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## Investigating cationic and zwitterionic successive multiple ionicpolymer layer coatings for protein separation by capillary electrophoresis

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1       **Investigating cationic and zwitterionic successive multiple ionic-**  
2               **polymer layer coatings for protein separation by capillary**  
3                       **electrophoresis**

4  
5

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15

16 **HIGHLIGHTS**

- 17 ● Coatings with higher molar mass PDADMAC lead to higher separation efficiency
- 18 ● Polyanion molar mass does not impact separation efficiency
- 19 ● PDADMAC/PSS multilayer coatings are more durable than a monolayer of PDADMAC
- 20 ● PDADMAC/PSS coatings with more than 5 layers lead to lower separation efficiency
- 21 ● EOF modulation was possible using coatings with a polyzwitterionic last layer

22

23 **ABSTRACT**

24

25 Successive multiple ionic-polymer layers (SMILs) have long since proved their worth  
26 in capillary electrophoresis as they ensure stable electroosmotic flow (EOF) and relatively  
27 high separation efficiency. Recently, we demonstrated that plotting the plate height ( $H$ )  
28 against the solute migration velocity ( $u$ ) enabled a reliable quantitative evaluation of the  
29 coating performances in terms of separation efficiency. In this work, various  
30 physicochemical and chemical parameters of the SMIL coating were studied and optimized  
31 in order to decrease the slope of the ascending part of the  $H$  vs  $u$  curve, which is known to  
32 be controlled by the homogeneity in charge of the coating surface and by the possible  
33 residual solute adsorption onto the coating surface. SMILs based on  
34 poly(diallyldimethylammonium chloride) (PDADMAC) and poly(sodium styrene sulfonate)  
35 (PSS) were formed and the effect of each polyelectrolyte molar mass and of the number of  
36 polyelectrolyte layers (up to 21 layers) was studied. The use of polyethylene imine as an  
37 anchoring first layer was considered. More polyelectrolyte couples based on PDADMAC,  
38 polybrene, PSS, poly(vinyl sulfate), and poly(acrylic acid) were tested. Finally, zwitterionic  
39 polymers based on the poly( $\alpha$ -L-lysine) scaffold were synthesized and used as the last layer  
40 of SMILs, illustrating their ability to finetune the EOF, while maintaining good separation  
41 efficiency.

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45 **Keywords:** Polyelectrolyte multilayers, protein separation, separation efficiency, capillary  
46 electrophoresis, zwitterionic polymers

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48

## 1. Introduction

Polyelectrolyte multilayers are thin films consisting of electrostatically bonded polyelectrolytes of alternating charges which find applications in many different fields ranging from healthcare to energy and materials [1]. As such, they may also be used in capillary electrophoresis (CE), where coatings are commonly used to limit solute interaction with the capillary wall [2-4]. Katayama et al. were the first to introduce these successive multiple ionic-polymer layers (SMILs) for the separation of basic proteins by CE [2]. A 3-layer polybrene (PB)/dextran sulfate (DS)/PB SMIL coating resulted in highly efficient peaks, similarly to more commonly used neutral coatings, as well as excellent endurance and stability to rinsing and changes in pH [2]. Since then, many different SMIL coatings have been developed and applied to protein separation, with some of the most commonly used polyelectrolytes being PB [2,5-8], polyethylene imine (PEI) [9,10], poly(diallyldimethylammonium chloride) (PDADMAC) [9,11,12], DS [2,5,8,10], and poly(sodium styrene sulfonate) (PSS) [5,9,11-13]. They are semipermanent coatings, which do not need to be present in the background electrolyte (BGE), like dynamic coatings, and are easier to prepare and regenerate than covalently bound ones.

Different coating stabilities and separation performances may be achieved depending on the nature of the polyelectrolyte couple [5,8,9]. A systematic study on the influence of polyelectrolyte nature has shown that both the polycation and polyanion have an impact on separation efficiency and coating stability. Over 20 couples were studied for the separation of model proteins in acetic acid MS compatible buffer, finding that PDADMAC was the best choice of polycation and poly(L-lysine citramide) (PLC) for the polyanion, with (PDADMAC/PLC)<sub>2.5</sub> coatings resulting in up to  $126 \times 10^3$  plates/m and excellent repeatability over 100 runs (%RSD( $t_m$ ) = 0.1%) [9]. Moreover, physico-chemical conditions such as construction buffer, background electrolyte (BGE) ionic strength and pH have been shown to impact both electroosmotic flow (EOF) and separation efficiency [9,11,14-16]. Adding salt to the construction buffer resulted in increased stability of the 5-layer PDADMAC/PSS SMIL coating as well as thicker layers [11,16,17]. Thick coatings are generally thought to be advantageous as they increase the distance between the solute and the silanol groups present in the silica wall [4], however they may also result in more solute

80 diffusion within the coating [18]. Coating thickness may also be varied by modifying the  
81 number of deposited layers, as was studied by Bekri et al., who found that 5-layer SMILs led  
82 to higher separation efficiency and repeatability than 1- and 21-layer ones for three  
83 different polyelectrolyte systems [9]. Coatings with a lower number of layers also displayed  
84 better stability to changes in pH [8].

85 Besides SMILs, other commonly used semipermanent capillary coatings are neutral  
86 polymers, including polyethylene oxide (PEO), poly(vinyl alcohol) (PVA), polyacrylamide, and  
87 cellulose derivatives [19]. Their hydrophilicity helps to reduce sample interaction with the  
88 capillary wall, and they also strongly suppress the EOF which can be useful for certain  
89 applications, but they are often less stable than charged polymers [20]. Another approach is  
90 to use zwitterions, molecules which contain an equal number of positive and negative  
91 charges, conferring them antifouling properties [21-23]. Semipermanent zwitterionic  
92 coatings may be based on double-chained zwitterionic surfactants which form stable  
93 bilayers on the capillary wall [24], or polyzwitterions may be synthesized and statically  
94 adsorbed onto the capillary. Polyzwitterions have been successfully applied onto planar  
95 surfaces and nanoparticles to limit protein adsorption for biomedical applications [25-27],  
96 but few cases of noncovalent polyzwitterionic coatings for use in CE have been reported  
97 [28,29]. Covalent polyzwitterionic coatings are more common, but require in situ chemical  
98 reactions to be implemented [30-34].

