coatings.

4. Polyelectrolyte nature

Since the pioneering work of Katayama et al. on SMIL coatings in 1998 for CE applications, many polyelectrolyte couples have been tested in various experimental conditions. Table 1 gathers around 70 couples reported from the literature classified in two categories (polycationic coatings with an odd number of layers, and polyanionic coatings with an even number of layers) and according to the chemical nature of the polycation. It shows that the nature of the polyelectrolyte couple strongly impacts the separation efficiency. It only reaches 4000 plates/m for $(PEI/DS)_{1.5}$ SMIL coating used to separate RNAse A and Cyt C in 10 mM phosphate buffer, pH 3,[37] but is as high as 626 000 plates/m for $(PB/PLC)_{2.5}$ SMIL coating used to separate TI, Myo, RNAse A and Lyz in 0.5 M acetic acid, pH 2.5.[5]

In MS-compatible acidic BGE (such as 0.5M acetic acid pH 2.5), Leclercq et al. found that PLC, then PMA and PSS, were the best polyanions when associated with most of the studied polycations.[21] For example, separation efficiencies of 293 000 plates/m and 145 000 plates/m were found for $(PB/PLC)_{2.5}$ and $(PB/PMA)_{2.5}$ coatings, respectively. The best separation efficiencies were observed with PLC polyanion, which has low linear charge density and amide enchainment. For example, a $(\varepsilon PLL/PLC)_{2.5}$ coating gave 336 000 plates/m.[21] Not only does the nature of the polyelectrolyte in the last layer affect the electroosmotic mobility of the SMIL coating and the separation efficiencies, but the underlying SMIL layers also have an impact. When comparing SMILs built from different polycations associated with the same polyanion (PSS), electroosmotic mobilities in 0.5 M acetic acid (pH 2.5) can be very different, depending on the nature of the polycation. For example,[21] μ_{EOF} was -62.6 TU for $(PB/PSS)_{2.5}$, -54.7 TU for $(PDADMAC/PSS)_{2.5}$, -51.2 TU for $(\varepsilon PLL/PSS)_{2.5}$, -46.4 TU for $(\alpha PLL/PSS)_{2.5}$ and -42.8 TU for $(DEAEDq/PSS)_{2.5}$. Another ranking can be given when replacing PSS by another polyanion, such as PAA. In both cases, the influence of the polycation nature is the same, i.e., εPLL, αPLL and DEAEDq always gave the lowest values of μ_{EOF}.[21] Similarly, Swords et al. found that PSS combined with PAH led to higher mobility (40 to 45 TU for $(PAH/PSS)_8$) compared to PSS with PDADMAC (35 to 42 TU for $(PDADMAC/PSS)_4$).[34]

Coatings for which μ_{EOF} is higher than -40 TU lead to shorter analysis times. The best couple was $(PB/PLC)_{2.5}$,[21] with 626 000 plates/m obtained for the separation of 4 proteins (TI, Myo, RNAse and Lyz) in 0.5 M acetic acid (pH 2.5). Some coatings have an intermediate μ_{EOF}, i.e. close to the electrophoretic mobilities of the most commonly studied model proteins (between -30 and -20 TU approximately for TI, Myo, RNAse and Lyz). The best results were obtained for $(PDADMAC/PSS)_{6.5}$,[16] with more than 800 000 plates/m, for Cyt C analysis in 20 mM phosphate buffer pH 4. In the negatively charged SMIL coatings, i.e. SMIL coatings finishing with a polyanion and thus with a positive μ_{EOF}, 680 000 plates/m were obtained for $(PB/PVS)_1$ for the analysis of insulin in 400 mM Tris phosphate buffer pH 7.[25] In general, EOF magnitude decreased in the order

PB>εPLL>DEAEDq, whatever the polyanion used.[21] Neutral coatings, with μ_{EOF} around 0 TU, are outside the scope of this review. Some brief examples include the µSIL DB-WAX capillary (a commercial capillary based on polyethylene glycol (PEG) coating) or PEG coatings obtained in ultra-acidic conditions (HCl 1M).[38],[10] Some comparisons have been made between neutral and charged coatings, with Katayama et al. proving that suppressing electroosmotic mobility using PEG led to better peak separation efficiency than with linear polyacrylamide (PAM) or poly(vinyl alcohol) (PVA) (335 000 plates/m for PEG *vs* 238 000 and 291 000 for PAM and PVA, respectively), for the analysis of a mixture of two proteins (α-lac and TI), in $NaHPO_4$-Na_2HPO_4 (I = 0.05 M) buffer (pH 7.4). Less protein adsorption was observed when using dextran sulfate (DS) as the last layer, leading to higher efficiencies (518 000 plates/m). It was also shown that SMIL coatings with DS polyanion gave more symmetrical peaks than neutral coatings.[10]

As shown in Table 1, most SMIL coatings have low RSD(μ_{EOF}), i.e., below 1%. Few systems have higher values, showing poorer stability of the corresponding SMILs. For example, $(DEAED/DS)_{2.5}$ gave RSD values over 20%. In terms of stability only, the worst SMIL was with αPLL. Indeed, $(\alpha PLL/HA)_{2.5}$, $(\alpha PLL/PSS)_{2.5}$, $(\alpha PLL/PAA)_{2.5}$ and $(\alpha PLL/PLCA12)_{2.5}$ led to RSD(μ_{EOF}) above 20%. Stability of SMIL coatings with PAA, such as $(PAH/PAA_{80}AM_{20})_{2.5}$, $(PAH/PAA_{20}AM_{80})_{2.5}$, and $(PDADMAC/PAA)_{2.5}$ was unsatisfactory, with RSD(μ_{EOF}) of 1-5%, 2-7% and 6%, respectively. In contrast, $(PAH/PAA)_{2.5}$, $(PDMAEMA/PAA)_{2.5}$ and $(PEI/PAA)_{2.5}$ are much more stable coatings (with RSD(μ_{EOF}) of 1%, 0.3% and 0.5%, respectively).

Table 1. Summary of all studied SMIL systems used in CE, according to the charge of the last layer (polycation *vs* polyanion) and according to the polycation type. Number of layers, separation efficiencies, EOF and analysis pH are indicated for each system.

Polycationic final layer								
Type	**Polycation**	**Polyanion**	**Reference**	**Solutes**	**Number of layers**	***N/l* (10^{-3})**	**μ_{EOF} (TU)**	**pH (BGE nature)**
Polyionene	PEI	DS	[37]	Cyt C, Rnase A	3	4 (pH 3) 273 et 198 (pH 6)	-37 (pH 3) -34 (pH 6)	4 (10 mM phosphate buffer pH 3 or pH 6) (with 0%, 10% or 20% v/v % of methanol)
		PMA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	22	-45.5 RSD 1%	2.5 (0.5M acetic acid)
		PAA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	24	-45.7 RSD 0.5%	2.5 (0.5M acetic acid)
	PB	DS	[10]	α-Chymo A, Cyt C, Lyz, RNAse A	3	298 - 440	-34 (pH 2-13)	3.0 (0.05 M phosphate)
			[32]	α-Chymo A, Cyt C, Lyz, RNAse A	3	416.6 - 666.6	-13.4	8 (100 mM Tris phosphate)
					3		-57.9	2.7 (175 mM acetic acid)
			[39]	α-Chymo A, Cyt C, Lyz, RNAse A	3	233.3 - 333.3	not mentioned	3.0 (50 mM acetic acide)
			[40]	AMPs	5	not mentioned	not mentioned	2.0 to 12.25 (Tris, NaOH, CH3COOH + constituants (41 BGE tested))
			[24]	Oxytocin acetate, rhGH, rhIFN- β	3	142.8	not mentioned	3 (50 mM acetic acid)
					3	428.8	not mentioned RSD 1.4%	3 (525 mM acetic acid)
			[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	67	-54.3 RSD 1.5%	2.5 (0.5M acetic acid)
			[30]	Mixture of 25 fluorobenzoic acid derivates	3	220	-29 (pH 3)	3.1 (5 mM TTAC in 50 mM phosphate)
			[41]	rhIFN-β	3	not mentioned	not mentioned	3 (50 mM acetic acid, adjusted with ammoniac)
			[42]	Immunogenic proteins (namely TB10.4 and Ag85B)	3	not mentioned	not mentioned	1.5 M acetic acid
			[43]	Citric acid, lactic acid, 3-hydroxybutyric acid, pyroglutamic acid	3	> 200	not mentioned	6 (200 mM sodium phosphate)
			[33]	Peanut allergens (Ara h 1, 2, 3, 6)	3	not mentioned	not mentioned	2.5 (100 mM phosphoric acid, 70 mM Tris, 3 g/L polysorbate 80)
		HA	[10]	α-Chymo A, Cyt C, Lyz, RNAse A	3	not mentioned	-34 (pH 2-13)	3.0 (0.05 M phosphate buffer)
		Alg	[10]	α-Chymo A, Cyt C, Lyz, RNAse A	3	not mentioned	-34 (pH 2-13)	3.0 (0.05 M phosphate buffer)
		PSS	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	103	-62.6 RSD 1.1%	2.5 (0.5M acetic acid)

		PMA	[5]	Lyz, Myo, RNAse A, TI	5	l_{eff} = 30 cm : 167 (Lys) l_{eff} = 50 cm : 207 (Myo) l_{eff} = 70 cm : 584 (Myo)	not mentioned	2.5 (0.5M acetic acid)
			[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	145	-55.3 RSD 0.5%	2.5 (0.5M acetic acid)
		PAMxAMPSy	Not published	Lyz, Myo, RNAse A, TI	5	not mentioned	-7	2.5 (0.5M acetic acid)
		PLC	[5]	Lyz, Myo, RNAse A, TI	5	l_{eff} = 70 cm : 626	not mentioned	2.5 (0.5M acetic acid)
			[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	293	-50.5 RSD 0.8%	2.5 (0.5M acetic acid)
Polyvinylic	PDMAEMA	PMA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	60	-52.2 0.2%	2.5 (0.5M acetic acid)
		PAA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	57	-51.6 0.3%	2.5 (0.5M acetic acid)
		PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	38	-51.9 3%	2.5 (0.5M acetic acid)
	PDMAEA	PSS	Not published	Lyz, Myo, RNAse A, TI	5	48	-85.8 RSD 2%	2.5 (0.5M acetic acid)
Allylic	PAH	PSS	[34]	None	3	not mentioned	-57 à -35 (pH 7.1 à 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	7	not mentioned	-50 à -37 (pH 7.1 à 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	15	not mentioned	-45 à -35 (pH 7.1 à 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
		PMA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	62	-54.8 RSD 0.2%	2.5 (0.5M acetic acid)
		PAA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	55	-53.7 RSD 1%	2.5 (0.5M acetic acid)
		$PAA_{80}AM_{20}$	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	55	-50.8 RSD 1-5%	2.5 (0.5M acetic acid)
		$PAA_{20}AM_{80}$	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	57	-50.2 RSD 2-7%	2.5 (0.5M acetic acid)
		PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	69	-50.0 RSD 1-4%	2.5 (0.5M acetic acid)
	PDADMAC	PSS	[34]	None	3	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
				None	7	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
				None	15	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
			[44]	α-lac, Cyt C, Lyz, Myo, RNAse A	5	not mentioned	-28.8 RSD 0.1 %	2.5 (100 mM Tris-phosphate)
					5	not mentioned	-22.5 RSD 0.16	7.0 (100 mM phosphate)
			[16]	α-Chymo A, Cyt C, Lyz, RNAse A	7 13	436.6 - 803.3	-37.9	4 (20 mM phosphate)
						516.6 - 786.6	not mentioned	6 (20 mM phosphate)

			[20]	α-Lac, Cyt C, Lyz, Myo, RNAse A		58 (Lys) 0.04% m/v 50 (Lys) 0.2 % m/v *I* = 1.5M ($N.t_m$)	-29.7 to -32.1 (concentration 0.04% m/v) RSD 0.07-0.8% -29.0 to -30.4 (concentration 0.2% m/v) RSD 0.18-0.25%	2.5 (100mM Tris-phosphate)
					5	50 (Lys) 0.04% m/v 55 (Lys) 0.2 % m/v *I* = 1.5M ($N.t_m$)	-28.2 to -31.2 (concentration 0.04% m/v) 0.2-0.46% -28.3 to -30.4 (concentration 0.2% m/v) RSD 0.22-0.42%	2.5 (100mM Tris-phosphate)
			[15]	β-lac A, Lyz, Myo, RNAse A, TI	11	85 / 194	-54.7 1-3% / -60.3	2.5 (0.5M acetic acid)
		PMA	[34]	None	5	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
				None	3	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
				None	7	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
			[15]	β-lac A, Lyz, Myo, RNAse A, TI	15	68	-55.0 RSD 0.2%	2.5 (0.5M acetic acid)
		PAMxAMPSy	Not published	Lyz, Myo, RNAse A, TI	5	not mentioned	not mentioned	2.5 (0.5M acetic acid)
		PAA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	25	-50.4 RSD 6%	2.5 (0.5M acetic acid)
		PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	126 188	-54.6 / RSD 1% -60.0	2.5 (0.5M acetic acid)
		dsDNA	[45]	Enantioseparation of BNP	5	not mentioned	not mentioned	4.0 (10mM phosphate)
		DS	[45]	Enantioseparation of BNP	3	not mentioned	not mentioned	4.0 (10mM phosphate)
Polysaccharide	DEAEDq	DS	[21]	β-lac A, Lyz, Myo, RNAse A, TI	3	87	-43.0 RSD 0.8%	2.5 (0.5M acetic acid)
		PSS	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	125	-42.8 RSD 0.4%	2.5 (0.5M acetic acid)
		PMA	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	139	-45.6 RSD 0.8%	2.5 (0.5M acetic acid)
		PAA	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	94	-39.5 RSD 1.2%	2.5 (0.5M acetic acid)
		PLC	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	136	-43.3 RSD 0.3%	2.5 (0.5M acetic acid)
	DEAED	DS	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	59	-42.4 RSD >20%	2.5 (0.5M acetic acid)
	PCD +	PCD -	Not published	Lyz, Myo, RNAse A, TI	5	not mentioned	-47.6 RSD 4%	2.5 (0.5M acetic acid)
Polyamide/Polypeptide	εPLL	PSS	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	58	-51.2 RSD 1.3%	2.5 (0.5M acetic acid)
		PMA	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	102	-50.3 RSD 1.0%	2.5 (0.5M acetic acid)