99 The objective of this work was to optimize the separation performance of  
100 PDADMAC/PSS SMIL coatings by changing polyelectrolyte molar mass and nature as well as  
101 the number of layers. PEI was tested as the first layer and newly synthesized polyzwitterions  
102 as the last layer to further reduce interactions between proteins and the capillary surface.  
103 All the coatings were systematically evaluated by plotting plate heights  $H$  against migration  
104 velocities  $u$  obtained by performing separations at different voltages, with  $n=5$  repetitions  
105 for each one. This provides valuable information about the kinetic behavior of the  
106 separations, enabling a ranking of coating performances. It also allows the impact of the  
107 different SMIL parameters described above on separation efficiency to be determined.

108

## 109 **2. Materials and Methods**

## 110 **2.1. Chemicals**

111 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES), acetic acid, and  
112 methyl 3-(dimethylamino)propionate were purchased from Sigma-Aldrich (Saint-Quentin  
113 Fallavier, France). *N,N*-dimethylglycine ethyl ester, 4-(dimethylamino)butyric acid  
114 hydrochloride, *N*-methylmorpholine (NMM) and 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-  
115 methylmorpholinium chloride (DMTMM) were purchased from Tokyo Chemical Industry  
116 (TCI). Propan-1,3-sultone from Thermo Fisher Scientific, and deuterated solvents from  
117 Deutero. All other solvents and chemicals were obtained from the usual commercial sources  
118 and used as received unless otherwise noted. The model proteins, Carbonic Anhydrase I  
119 from human erythrocytes (CA, purity not indicated by the supplier), Trypsin Inhibitor from  
120 Glycine max (soybean) (TI, purity not indicated by the supplier), Myoglobin from equine  
121 skeletal muscle (Myo, purity  $\geq 95\%$ ), Ribonuclease A from bovine pancreas (RNase A, purity  
122  $\geq 60\%$ ),  $\beta$ -lactoglobulin A from bovine milk ( $\beta$ -lac A, purity  $\geq 90\%$ ), and Lysozyme from  
123 chicken egg white (Lyz, purity  $\geq 90\%$ ) were purchased from Sigma-Aldrich (Saint-Quentin  
124 Fallavier, France). Ultrapure water was obtained using a MilliQ system from Millipore  
125 (Molsheim, France). Poly( $\alpha$ -L-lysine) hydrochloride (PLL; average MW  $66 \times 10^3$  g/mol) with a  
126 polydispersity index (PDI) between 1.0 – 1.2 was purchased from Alamanda Polymers.  
127 Poly(diallyldimethylammonium chloride) (PDADMAC, high molar mass MW  $4 \times 10^5$  –  $5 \times 10^5$   
128 g/mol, and low molar mass MW  $1 \times 10^5$  g/mol), 20% m/m and 35% m/m in water,  
129 respectively, were purchased from Aldrich (Lyon, France). Poly(styrene sulfonate) (PSS, MW  
130  $5 \times 10^3$  g/mol;  $29 \times 10^3$  g/mol;  $7 \times 10^4$  g/mol;  $148 \times 10^3$  g/mol;  $330 \times 10^3$  g/mol) were  
131 purchased from Acros Organics (Geel, Belgium). PB (MW  $15 \times 10^3$  g/mol), PAA (MW  $2 \times 10^4$   
132 g/mol and  $97 \times 10^3$  g/mol), PVS (MW  $17 \times 10^3$  g/mol), and PEI (MW  $75 \times 10^4$  g/mol) were  
133 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

134

## 135 **2.2. Zwitterion synthesis**

136 Briefly, the zwitterionic building blocks equipped with a carboxylic acid function were  
137 synthesized by quaternization of the tertiary amino function of set of  $\omega$ -dimethylamino  
138 carboxylic acids with systematically increasing chain length using propan-1,3-sultone. For  
139 this, the methyl or ethyl ester protected  $\omega$ -dimethylamino carboxylic acids (1 eq.) were

140 dissolved in acetone. Upon addition of propan-1,3-sultone (1.1 eq.) the mixture was heated  
141 to 50 °C overnight producing the zwitterions as a colorless precipitate, respectively. To  
142 generate the free carboxylic acid function, the esters were hydrolyzed in 6 M hydrochloric  
143 acid at 70 °C yielding the zwitterionic building blocks as quaternary ammonium chlorides  
144 fully characterized by <sup>1</sup>H- und <sup>13</sup>C-NMR spectroscopy as well as CE-MS analysis. <sup>1</sup>H- and <sup>13</sup>C-  
145 NMR spectra were recorded on Bruker DRX-500 NMR spectrometer.

146

### 147 **2.3. Functionalization of the PLL**

148 Briefly, PLL was dissolved in H<sub>2</sub>O/MeOH 1:1 v/v at a concentration of 10 mg/mL. The  
149 zwitterions were also dissolved in H<sub>2</sub>O/MeOH 1:1 v/v and deprotonated with NMM (2 eq.).  
150 The solution of the deprotonated zwitterion and the coupling reagent DMTMM (1:1 molar  
151 ratio) were added to the PLL solution in the desired stoichiometric ratio under vigorous  
152 stirring. The reaction was allowed to proceed overnight at room temperature followed by  
153 dialysis against 0.5 M sodium perchlorate for 48 hours. The dialyzed polymer was lyophilized  
154 for 48 hours and obtained as colorless solid. The degree of functionalization was determined  
155 from the ratio of the integrals of the signals of the α-CH protons of the Lys residues of PLL  
156 and the signals of the zwitterionic moieties by <sup>1</sup>H NMR. The final derivatized polymers are  
157 represented in Fig. 1.

158

### 159 **2.4. Capillary coating procedure**

160 Acetic acid 2 M (pH 2.2) was used as the BGE and 20 mM HEPES with 10 mM NaOH  
161 (pH 7.4) as the construction buffer. Polyelectrolyte and zwitterion solutions (3 gL<sup>-1</sup>) were  
162 prepared by dissolving or diluting the polyelectrolytes in HEPES, at least one night before  
163 the first use. The polycation and zwitterion solutions were kept in the freezer while the  
164 polyanions could be kept in the fridge. The protein mix was prepared from individual  
165 solutions of proteins stored in water at a higher concentration, which can be kept in the  
166 freezer for several months without degradation. Equal amounts of each stock solution were  
167 mixed, adding acetic acid at a higher concentration in order to obtain the final mix in acetic  
168 acid 2 M, where each protein is at 0.2 gL<sup>-1</sup>. The protein mix was placed in an oven for 30



169 minutes at 37°C in order to reach more stable protein conformations [35]. The SMIL  
170 coatings were prepared by preconditioning the silica capillary with 1 M NaOH for 10 min,  
171 then flushing with water for 5 min and HEPES for 10 min. Next, the polyelectrolyte solutions  
172 were flushed for 7 min each, followed by 3 min of HEPES, starting with the polycation and  
173 alternating with the polyanion, until the desired number of layers was reached. After the  
174 last HEPES flush, wait 5 min and flush with water for 3 min, with BGE for 10 min and wait for  
175 another 5 min before starting analyses. All flushes were performed at 930 mbar. Using  
176 different HEPES vials after the polycation and the polyanion rinses helps to reduce cross-  
177 contamination between polyelectrolytes. The vials should be filled to a level such that the  
178 electrodes do not come into contact with the polyelectrolyte solutions. For the zwitterionic  
179 coatings, the final polycation rinse was replaced with a polyzwitterion solution rinse for the  
180 same amount of time. SMILs are designated by (A/B)<sub>i</sub>, where A refers to the polycation, B  
181 the polyanion, and *i* the number of bilayers.

182

## 183 **2.5. Capillary electrophoresis**

184 Analyses were performed on Agilent 7100 CE (Waldbronn, Germany). Fused silica  
185 capillaries of 50 µm in inner diameter, 357 µm in outer diameter and cut to 40 cm total  
186 length (31.5 cm to the detector) or 60 cm total length (51.5 cm to the detector) were from  
187 Composite Metal Servies (Photon Lines, France). Applied voltages were between -25 kV and  
188 -10 kV for the 40 cm capillaries, and between -30 kV and -10 kV for the 60 cm ones. A 5 min  
189 flush with BGE was done between each run. 0.001% v/v DMF in acetic acid 2 M was injected  
190 before the proteins to serve as the EOF marker. Then, the protein mix with each protein at  
191 0.2 gL<sup>-1</sup> in BGE was injected. The injection parameters were adjusted depending on the  
192 capillary length (30 mbar, 4 s for the 40 cm capillaries, and 30 mbar, 6 s for the 60 cm ones).  
193 The cassette and tray temperatures were set at 25 °C and the detection wavelength was 214  
194 nm. Plate heights *H* were calculated from the capillary length to the detector *l*, the  
195 migration times *t<sub>m</sub>*, and the widths at half height *δ* measured with the CEval software  
196 available at [<https://echmet.natur.cuni.cz/>] [36]:

197

$$H = \frac{l}{5.54 \left( \frac{t_m}{\delta} \right)^2} \quad (1)$$

198

### 199 **3. Results and discussion**

#### 200 **3.1. Influence of polyelectrolyte molar mass**

##### 201 **3.1.1. PDADMAC molar mass**

202 Polyelectrolyte molar mass has not been as extensively studied as other  
203 experimental conditions, yet it is an important aspect to consider. In the current study, a  
204 mix of five model proteins was separated on (PDADMAC/PSS)<sub>2.5</sub> coated capillaries using  
205 either PDADMAC MW  $4.5 \times 10^5$  g/mol or MW  $1 \times 10^5$  g/mol, both with PSS MW  $7 \times 10^4$   
206 g/mol. Separations were done at five electric field strengths and the resulting plate heights  
207  $H$  were plotted against solute migration velocity  $u$  following a previously described protocol  
208 [12,35,37]. Briefly, this method allows to evaluate and to rank the coating performances in  
209 terms of separation efficiency. As in chromatography, the flatter the  $H$  vs  $u$  curve, the better  
210 the separation efficiency and performances. The following equation was used to model the  
211 experimental points:

$$212 \quad H = A + \frac{B}{u} + pu \quad (2)$$

213 where  $A$  is a constant term which includes extra-column effects such as injection, detection,  
214 and capillary coiling;  $\frac{B}{u}$  is due to axial diffusion and can be written as  $\frac{2D}{u}$ , where  $D$  is the  
215 analyte diffusion coefficient determined by Taylor dispersion analysis [35]; and  $p$  is the slope  
216 in the ascending linear part of the curve.  $p$  is controlled both by possible analyte adsorption  
217 on the coating surface and by electroosmotic fluctuations along the capillary due to charge  
218 inhomogeneity [38].

219 As seen in Fig. 2 for the analysis of a mixture of 5 proteins (CA, TI, Myo, RNase and  
220 Lyz) dissolved in the BGE, the coatings made from high molar mass PDADMAC (MW  $4.5 \times$   
221  $10^5$  g/mol; average  $p(10^{-3}s) = 1.92 \pm 0.28$ ) led to much flatter  $H$  vs  $u$  curves than those made  
222 from lower molar mass PDADMAC (MW  $1 \times 10^5$  g/mol; average  $p(10^{-3}s) = 15.75 \pm 2.23$ ),  
223 indicating lower dependence on electric field strength and overall better separation  
224 efficiency. Plate heights below  $8 \mu\text{m}$  correspond to at least  $125 \times 10^3$  plates/m. One possible  
225 explanation is that the polycation molar mass can affect the structure of the SMIL coating

226 and its homogeneity in charge. This behavior was observed for all the proteins, which seems  
227 to exclude the possible contribution of solute adsorption to the slope. Recently, we  
228 demonstrated that the presence of surface defects and / or charge inhomogeneities  
229 disrupts locally the EOF which generates hydrodynamic perturbations, and consequently  
230 leads to higher  $H$  vs  $u$  slopes [38].