		PAA	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	115	-48.0 RSD 2.2%	2.5 (0.5M acetic acid)
		PLC	[21]	β-lac A, Lyz, Myo, RNAse A, TI	5	336	-53.9 RSD 0.4%	2.5 (0.5M acetic acid)
	Plys	HA	[15]	β-lac A, Lyz, Myo, Rnase A, TI	5	13	-48.7 RSD >20%	2.5 (0.5M acetic acid)
		PSS	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	17	-46.4 RSD 18%	2.5 (0.5M acetic acid)
		PAA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	76	-43.1 RSD >20%	2.5 (0.5M acetic acid)
		PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	81	-54.6 RSD 0.5-2%	2.5 (0.5M acetic acid)
		PLCAI	Not published	Lyz, Myo, RNAse A, TI	5	not mentioned	-52.7 RSD 0.36%	2.5 (0.5M acetic acid)
		PLCA12	Not published	Lyz, Myo, RNAse A, TI	5	not mentioned	-49.7 RSD 17%	2.5 (0.5M acetic acid)
		PGLU	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	not mentioned	-54.9 RSD 1.5%	2.5 (0.5M acetic acid)
	P(lys,ser)	HA	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	8	-45.4 RSD >20%	2.5 (0.5M acetic acid)
		PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	54	-53.7 RSD >20%	2.5 (0.5M acetic acid)
	P(lys,tyr)	PLC	[15]	β-lac A, Lyz, Myo, RNAse A, TI	5	74	-51.8 RSD 0.5%	2.5 (0.5M acetic acid)
Other	PEI + PRT	DS	[37]	Cyt C, RNAse A Enantioseparation of BNP	5	36 - 119	-51 (pH 3)	4 (10 mM phosphate buffer pH 3 or pH 6) (with 0%, 10% or 20% v/v % of methanol)
		ssDNA	[45]	Enantioseparation of BNP	3	2.0 - 2.2	-33	(10 mM phosphate)
		dsDNA	[45]	Enantioseparation of BNP	3	0.14 - 0.16	-23	(10 mM phosphate)
	PEI + PA	DS	[37]	Cyt C, RNAse A Enantioseparation of BNP	3	129 - 169	-46 (pH 3)	4 (10 mM phosphate buffer pH 3 or pH 6) (with 0%, 10% or 20% v/v % of methanol)
	PEI + PLA	DS	[45]	Enantioseparation of BNP	3	not mentioned	-37.5	4.0 (10 mM phosphate)
		ssDNA		Enantioseparation of BNP	3	0.68 - 1.1	-31	
		dsDNA		Enantioseparation of BNP	3	1.4 - 2.1	-29	
	PEI + PDL	ssDNA	[45]	Enantioseparation of BNP	3	0.53 - 0.91	-39	10 mM phophate
		dsDNA		Enantioseparation of BNP	3	1.8 - 2.8	-38	
	PEI + PLL	dsDNA	[45]	Enantioseparation of BNP	3	not mentioned	not mentioned	10 mM phophate
	PEI (ssDNA - Prt)x	dsDNA	[45]	Enantioseparation of BNP	3	6.5 - 10.4	not mentioned	10 mM phophate
				Enantioseparation of BNP	(x = 1) 3	2.2 - 2.7	not mentioned	
				Enantioseparation of BNP	(x = 2) 5	0.82 - 0.86	not mentioned	
	PDADMAC (80) -	PSS	[36]	None	(x = 3) 7	not	-38 (pH 2.78)	2.78-7.30 (citric buffer 10 mM)

	PDADMAC-co-PAA(0.64/0.36)					mentioned	32 (pH 7.3)	

Polyanionic final layer								
Polyionene	PB	DS	[28]	α-lac, DHFR, TI	2	518 - 191	35	7.4 (phosphate)
			[46]	benzoin (flavoprotein as chiral selector)	2	129	not mentioned	7 (phosphate)
			[31]	None	2	not mentioned	20 - 40	3 - 10 (phosphate)
			[47]	Diflunisal, Ibuprofen, Indomethacin, Ketoprofen, Phenprocounom, Warfarin	2	not mentioned	not mentioned	7.4 (66.7 mM sodium phosphate, NaOH)
			[48]	TRI-1144	2	not mentioned	not mentioned	6 (150 mM ammonium formate)
		CC	[49]	Chloroquine diphosphate, Diltiazem hydrochloride, Laudanosine, Primaquine diphosphate, Propanolol hydrochloride	2	not mentioned	20 - 40	2.9 (20 mM PBS)
		PVS	[25]	α-lac, β-lac A and B, Albumin, CA, In, Myo, Ovalbumin, rhIFN-α	2	240 (insulin)	not mentioned	7 (25 mM sodium phosphate)
					2	300 (insulin)	not mentioned	7 (50 mM sodium phosphate)
					2	340 (insulin)	not mentioned	7 (100 mM sodium phosphate)
					2	320 (insulin)	not mentioned	7 (200 mM tris phosphate)
					2	500 (insulin)	not mentioned	7 (300 mM tris phosphate)
					2	680 (insulin)	not mentioned	7 (400 mM tris phosphate)
			[50]	10 amino acids in human urine (lysine, arginine, valine, phenylalanine, tyrosine, methionine, alanine, glutamic acid, isoleucine, leucine)	2	40-50	not mentioned	1.8 (1 M formic acid)
			[9]	Imidazole, *N*-benzylmethylamine, phenylpropanolamine, tertbutaline	2	600	49	2 (25 mM NaCl in 5 mM KH_2PO_4)
			[41]	rhGH	2	not mentioned	not mentioned	8.5 (75 mM ammonium formate, adjusted with formic acid)
			[43]	Amino acids (alanine, arginine, glutamic acid, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, valine)	2	42 to 250	not mentioned	1.8 (1M formic acid)
		PSS	[51]	β-lac A,β-lac B,Albumin, In, Lyz, Ovomucoid, Pancreatin, Pepsin	2	470	35.1 - 44.3	7.5 (400 mM Tris-phosphate)
Polyallylic	PAH	PSS	[34]	None	4	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)

				None	8	not mentioned	42 (pH 7.1 to 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	16	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
	PDADMAC	PSS	[34]	None	4	not mentioned	40 to 38 (pH 7.1 to 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	8	not mentioned	42 (pH 7.1 to 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	16	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
			[44]	CA, Myo, RNAse A	4	715 ($N.t_m$)	not mentioned	9.3 (Borate 100 mMM)
			[16]	α-Chymo A, Cyt C, Lyz, RNAse A	14	not mentioned	41 (pH 4) 42 (pH 6) 44 (pH 8)	pH 4, 6 and 8 (20 mM phophate buffer)
			[26]	Pheniramine	12	111.6	29.6 (pH 4.5)	4.5 (100 mM sodium phosphate)
					12	not mentioned	31.9 (pH 9.5)	9.5 (100 mM sodium borate)
		PMA	[34]	None	4	not mentioned	43 - 27 (pH 7.1 to 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	8	not mentioned	45 - 30 (pH 7.1 to 5.1)	7.1 - 5.1 (10 mM MES with NaCl)
				None	16	not mentioned	not mentioned	7.1 - 5.1 (10 mM MES with NaCl)
		GO -	[52]	PAHs, Thiourea (mixture)	2	32.5 - 2.5	11.38 - 11.47	7.5 (5 mM PBS)

5. Polyelectrolyte concentration and molar mass

5.1. Polyelectrolyte concentration

Polyelectrolyte concentration may have an impact on coating stability and separation efficiency. Indeed, outside of CE applications, thickness of polyelectrolyte multilayers (PEMs) as well as the total amount of polyelectrolyte deposited have been shown to increase as polyelectrolyte concentration increases, notably through studies of cationic high molar mass PAM adsorption on silica surfaces.[53],[54]

These findings were further investigated with SMIL coatings by Nehmé et al. using 0.04 and 0.2% m/v solutions of PDADMAC and PSS, and ionic strengths of 0.01 and 1.5 M.[20] In the case of $(PDADMAC/PSS)_5$ and $(PDADMAC/PSS)_{11}$ coatings, low concentrations of polyelectrolyte (0.04%) resulted in stable EOFs only at 1.5 M, whereas 0.2% polyelectrolyte coatings were always stable, but required a 10 min wait before analyses. The most repeatable option in terms of migration times was the 0.2% polyelectrolyte coating at 1.5 M ionic strength (RSD<0.5%, *n*=13). As shown in Figure 5, polyelectrolyte concentration had little influence on separation efficiency, with only slight improvement in performance using the 0.2% polyelectrolytes at 0 M ionic strength. Overall, the higher polyelectrolyte concentration led to better results.

Likewise, Cordova et al. demonstrated that sufficient polyelectrolyte concentration was needed to obtain repeatable separations.[55] At first, a 0.05% m/v PB solution in 25 mM Tris-192 mM glycine (Gly) buffer (pH 8.3) was used as a capillary coating to analyze a lysozyme charge ladder, which was successfully detected but required recoating after each run. Next, the PB concentration was increased to 7.5% m/v, corresponding to its solubility limit, and this led to stable EOF, high separation resolution and reproducibility, without any recoating. The same conditions were applied to other cationic polymers (poly(methoxyethoxyethyl)ethylenimine, PEI and PDADMAC). Therefore, higher capillary coating polyelectrolyte concentration was shown to provide better results in CE.

5.2. Polyelectrolyte molar mass

Multilayers made with a weak polyacid and a strongly dissociated polycation were also studied by Dubas et al., who found molar mass and polymer concentration had little impact on the thickness of the deposited layers, an exception being PAA which formed thicker films at higher molar mass at pH 11.[56] The study concludes that the number of extrinsic (i.e. free), and intrinsic (or bound with a counter-ion) charges in the polymer layer does not depend on molar mass.

The effects of polyelectrolyte concentration and molar mass used as coatings in CE were examined on a monolayer

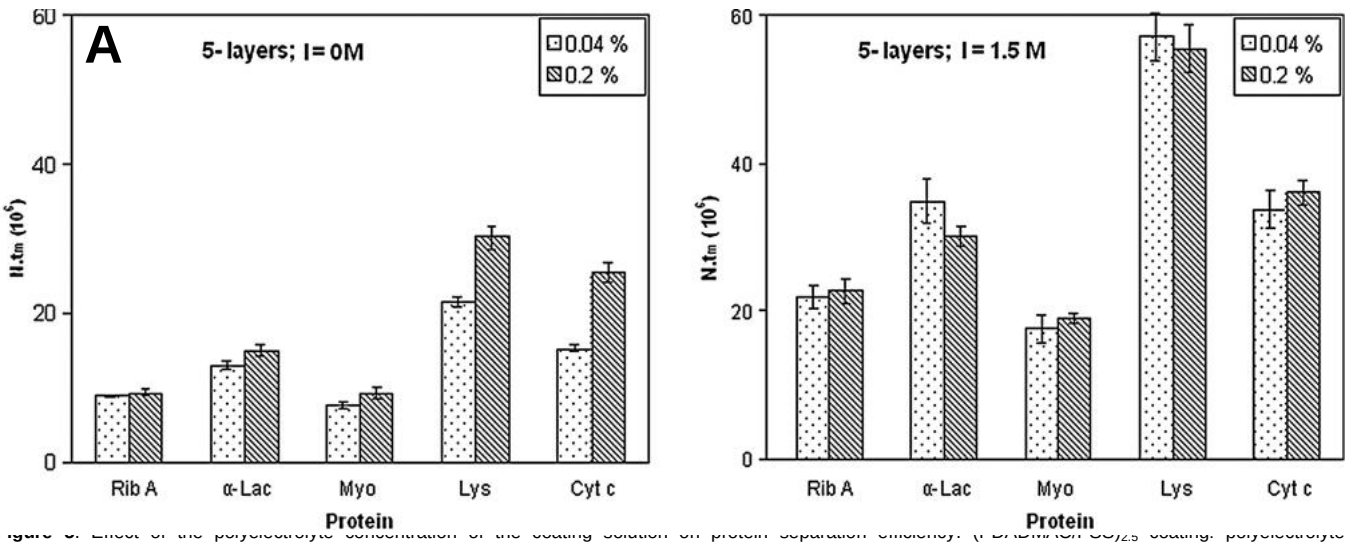


Figure 5. Effect of the polyelectrolyte concentration of the coating solution on protein separation efficiency. $(PDADMAC/PSS)_{2.5}$ coating. polyelectrolyte concentration 0.04% (A) and 0.2% m/v (B). Error bars indicate the SD on N.tm for 13 different experiments. Reprinted from Electrophoresis, Vol 30, Reine Nehmé,,Catherine Perrin, Hervé Cottet, Marie-Dominique Blanchin, Huguette Fabre, Influence of polyelectrolyte capillary coating conditions on protein analysis in CE, Pages No. 1888-1898, Copyright (2009), with permission from Wiley.

$(PDADMAC)_1$ coating by Wang et al., with varying ionic strengths of 0.5 and 0.005 M (Tris buffer), polymer

concentrations of 0.1 and 20 g/L, and M_r of 30 and 428 kDa at pH 8.[57] Only the high ionic strength and high molar mass polymer coating resulted in a pH-independent EOF, and the same EOF was obtained for both 0.1 g/L and 20 g/L 428 kDa PDADMAC monolayer coatings.

Adding onto these initial studies, Pei et al. compared $(PDADMAC)_1$ coatings with 5 different molar masses (8.5, 76, 100-200, 200-350 and 400-500 kDa) at pH 9.5 in 20 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) solution.[58] All PDADMAC monolayer coatings led to the same EOF on the first run, but only the two highest molar mass polymers presented stable EOF after several rinse cycles. They were also tested in terms of performance for the separation of inorganic anions (Br^-, CrO_4^{2-}, IO^{3-}, benzoate), where the 400-500 kDa M_r PDADMAC achieved better reproducibility, efficiency and baseline stability than the 8.5 kDa PDADMAC.

Therefore, capillary coatings seem to be more stable with higher molar mass polyelectrolytes, though short polyelectrolytes such as PLC (M_r =39 000 g/mol) and εPLL (M_n=5000 g/mol) have also led to stable and efficient coatings.[[5] Practical considerations should also be taken into account, including the fact that a high molar mass polymer solution may be too viscous to enter the capillary.

6. Coating stability and long-term behavior

6.1. Chemical stability

An interesting parameter to examine in SMIL coatings is their durability and resistance over time. In particular, their chemical stability may be evaluated by rinsing them with different solutions and repeating analyses, as was conducted by Katayama et al. with $(PB/DS)_1$ coated capillaries.[28] EOF was measured before (EOF_1) and after (EOF_2) rinsing for 15 minutes with different solutions or solvents (0.1 M HCl, 1 M NaOH, CH_3CN, CH_3OH, 5 M urea) and degradation ratios were determined (ratio = $(EOF_1-EOF_2)/EOF_1$). These were above 10% for the PB monolayer coating rinsed with HCl and CH_3CN, while the NaOH rinse detached the coating completely. The $(PB/DS)_1$ coating resisted well to all solvents except HCl, showing stronger stability which may be due to a higher number of layers than the PB monolayer coating.