231 These results are consistent with data from the literature which found that coating  
232 capillaries with high molar mass PDADMAC (MW  $4.28 \times 10^5$  g/mol) and high ionic strength ( $I$   
233 = 0.5 M) led to optimal surface coverage, as indicated by the more stable and pH-  
234 independent EOF compared to MW  $3 \times 10^4$  g/mol PDADMAC at  $I = 0.005$  M [39]. The EOF  
235 magnitude was much lower when MW  $1 \times 10^4$  g/mol PDADMAC was used [40]. Similarly, Pei  
236 and Lucy studied coatings prepared from 5 different molar masses of PDADMAC, finding  
237 that only high molar mass PDADMAC (MW  $2-3.5 \times 10^5$  g/mol and MW  $4-5 \times 10^5$  g/mol)  
238 maintained a stable EOF through buffer rinse cycles (pH 9.5), while the lower molar masses  
239 (MW  $1-2 \times 10^5$  g/mol,  $75 \times 10^3$  g/mol and  $85 \times 10^2$  g/mol) quickly desorbed from the  
240 capillary wall [41].

### 241 **3.1.2. PSS molar mass**

242 Much less attention has been paid to the molar mass of the polyanions used in SMILs  
243 compared to polycations. To complete the study, five different molar masses of PSS were  
244 used in (PDADMAC/PSS)<sub>2,5</sub> SMILs with MW  $4.5 \times 10^5$  g/mol PDADMAC. Fig. 3 reveals that  
245 the  $H$  vs  $u$  plots for each PSS molar mass are quite similar, with variations in slope below  
246 30% for CA. Similar results were found for the other proteins (see Table S11). Therefore, it is  
247 apparent that polyanion molar mass has much less impact on separation efficiency  
248 compared to polycation molar mass. This may be because the polyanion is only used in  
249 intermediate layers and not as the first layer, which may impact film stability, or as the last  
250 layer, which is in contact with the proteins. More investigations about polycation and  
251 polyanion nature are discussed in Section 3.3.

252

## 253 **3.2. Impact of number of layers of PDADMAC/PSS coatings**

### 254 **3.2.1. Comparing SMIL and monolayer coatings**

255 To highlight the advantage of SMIL coatings over single-layer coatings, a durability  
256 study was carried out, whereby five successive series of separations (125 runs) were  
257 performed on the same capillary without regeneration. Both separation efficiency and intra-  
258 capillary reproducibility were higher for the PDADMAC/PSS SMIL coating compared to the  
259 monolayer PDADMAC one (see Fig. 4 and Table SI2). This is also the case for PB/PSS SMILs  
260 compared to PB (see Fig. 6B). Thus, it appears that SMIL coatings are most robust to  
261 successive rinses compared to monolayer ones, which lead to more variations in plate  
262 heights across several series of measures. This is coherent with other work which showed  
263 that multilayer coatings exhibit much higher separation efficiency [2,11], repeatability  
264 [17,41,42], and stability to alkaline rinses [13,16] than monolayers.

### 265 **3.2.2. Impact of number of layers and nature of the first layer of SMILs**

266 Some papers dealing with the structure of polyelectrolyte multilayers report that  
267 using a high molar mass polyelectrolyte with high charge density such as PEI as the first  
268 layer of a SMIL coating is advantageous as it serves to anchor the coating [43]. Hence, PEI  
269 was tested as the first layer in 5-, 11-, and 21-layer PDADMAC/PSS coatings.  $H$  vs  $u$  curves  
270 were determined for each system, showing that the (PDADMAC/PSS)<sub>2.5</sub> coating led to the  
271 highest separation efficiency (see Fig. 5). Using PEI as the first layer, as well as increasing the  
272 number of layers to 11, doubled the average slopes. (PDADMAC/PSS)<sub>10.5</sub> was not able to  
273 form a stable film (the slopes were at least 20 times higher than the for the other coatings,  
274 see Table SI3). For 11- and 21-layer coatings, though, the use of PEI as the first layer did  
275 improve separation efficiency as well as repeatability (see Fig. SI1), perhaps because the  
276 anchoring effect prevailed over polyelectrolyte nature. Indeed, when used as monolayer  
277 coatings, PDADMAC led to more stable EOF compared to PEI [16], and PEI was found to be  
278 the worst out of seven polycations used in combination with a range of polyanions in terms  
279 of separation efficiency [9]. Bekri et al. achieved stable coatings with 21 layers using  
280 different polyelectrolytes, but also determined the 5-layer coating to be the best in terms of  
281 separation efficiency and migration time repeatability [9].

282

### 283 **3.3. Studying different polyelectrolyte couples**

284 Next, SMILs made from different combinations of polycations and polyanions were  
285 tested. Fig. 6A compares the  $H$  vs  $u$  plots for different polyanions used in combination with  
286 PDADMAC. The nature of polyanion has a very low impact on the  $H$  vs  $u$  plots, with a  
287 relatively low 17% variation of the slopes between the couples (see Table S14), and with a  
288 slightly better performance for PSS. These results indicate that the coatings lead to similar  
289 surface homogeneity. According to our previously developed model, this corresponds to just  
290 0.9% of coating defects, assuming that the defects are of opposite charge to the rest of the  
291 coating, and to 0.6% assuming that one of the zones is neutral [38]. As for PSS, PAA molar  
292 mass does not seem to have a significant influence on separation efficiency. The similarity  
293 between the different polyanions used with PDADMAC is coherent with the previous section  
294 which showed that polyanion molar mass had little effect on CE performance.