Likewise, Isemura et al. examined the stability of their optimized $(PB/DS)_{1.5}$ coating for the analysis of three fluorobenzoic acids (tetrafluorophthalic acid, 2,4,5-trifluorobenzoic acid, and 3,4-difluorobenzoic acid).[30] Figure 6 shows the evolution of EOF and the acids electrophoretic mobilities over 60 runs, the BGE being changed every 15 runs. It can be seen that the EOF was stable for 30 runs and then gradually decreased, perhaps because of a minor detachment of the final PB layer. On the other hand, the electrophoretic mobilities increased slightly over 15 runs and returned to their initial value when the BGE was changed, likely due to the volatilization of the organic modifier acetonitrile (ACN), they can therefore be considered as stable.

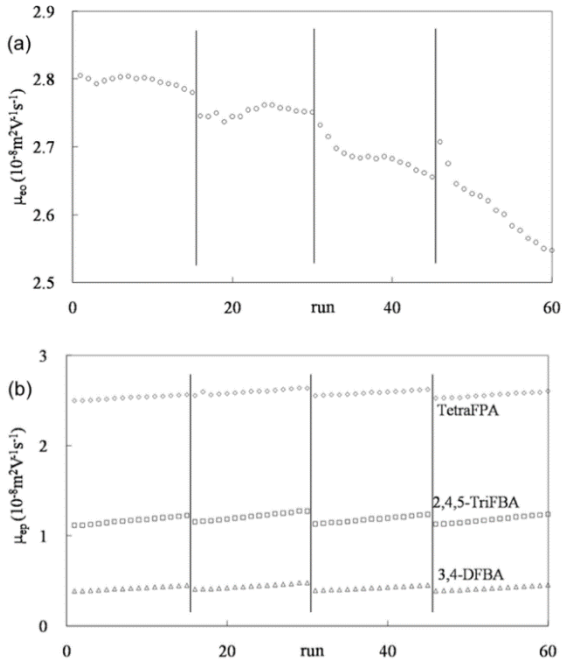


Figure 6. Stabilities of (a) EOF and (b) electrophoretic mobility of the PB-DS-PB SMIL capillary. Vertical lines show the change of BGE; EOF marker, benzyl alcohol; samples, 2,4,5-TriFBA, TetraFPA, and 3,4-DFBA; rinse between runs, buffer solution; separation with the optimized condition: triple layered (PB/DxS/PB) SMIL coating capillary (50 µm id x 57 cm (effective length 50 cm)); separation solution, 5 mM Tetradecyl trimethyl ammonium chloride (TTAC) in 50 mM phosphate buffer (pH 3.1) containing 30 vol.% ACN; applied voltage, – 20 kV; detection, 200 nm; temperature, 258C; injection time 3 s. Reprinted from Journal of Separation Science, Vol 32, Tsuguhide Isemura, Fumihiko Kitagawa, Koji Otsuka, Separation of complex mixtures of fluorobenzoic acids by capillary electrophoresis, Pages No. 381-387, Copyright (2009), with permission from Elsevier.

PDADMAC/PSS coatings were studied in the same way. Graul et al. showed that rinsing $(PDADMAC/PSS)_{7.5}$ coated capillary (built in 20 mM phosphate at pH 4) either with 0.01 M NaOH (pH 12) or 0.01 M HCl (pH 2) for 10 min had very little effect on the mobility, even when the rinses were repeated, confirming the stability of SMIL coatings for varying pH and ionic strengths.[16] Nehmé et al. similarly examined $(PDADMAC/PSS)_2$ and $(PDADMAC/PSS)_{2.5}$ coatings when subjected to destabilizing treatments (1 M HCl, 1 M NaOH, CH_3CN, CH_3OH, 1 M HCl/1 M NaOH, 1 M HCl/1 M NaOH/CH_3CN/CH_3OH for 30 minutes in a Tris-phosphate 100 mM buffer (pH 2.5)). Both coatings were stable to all the rinses, with less than 0.4% variation in mobility for the cationic coatings and less than 2% for the anionic coatings for the alkaline rinse in particular, which destabilized the PDADMAC monolayer coating. Successive alkaline, acidic and organic rinses had more of an effect (up to 46%) but were still more stable than the monolayer which varied up to 101%.[29]

Some of the same rinses were applied to a (PB/chondroitin sulfate C)$_1$ (CC) coated capillary made by Du et al.[59] In a phosphate buffer (pH 3), EOF was measured before and after rinsing with a solvent for 15 minutes (0.01 M NaOH, 0.1 M NaOH, 1 M HCl, CH_3OH, CH_3CN), leading to degradation ratios below 1% for all but 0.1 M NaOH, showing the stability of the coating over a wide range of pH.

Zheng et al. studied $(PB/PSS)_X$ coating in the same way and showed good stability against 1 M NaOH, CH_3CN and CH_3OH rinses (degradation ratios below 0.6%), and slight change after rinsing with 0.1 M HCl (4%).[51]

6.2. Endurance and regeneration

Endurance of the SMIL coating is also an important aspect to take into account, as noncovalent bonding may deteriorate over time. Katayama et al. studied the endurance of a SMIL-DS coated capillary with successive analyses of three model proteins (α-lac, β-lac A and B), showing good resistance up to 100 runs, and 60% EOF after 200 runs.[10] Regeneration of the coating was implemented by rinsing with 0.1 M HCl and repeating the coating procedure, restoring the initial performance. This highlights the advantage of using noncovalent coatings which can be easily regenerated.

The long term reproducibility of PDADMAC/PSS coatings was also investigated, with Graul et al. repeating 100 separations on a $(PDADMAC/PSS)_{7.5}$ coating, 20 of which were of a basic protein combination (α-chymo A, RNAse A, Cyt C, Lyz), over six days.[16] These were done at pH 4 and 6 in up to 100 mM phosphate buffer and between 10 and 15 kV and presented no variation in electroosmotic mobility. After 6 days, another layer of PSS was applied and 22 more runs at pH 4-8 were conducted, giving once again stable results. Next, the capillary was dried out over two weeks after which 5 runs were carried out at pH 6 in 20 mM phosphate, with less than 1% variation in mobility, and drying for another 34 days resulted in less than 2% variation. This confirms the great stability of the SMIL coating over a long period of time, as opposed to a PDADMAC monolayer coating which had to be reapplied before each run in order to conserve the EOF.

The benefits of regeneration were also shown by Nehmé et al. who found that it was necessary to reapply a layer of PDADMAC between runs on a $(PDADMAC/PSS)_{2.5}$ coated capillary at pH 7 to maintain a stable EOF and perform successful protein analysis.[29] On the other hand, $(PDADMAC/PSS)_{2.}$ coatings in a 100 mM borate BGE (pH 9.3) were stable even without regeneration at pH 7, due to repulsion between negatively charged proteins and the anionic coating.

Du et al.'s $(PB/CC)_1$ coating was evaluated through repeated runs at pH 3, showing a very stable EOF and migration time of one analyte (primaquine) after 100 runs.[59] Bekri et al. studied $(PDADMAC/PLC)_{2.5}$ coating in the same way and showed great repeatability over 100 runs (RSDs of 0.1% for the EOF and 10% for *N/I*).[15] Reproducibility was also examined on three capillaries, leading to 1.2% RSD(t_m) and 7% RSD(*N/I*).

Qu et al. showed that (PDADMAC/graphene oxide)$_1$ (GO) coating can endure 60 runs with only slight variation in migration times of the analytes (RSD 0.52%), and another 200 runs were performed with little change.[60] Moreover, the number of theoretical plates decreased only slightly for each analyte. Day-to-day and column-to-column reproducibilities were also excellent (RSDs of 0.96% and 1.86% respectively).

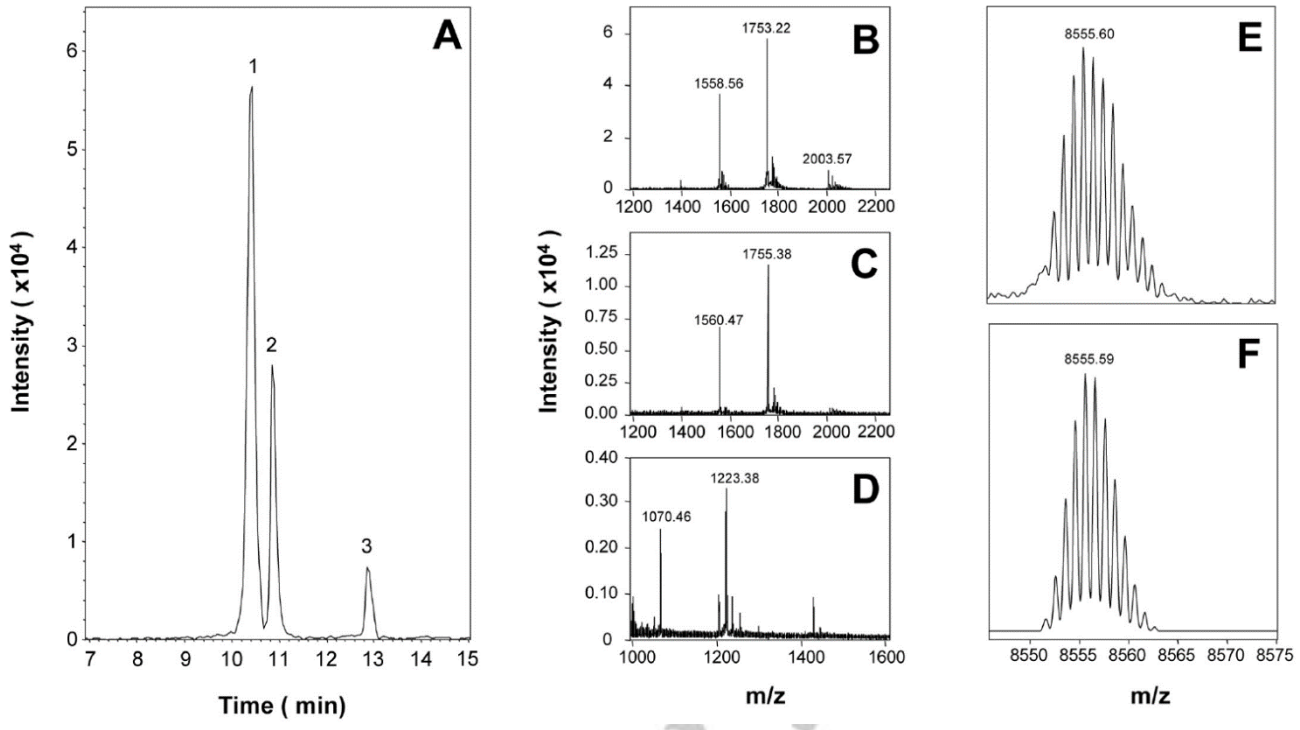


Figure 7. CE-ESI-TOF-MS of 14 µM VHH-A52. (A) Base-peak electropherogram constructed in the mass range m/z 1000-3000. (B-D) Average mass spectra obtained from peaks 1, 2, and 3, respectively. (E) Deconvoluted mass spectrum of peak 3. (F) Theoretically calculated mass spectrum of S. cerevisiae ubiquitin ($C_{375}H_{625}N_{105}O_{120}S_1$). Conditions: capillary coating, PB-DS-PB; capillary length, 100 cm; BGE, 175 mM acetic acid (pH 2.7). Reprinted from Journal of Separation Science, Vol 32, Rob Haselberg, Gerhardus J. de Jong, Govert W. Somsen, Capillary electrophoresis of intact basic proteins using noncovalently triple-layer coated capillaries, Pages No. 2408-2415, Copyright (2009), with permission from Elsevier.

Furthermore, storing and reusing SMIL coated capillaries may be considered, which requires long term stability of the capillary in the storage medium. The longevity $(PB/DS)_1$ coating was tested by comparing its EOF before and after storage in water overnight, leading to a 9.2% RSD (n=3 days), as opposed to the much lower 0.6% RSD run to run (n=5).[47] Next, the capillary was rinsed with 66.7 mM phosphate buffer at pH 7.5 containing 0.01% m/v DS between runs, improving day-to-day reproducibility (RSD 0.8%, n=3 days) and offering a satisfying option for capillary storage.

7. Applications

7.1. Limiting solute adsorption in CZE

CE is an efficient tool to analyze therapeutic compounds, as is shown by Isemura et al. for the analysis of drug intermediates.[30] Indeed, fluorobenzoic acids (FBAs) are important intermediates in antibacterial drug synthesis and require reproducible and efficient separation which can be provided by CE. 25 derivatives were analyzed with phosphate buffers (pH 2.5-7) and a $(PB/DS)_{1.5}$ coated capillary. Organic modifiers, methanol or ACN, were shown to improve separation of the FBA mix, and notably 24 peaks were observed at pH 3.1 with 30 vol.% ACN. Under these conditions, a purity analysis of 3-Chloro-4-fluoro-6-nitrobenzoic acid, an intermediate of quinolone antibacterials, was conducted by CE, and several impurities were separated with 110 000 theoretical plates for the main peak, compared to 4000 plates by high-performance liquid chromatography (HPLC). Thus, CE, along with SMIL coatings, was demonstrated to be a highly efficient separation technique for the analysis of pharmaceutical intermediates.

One example of therapeutic drugs which has gained a lot of media attention is monoclonal antibodies (mAbs), large proteins which show great promise for the treatment of rare and/or immune diseases and cancers. It is therefore interesting to see if SMIL coated capillaries are able to separate the different mAbs isoforms (either variant in charge or in mass). Such a study was conducted by Haselberg et al. using a $(PB/DS)_{1.5}$ coated capillary to profile recombinant humanized mouse monoclonal immunoglobulin G1 as well as two llama antibodies.[32] Four basic model proteins (α-chymo A, RNAse A, Cyt C, and Lyz, with pIs between 9 and 11) were initially analyzed in Tris-phosphate BGEs at different pHs (3, 4, 5, 6, 7, 8) in which they were positively charged, leading to the detection of some minor

impurities with very repeatable migration times (RSD < 0.85%) and good separation efficiencies (> 125 000 plates). The IgG1 antibody (pI 9) was analyzed in a 50 mM phosphate BGE (pH 6.8) and showed a repeated pattern of bands which correspond to different glycoforms coming from two glycosylation sites. Components may be identified when using CE coupled with MS, as was demonstrated for the study of llama antibodies, VHH-152 and VHH-R2, which are particularly stable and offer good nanomolar affinity, useful for antibody engineering. CE alone allowed the repeatable analysis of both antibodies and showed the long-term stability of the $(PB/DS)_{1.5}$ coating with a 175 mM acetic acid BGE (pH 2.7). VHH-R2 showed no impurities, while three peaks were resolved for VHH-A52. When coupled with MS, mass spectra of VHH-A52 was deconvoluted and the peaks 1-3 were identified as is shown in Figure 7, corresponding to a VHH-A52 form (peak 2), its modification through a transition of N-terminal glutamine to pyroglutamic acid (peak 1), and ubiquitin from *S. cerevisiae* (peak 3). This example illustrates the strength of CE and MS hyphenation, which combines fast and efficient separation of analytes with mass information. A summary of recent advances in CE-MS instrumentation and methodology,[61] as well as the use of different kinds of capillary coatings in CE-MS,[62] may be found elsewhere.