295 When PB is used as the polycation, the variations in the plate height between  
296 polyelectrolyte couples increase, namely due to the higher slope of the (PB/PAA MW  $97 \times$   
297  $10^3$  g/mol)<sub>2.5</sub> system (see Fig. 6B). Other work had shown that PAA was one of the worst  
298 polyanions in terms of separation efficiency [5,9]. Only the high molar mass PAA was used  
299 since polyanion molar mass does not appear to be significant. However, the slopes remain  
300 relatively low compared to the coatings made from PDADMAC MW  $1 \times 10^5$  g/mol shown in  
301 Fig. 2B, even though the molar mass of PB is much lower (MW  $1.5 \times 10^4$  g/mol). This may be  
302 due to differences in structure between PDADMAC and PB, whose higher charge density  
303 causes it to form thinner films [44]. Likewise, the polyanions cover a range of molar masses  
304 (MW  $2 \times 10^4$  g/mol for PAA to over MW  $17 \times 10^4$  g/mol for PVS) but this does not affect  
305 separation efficiency much, perhaps because the experimental conditions have been  
306 previously optimized, leading to high CE performance regardless of the choice of  
307 polyelectrolyte couple. Some of the main differences compared to earlier studies is placing  
308 the injected samples in BGE rather than water, ensuring proper protein conservation, and  
309 limiting cross-contamination between vials [35].

310

### 311 **3.4. Testing zwitterionic polyelectrolytes as the last layer of SMILs**

312 To further investigate the impact of polyelectrolyte nature on CE performance, novel  
313 coatings based on zwitterionic polymers were implemented. Three zwitterions of the

314 sulfobetaine family were synthesized (see experimental section) and grafted onto the  $\epsilon$ -  
315 amino functions of the Lys residues of PLL in different amounts. The resulting compounds  
316 (13 in total) were used as the last layer of PDADMAC/PSS coatings and tested for the  
317 separation of model proteins using the usual model. Fig. 7A shows the EOFs obtained for the  
318 different coatings, which decrease as the % derivatization increases, while the different  
319 zwitterion types (1 to 3) behave similarly independent from the length of alkyl chain. This is  
320 consistent with the idea that the overall surface charge is reduced as more zwitterions,  
321 containing positive and negative charges, are introduced into the last polyelectrolyte layer.  
322 The EOF magnitude of the 70% derivatized zwitterion 1 was very close to the proteins'  
323 electrophoretic mobilities (24 to 32 TU, where  $1 \text{ TU} = 10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$ ), so no proteins were  
324 visible in positive or negative polarities. For the 88% and 99% derivatized zwitterions, the  
325 EOF was very slow, and was measured with a negative polarity, while the protein  
326 separations had to be performed with positive electric fields in order to be visible.

327 The zwitterionic coatings' separation abilities were evaluated in Fig. 7B, which shows  
328 results close to the  $(\text{PDADMAC/PSS})_{2.5}$  coating except for the higher percentages of  
329 derivatization. Zwitterion 3-63%, Zwitterion 1-88%, and Zwitterion 3-99% led to much higher plate heights  
330 (see Fig. S12), while Zwitterion 1-70% could not analyze any proteins. For these highly zwitterionic  
331 coatings, the polarity had to be changed and the order of proteins was inverted, and so CA,  
332 appearing last, was not able to be detected in a reasonable amount of time. This work  
333 demonstrates the ability of zwitterionic derivatization to modulate the EOF, which could be  
334 interesting for certain applications. Indeed, capillary coatings have been used in CE-mass  
335 spectrometry (MS), where slower EOF is preferred to achieve better resolution [4]. For  
336 example, a quaternized diethylaminoethyl dextran (DEAEDq)/PLC coating generating an EOF  
337 of -43.3 TU was used to separate and identify four glycoforms of ribonuclease B [5].  
338 Therefore, zwitterionation of cationic coatings offers an alternative way of reducing the EOF.

339 The zwitterionic polymers were also used in all the layers of the SMIL coating instead  
340 of PDADMAC, but this led to unstable films with poor migration time repeatability (not  
341 shown). When PDADMAC was used as the first layer,  $\text{PDADMAC}(\text{PSS/zwitterion})_2$  led to similar  
342 results to the  $(\text{PSS/zwitterion})_{2.5}$  coatings with only slightly modified EOF. This reinforces once  
343 again the higher importance of both the first and last layer compared to the intermediate  
344 layers in the SMIL coating. In another approach, several polycations were modified with

345 poly(ethylene glycol) (PEG) and added on top of (PDADMAC/PSS)<sub>2</sub> coatings, effectively  
346 reducing the EOF to as low as 2 TU with 11% derivatized MW  $5 \times 10^3$  g/mol poly(allylamine  
347 hydrochloride) (PAH) [37]. The modified polymers led to similar separation efficiency  
348 compared to the unmodified ones ( $200 \times 10^3$  plates/m and  $263 \times 10^3$  plates/m, respectively,  
349 for Lyz) [37]. The difference is even lower between (PDADMAC/PSS)<sub>2</sub>zwitter3-11%<sub>0.5</sub> and  
350 (PDADMAC/PSS)<sub>2.5</sub> for the same protein ( $316 \times 10^3$  plates/m and  $311 \times 10^3$  plates/m  
351 respectively). The best zwitterionic coating in the current work, (PDADMAC/PSS)<sub>2</sub>zwitter1-  
352 17%, led to plate heights as low as 2.7  $\mu\text{m}$ , which is equivalent to  $375 \times 10^3$  plates/m. Hence,  
353 using polymers with up to 20% of zwitterions as the last layer of (PDADMAC/PSS)<sub>2.5</sub> coatings  
354 provides separations as efficient as the typical SMILs while reducing the EOF by up to 3 TU.

355

## 356 **Conclusions**

357 In this paper, SMIL coating characteristics were examined in order to determine the  
358 optimal conditions for protein separation by CE. A large amount of data, 975 runs in total  
359 performed on 31 different capillaries, enabled *H vs u* curves to be plotted, leading to  
360 quantitative comparisons between coatings. The widely used PDADMAC/PSS combination  
361 was assessed, varying polyelectrolyte molar mass and the number of deposited layers.  
362 PDADMAC molar mass was shown to greatly impact separation efficiency, while that of PSS  
363 had little influence. The best number of layers in the coating was 5, above which separation  
364 efficiency decreased, and under which coating durability was affected. The use of PEI as the  
365 first layer improved coating stability when the coating was more than 11 layers thick but did  
366 not improve the separation for a 5-layer coating. The effect of polyelectrolyte nature was  
367 also investigated, with different combinations of PB or PDADMAC with the polyanions PAA,  
368 PVS, and PSS leading to similar separation efficiencies, with the exception of (PB/PAA)<sub>2.5</sub>  
369 which was notably worse. Overall, (PDADMAC/PSS)<sub>2.5</sub> appears to be the best choice in terms  
370 of separation efficiency and preparation time, reaching  $326 \times 10^3$  plates/m. In addition,  
371 novel zwitterionic polymers were implemented as the last layer of PDADMAC/PSS SMILs,  
372 leading to similar separation efficiency when the percentage of derivatization was not too  
373 high. Most interestingly, they were able to modulate the EOF while preserving good CE  
374 performance, which could be promising for improving resolutions in CE-MS applications.

375

## 376 **Declaration of competing interest**

377 The authors declare no conflict of interest.

378

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381 from the University of Aalen, Germany (ANR-DFG SMIL E, ANR-20-C E 92-0021-01).

382

## 383 **Supporting Information**

384 Please find supplementary material provided with this file.

385

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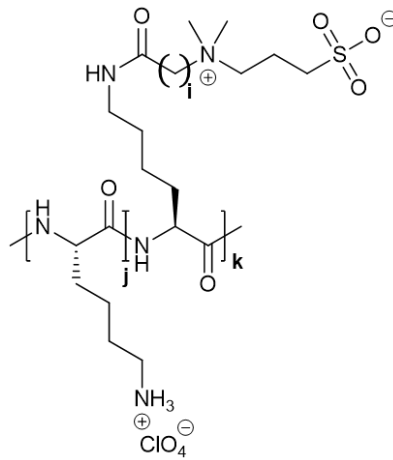
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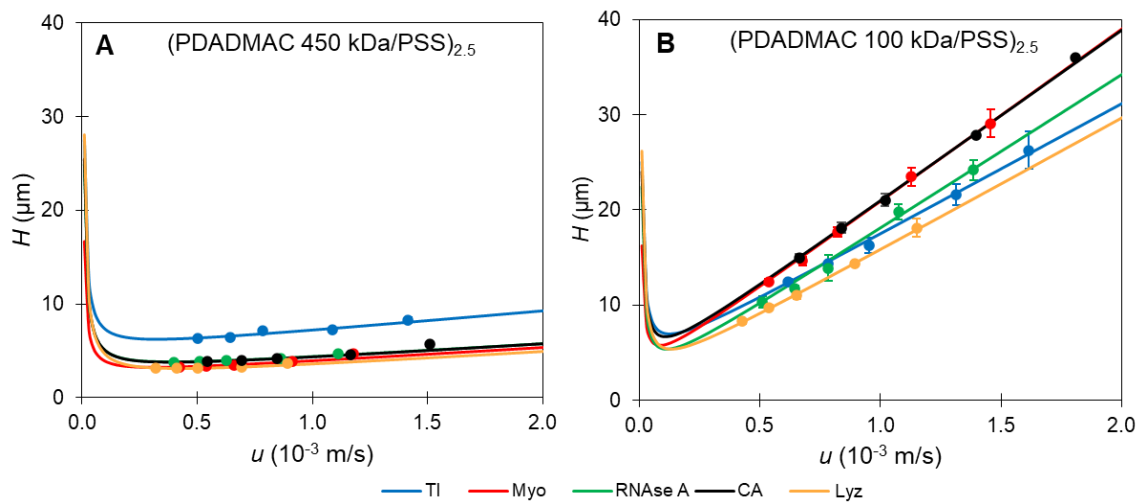
### FIGURES



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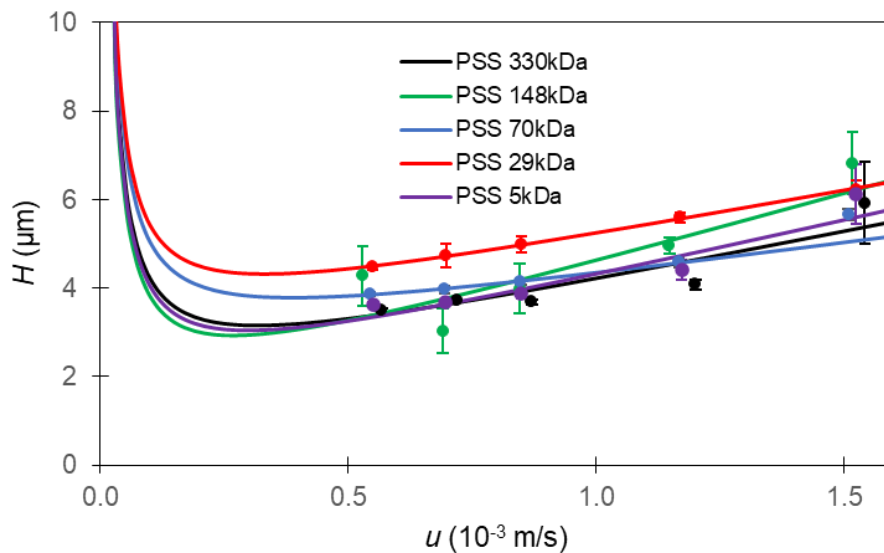
532 **Figure 1.** Structure of PLL derivatized with zwitterionic moieties.  $i = 1$  to 3, designates the type of  
 533 zwitterion 1 to 3.  $j = 400 - k$  and  $k$  ranging from 32 - 396 indicate the composition of the randomly  
 534 derivatized PLL. The counterion chloride was exchanged against perchlorate.