In addition, CE may be used to detect degradation products of pharmaceutical compounds, as was shown by Haselberg et al. with $(PB/PVS)_1$ and $(PB/DS)_{1.5}$ coated capillaries.[24] Specifically, capillary electrophoresis-electrospray ionization time-of-flight–mass spectrometry (CE-TOF-MS) was employed to detect degradation products resulting from heat stress and/or prolonged storage of recombinant human growth hormone (rhGH), used to treat growth abnormalities, and oxytocin, a hormone drug used in labor. Potential modifications could be seen by comparing compounds' molar masses and migration times to the original product. The anionic $(PB/PVS)_1$ coating was used with a medium to high-pH BGE (ammonium formate at pH 8.5) to analyze acidic rhGH, whereas the cationic $(PB/DS)_{1.5}$ coating was used with acetic acid at pH 3 for basic oxytocin. Analyses showed that exposing rhGH to heat resulted in oxidations, sulfonate formation as well as deamidation. On the other hand, oxytocin was highly deamidated at low pH, but showed more dimers and trisulfides at medium and high pH. Recombinant human interferon-β-1a (rhIFN-β), an N-glycosylated protein used to treat multiple sclerosis, was also analyzed and at least ten glycoforms were detected. These resolved peaks could be quantified through their relative areas and give a profile, which is useful for quality control.

Likewise, the degradation of a therapeutic peptide drug that acts as an inhibitor towards HIV-1, TRI-1144, was studied by CE-tandem mass spectrometry (CE-MS/MS).[48] MS/MS consists of two mass analyzers which split ions into fragments and allows the identification and separation of ions that have similar m/z ratios, resulting in a high-resolution tool for the detection of slight peptide modifications. Indeed, stressed samples of TRI-1144 were analyzed with a $(PB/DS)_1$ coating in a 150 mM ammonium formate BGE (pH 6) and 14 peaks corresponding to deamidated and deacetylated species were resolved.

The analysis of degradation products by CE-MS may also be useful for vaccine development. Indeed, Tengattini et al. analyzed tuberculosis antigens TB10.4 and Ag85B and their glycoconjugates with a $(PB/DS)_{1.5}$ coating and a 1.5 M acetic acid BGE, showing several compounds corresponding to deamidation and truncation products.[42] Exposed to glycosylation conditions, TB10.4 presented eight additional peaks and Ag85B two additional peaks, which were able to be resolved and identified, highlighting the strength of the method.

Haselberg et al. analyzed erlotinib, a kinase-inhibiting drug used to treat renal diseases which can bind to lysozyme.[24] An erlotinib-universal linkage system (ULS)-N-(tert-Butoxycarbonyl)-L-methionine (BOCmet)-Lyz solution was analyzed in an acidic BGE with a PEI monolayer coated capillary, and base peak electropherograms obtained from sheathless CE-MS were deconvoluted to show 6 peaks assigned to different drug conjugates.

In addition to analyzing therapeutic compounds, SMIL coatings in CE may be used to screen for biomarkers which allows the detection of illnesses such as diabetes and kidney disease.[63] Indeed, Stolz et al. achieved the baseline separation of human hemoglobin proteoforms with a $(DEAED/PMA)_{2.5}$ coating in a 2 M acetic acid BGE. Coupled with MS, identification of the peaks was possible, as well as relative quantification of the glycated and carbamylated species, showing the effectiveness of CZE-MS for this kind of application.

Similarly, the analysis of free amino acids in human urine may lead to the detection of deficiencies, as was shown by Ramautar et al. using a $(PB/PVS)_1$ coating in CE-TOF-MS.[50] Repeated analyses of urine samples at pH 1.8 in a 1 M formic acid BGE successfully separated eight of ten amino acids and were able to differentiate healthy controls from patients suffering from urinary tract infections. The authors also conducted the analysis of

cerebrospinal fluid in the same conditions, which could lead to the metabolic profiling of complex regional pain syndrome.[43] The applications and future developments of CE-MS to metabolomics have been summarized in another review.[64]

Moreover, Zheng et al. studied the stability of allergenic proteins in simulated gastric fluid (SGF) and intestinal fluid (SIF) and observed a degradation product, improving on the previously used method, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),[51] which was unable to detect it. This is interesting to investigate since protein stability in simulated gastrointestinal fluids gives information about digestion resistance and therefore food allergies. A PB-PSS bilayer coating was used to test four proteins, BSA, OVM, β-lac and Lyz in different BGEs (KH_2PO_4, $Na_2B_40_7$-HCl, Tris-H_3PO_4, KH_2PO_4) and at different pH (2.5-8), and stability was evaluated by comparing peak areas after 60 min of incubation. Finally, it was shown that three out of the four proteins presented low resistance to the pepsin in SGF, while 3 out of 4 were stable in SIF with degradation rates under 25%. Therefore, SMIL-coated capillaries used in CE gave new information on the digestibility of certain proteins.

Another allergen, recombinant birch pollen Bet v 1a, was analyzed by CZE by Stock et al. using a 4-layer anionic SMIL coating.[65] The separation was conducted in a 75 mM $Na_2HPO_4 \cdot 2H_2O$ BGE (pH 6.5) and two coatings were compared, $(PDADMAC/DS)_{1.5}$-poly(acrylamide-co-2-acrylamido-2-methyl-1-propanesulfonate)$_1$ (PAMAMPS) (at 55%) and $(PDADMAC/DS)_2$, the first resulting in separation of minor allergen variants, unlike the latter which only showed the three major peaks corresponding to Bet v 1a, single- and double-carbamylated Bet v 1a. Moreover, it was shown that dry storage of the capillary overnight modified the peak shape, which could be restored after rinsing with the BGE. Other techniques were used to assess properties of the SMIL coating, notably AFM and topography and recognition imaging (TREC) which showed the homogeneous surface, charge distribution, and reconstruction ability of the SMIL-55% PAMAMPS. Similarly, protein mapping of peanut extract was conducted with CZE by Villemet et al.,[33] resulting in the characterization of major peanut allergens (Ara h1, Ara h2, Ara h3, Ara h6). A $(PB/DS)_{1.5}$ coating was used with a BGE made up of 100 mM phosphoric acid, 70 mM tris, and 3 g/L PS at pH 2.5, and the peaks were identified by comparison with Ara h standards.