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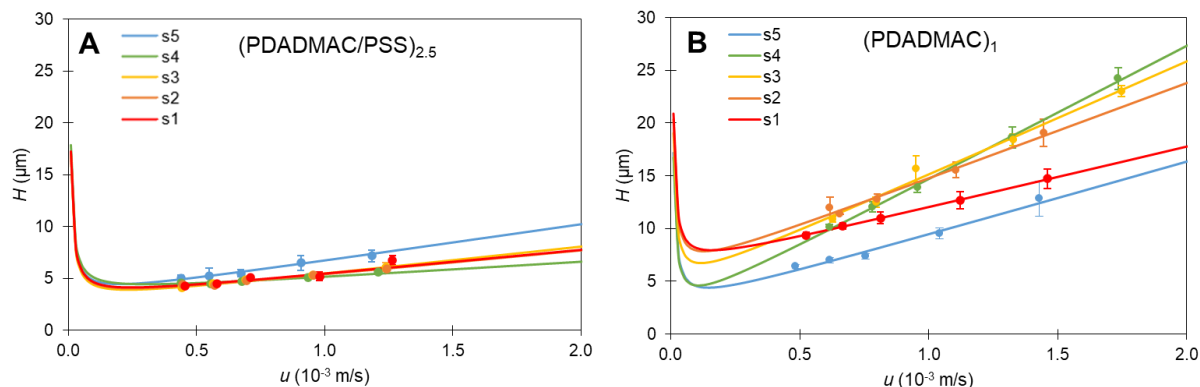
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537 **Figure 2.** Influence of PDADMAC MW  $4.5 \times 10^5$  g/mol (A) and MW  $1 \times 10^5$  g/mol (B) on the  $H$  vs  $u$   
538 representations for the separation of 5 model proteins on a 5-layer PDADMAC/PSS SMIL coating,  
539 using PSS MW  $7 \times 10^4$  g/mol. Error bars are  $\pm$  one SD on  $n = 5$  repetitions. Experimental conditions: 5-  
540 layer SMIL coated capillary terminating with the polycation PDADMAC. Capillary: 40 cm (31.5 cm to  
541 the detector)  $\times$  50  $\mu$ m I.D. BGE: 2 M acetic acid, pH 2.2. Flush before each run: BGE 1 bar, 5 min.  
542 Hydrodynamic injection: 30 mbar, 4 s. Sample mixture: 0.2 g/L of each protein in BGE. Hydrodynamic  
543 co-injection of 0.001% v/v DMF in BGE: 30 mbar, 3 s. Temperature: 25°C. Applied voltages: -25 kV, -  
544 20 kV, -15 kV, -12.5 kV, -10 kV. For the coating procedure, see section 2.4.  $p$  and  $constant$  are  
545 calculated from average values of  $H$  and  $u$  over 5 runs for each voltage. Error is  $\pm$  one standard  
546 deviation of values determined with the jackknife resampling method. For PDADMAC MW  $4.5 \times 10^5$   
547 g/mol, CA:  $p(10^{-3}s) = 1.94 \pm 0.28$ ,  $constant (\mu m) = 2.32 \pm 0.22$ ; TI: :  $p(10^{-3}s) = 2.35 \pm 0.13$ ,  $constant$   
548  $(\mu m) = 4.69 \pm 0.12$ ; Myo: :  $p(10^{-3}s) = 1.97 \pm 0.27$ ,  $constant (\mu m) = 2.02 \pm 0.16$ ; RNase A:  $p(10^{-3}s) =$   
549  $1.62 \pm 0.07$ ,  $constant (\mu m) = 2.58 \pm 0.04$ ; Lyz:  $p(10^{-3}s) = 1.72 \pm 0.13$ ,  $constant (\mu m) = 1.74 \pm 0.06$ . For  
550 PDADMAC MW  $1 \times 10^5$  g/mol, CA:  $p(10^{-3}s) = 17.45 \pm 0.14$ ,  $constant (\mu m) = 3.12 \pm 0.13$ ; TI:  $p(10^{-3}s) =$   
551  $13.32 \pm 0.16$ ,  $constant (\mu m) = 3.63 \pm 0.20$ ; Myo:  $p(10^{-3}s) = 17.89 \pm 0.64$ ,  $constant (\mu m) = 2.63 \pm 0.47$ ;  
552 RNase A:  $p(10^{-3}s) = 16.71 \pm 0.56$ ,  $constant (\mu m) = 0.95 \pm 0.46$ ; Lyz:  $p(10^{-3}s) = 13.37 \pm 0.93$ ,  $constant$   
553  $(\mu m) = 2.10 \pm 0.81$ .



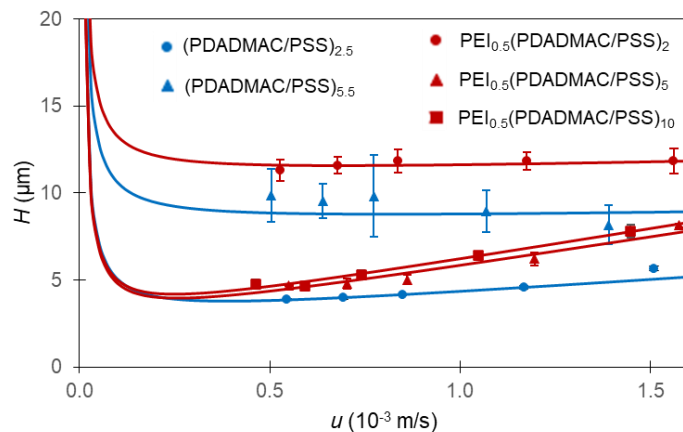
554  
555 **Figure 3.** Influence of PSS molar mass on the  $H$  vs  $u$  representations for CA on a 5-layer  
556 PDADMAC/PSS SMIL coating, using PDADMAC MW  $4.5 \times 10^5$  g/mol. Experimental conditions as in  
557 Fig. 2.  $p$  and  $constant$  are calculated from average values of  $H$  and  $u$  over 5 runs for each voltage.  
558 Error is  $\pm$  one standard deviation of values determined with the jackknife resampling method. For  
559 PSS MW  $5 \times 10^3$  g/mol:  $p (10^{-3} s) = 2.40 \pm 0.61$ ,  $constant (\mu m) = 1.79 \pm 0.55$ ; PSS MW  $29 \times 10^3$ :  $p (10^{-3}$   
560  $s) = 2.09 \pm 0.05$ ,  $constant (\mu m) = 2.92 \pm 0.04$ ; PSS MW  $70 \times 10^3$  g/mol:  $p (10^{-3} s) = 1.94 \pm 0.28$ ,  
561  $constant (\mu m) = 2.32 \pm 0.22$ ; PSS MW  $148 \times 10^3$  g/mol:  $p (10^{-3} s) = 3.25 \pm 0.79$ ,  $constant (\mu m) = 1.21 \pm$   
562  $0.78$ ; PSS MW  $330 \times 10^3$  g/mol:  $p (10^{-3} s) = 3.81 \pm 1.47$ ,  $constant (\mu m) = 0.53 \pm 1.07$ .