TREC method was also used by Leitner et al. to obtain adhesion maps of different SMIL coatings, showing the strength of TREC as a nanoscale characterization technique.[66] Thus, differences in the charge distributions of $(PDADMAC/DS)_{1.5}$-55% $(PAMAMPS)_1$ and $(PDADMAC/DS)_2$ as well as electrostatic interactions between proteins and the capillary wall were demonstrated. Similarly, Haselberg et al. used AFM to determine the layer thickness and morphology of mono-, 2- and 3-layer coatings made from PB, DS and PVS, finding that the single layer coating does not fully recover the silica surface.[67] The coatings were also studied after exposure to proteins, allowing an evaluation of their effectiveness to reduce protein adsorption according to the variation in layer thickness.

7.2. Using SMIL coatings for CEC separations

CEC is an analytical technique which combines HPLC and CE, using capillaries filled with HPLC stationary phase under high voltage. Analytes interact with both the mobile and stationary phases, leading to their separation. The stationary phase may be prepared through physical adsorption, turning the otherwise unwanted phenomena of adsorption into an advantageous one.[68] Indeed, basic chiral selectors can be made into the stationary phases for open-tubular CEC (OTCEC). Generally, chiral compounds may be analyzed by different chromatographic and electrophoretic techniques, such as liquid chromatography (LC), supercritical fluid chromatography (SFC), gas chromatography (GC) and CE, as was described in several recent reviews.[69]-[70]

In particular, SMIL coated capillaries find several applications for chiral separations.[26],[45],[46] Katayama et al. used a dovetailed capillary (an assembly of capillary segments with different EOF magnitudes and directions, resulting in a unit with a perfectly controlled EOF) to perform enantiomeric separations.[46] The segments consisted of uncoated, PB or DS coated, and neutral µSIL DB-WAX capillaries. First, cyclodextrin Capillary Zone Electrophoresis (CD-CZE) analysis of chlorprenaline with a neutral coating (dovetail ratio 100%) resulted in baseline separation of the (+) and (-) enantiomers, which was further improved with a 50% dovetailed capillary (equally long PB and neutral segments). CD and 2,6-Di-*O*-methyl-ß-CD (DMCD) served as chiral selectors. Chiral compounds such as proteins and antibiotics may also be used as chiral selectors in partial filling affinity electrokinetic chromatography (EKC). This method, along with a dovetailed capillary (DS and neutral coated), was successfully applied to the chiral separation of benzoin with

flavoprotein as a chiral selector in a phosphate buffer (pH 7). These studies illustrate the importance of controlling the EOF for both EKC and CD-CZE. However, the use of non-uniform charge density along the capillary surface is generating peak broadening due to Taylor dispersion.[71] Another approach for the modulation of the EOF is to use an homogeneous coating of controlled charged density,[72][73] as exemplified in the case of peptide separations for a $(PDADMAC/PAMAMPS)_1$ in phosphate buffer at pH 2.5. The charge density was controlled by the molar content in AMPS in the PAMAMPS statistical copolymer.

Another application of CE for chiral separation was implemented by Currie et al., who used heparin, a strongly anionic biopolymer, as a chiral selector.[26] The SMIL coating consisted of strong polyelectrolytes, PDADMAC and PSS, in order to limit the adsorption of heparin onto the capillary walls, and was built up to 12 layers, ending in PSS. This system was applied to the analysis of antihistamine pheniramine, where a pH of 4.2, a heparin concentration of 4% and phosphate buffer concentration of 100 mM were found to be optimal in terms of separation efficiency, reaching 55 800 plates. However, the bare fused capillary achieved a higher resolution between the two peaks.

Likewise, Kitagawa et al. prepared SMIL coated capillaries for the analysis of binaphthyl enantiomers using CEC.[45] The coatings were made up of three different polymers, with PEI as the first layer, anionic DS or DNA as the second layer, and a cationic polymer or polypeptide such as protamine (Prt) as the third layer. Good chiral separation was obtained only with DNA as the second layer, due to the formation of DNA-polypeptide complexes, and single stranded DNA (ssDNA) was found to give a more stable EOF and reproducible results than double stranded DNA (dsDNA). Finally, the PEI-ssDNA-Prt coating yielded the best chiral separation of 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BNP). An optical purity test was carried out using this coating and was able to evaluate the amount of each enantiomer, showing an enantiomeric excess of (*R)*-BNP over (*S)*-BNP of 97.9%. The effect of the number of layers on this chiral separation was also evaluated.[45] Three combinations were tested: $PEI–(ssDNA–Prt)_n$ with n=1, 2, 3. As the number of layers increased, the retention factors increased, while the plate numbers, the selectivity factors and the resolutions decreased. Peak tailing was also amplified at the higher numbers of layers and repeated injections led to capillary clogging, showing that thinner coatings are better suited for this kind of application.

All in all, SMIL coated capillaries in CE offer many applications for the analysis of pharmaceutical compounds and separations requiring high efficiency and reproducibility and are promising tools as these needs continue to grow.

Conclusion

In a previous review about applications of capillary electromigration methods, protein adsorption on the capillary wall was described as a critical issue.[74] In this paper, it was shown that a huge research work, from the end of the 20th century until today, was dedicated to diminish this phenomenon, particularly using SMIL systems. Different parameters were analyzed to understand how these multilayers were affected. Adding salt to the coating buffer solution was shown to have a great impact on separation efficiency, with different optimal concentrations depending on the system. The effect of pH was also noticeable in the case of weak PE coatings since their ionization state depends on pH. Thus, a pH between the polycationic and polyanionic pKa was the best option. BGE composition and nature also proved to be also important factors to control solute interaction with the capillary all. Indeed, increasing BGE ionic strength resulted in better separation efficiency for protein separation. The number of deposited layers also affects SMIL performances since a thicker film leads to greater diffusion of the solute and lower separation efficiency. On the other hand, a single or very low number of layers can leave an access to the silica surface. Therefore, a minimum number of layers (typically 5) is generally required for optimal separation. In addition, a great number of studies was devoted to PE nature, showing a prominent impact on μ_{EOF}, therefore t_m, from one SMIL to the next. The best polyelectrolyte couples in terms of separation efficiencies reported in literature were PDADMAC/PSS, PB/PSS, PB/PMA, PB/PLC, DEAEDq/PMA, DEAEDq/PSS and εPLL/PLC. Therefore, varying BGE and PE natures led to a great number of possibilities to optimize analysis (as seen in Table 1). In terms of PE characteristics, higher concentration and molecular mass led to more stable films and better overall performance. Coating stability is another important factor to consider. Chemical stability was evaluated by rinsing the capillaries with different solutions, showing that SMIL coatings are generally quite stable. They were found to be able to endure up to 100 runs with little loss in separation efficiency, as well as easy to regenerate and possibly to store. Finally, various applications of SMIL coatings were presented, showing particular promise for the analysis of pharmaceutical compounds

including proteins, whether to check for impurities, the presence of variants or possible degradation products, as well as chiral separations. All in all, it can be claimed that SMIL coatings used in CE, especially coupled with MS, have a bright future as their applications continue to grow.

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Keywords: electro-osmotic flow • electrophoresis • polyelectrolyte multilayers • proteins • separation efficiency

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Entry for the Table of Contents

This review describes the effects of various parameters on the performance of SMIL coatings (alternation between polycationic and polyanionic layers, with the objective of reducing interactions between silanol groups and proteins), covering the construction buffer ionic strength and pH, the BGE, the number of layers, polyelectrolyte nature, polyelectrolyte concentration and molar mass, coating stability and long-term behavior. It also presents some applications of these coatings, both in CZE and CEC, paying particular attention to the increasingly important biopharmaceutical applications.