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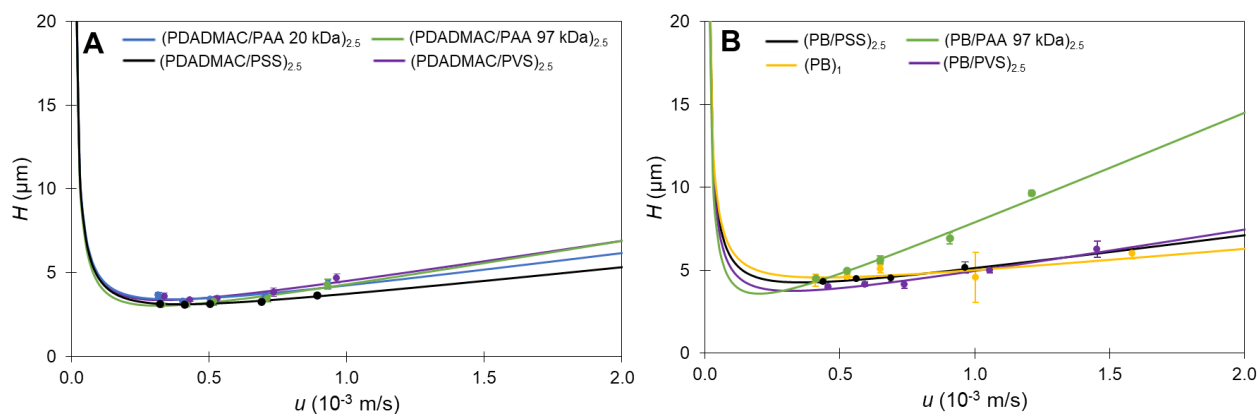
565 **Figure 4.** Superposition of five successive series of separations of five model proteins on a 5-layer  
 566 PDADMAC/PSS SMIL coating (A) and a single layer PDADMAC coating (B). PDADMAC MW  $4.5 \times 10^5$   
 567 g/mol and PSS MW  $7 \times 10^4$  g/mol were used.  $H$  vs  $u$  curves shown for Myo. Error bars are  $\pm$  one SD  
 568 on  $n = 5$  repetitions. Experimental conditions as in Fig. 2.  $p$  and  $constant$  are calculated from average  
 569 values of  $H$  and  $u$  over 5 runs for each voltage. Error is  $\pm$  one standard deviation of values  
 570 determined with the jackknife resampling method. For (PDADMAC/PSS)<sub>1</sub>, CA:  $p$  ( $10^{-3} \text{ s}$ ) =  $2.38 \pm 0.56$ ,  
 571  $constant$  ( $\mu\text{m}$ ) =  $4.02 \pm 0.42$ ; TI:  $p$  ( $10^{-3} \text{ s}$ ) =  $2.85 \pm 0.28$ ,  $constant$  ( $\mu\text{m}$ ) =  $6.35 \pm 0.19$ ; Myo:  $p$  ( $10^{-3} \text{ s}$ ) =  
 572  $1.55 \pm 0.38$ ,  $constant$  ( $\mu\text{m}$ ) =  $3.95 \pm 0.21$ ; RNase A:  $p$  ( $10^{-3} \text{ s}$ ) =  $0.79 \pm 0.14$ ,  $constant$  ( $\mu\text{m}$ ) =  $4.67 \pm$   
 573  $0.14$ ; Lyz:  $p$  ( $10^{-3} \text{ s}$ ) =  $1.09 \pm 0.18$ ,  $constant$  ( $\mu\text{m}$ ) =  $3.13 \pm 0.12$ . For (PDADMAC/PSS)<sub>2.5</sub>, CA:  $p$  ( $10^{-3} \text{ s}$ ) =  
 574  $1.87 \pm 0.32$ ,  $constant$  ( $\mu\text{m}$ ) =  $2.53 \pm 0.26$ ; TI:  $p$  ( $10^{-3} \text{ s}$ ) =  $2.35 \pm 0.13$ ,  $constant$  ( $\mu\text{m}$ ) =  $4.69 \pm 0.12$ ;  
 575 Myo:  $p$  ( $10^{-3} \text{ s}$ ) =  $1.97 \pm 0.27$ ,  $constant$  ( $\mu\text{m}$ ) =  $2.02 \pm 0.16$ ; RNase A:  $p$  ( $10^{-3} \text{ s}$ ) =  $1.33 \pm 0.67$ ,  $constant$   
 576 ( $\mu\text{m}$ ) =  $2.83 \pm 0.57$ ; Lyz:  $p$  ( $10^{-3} \text{ s}$ ) =  $1.72 \pm 0.13$ ,  $constant$  ( $\mu\text{m}$ ) =  $1.74 \pm 0.06$ .



577

578 **Figure 5.** Influence of the number of layers and the use of PEI as the first layer on the  $H$  vs  $u$   
 579 representations for the separation of 5 model proteins on PDADMAC/PSS SMIL coatings, shown for  
 580 CA. PDADMAC MW  $4.5 \times 10^5$  g/mol and PSS MW  $7 \times 10^4$  g/mol were used. Experimental conditions as  
 581 in Fig. 2.

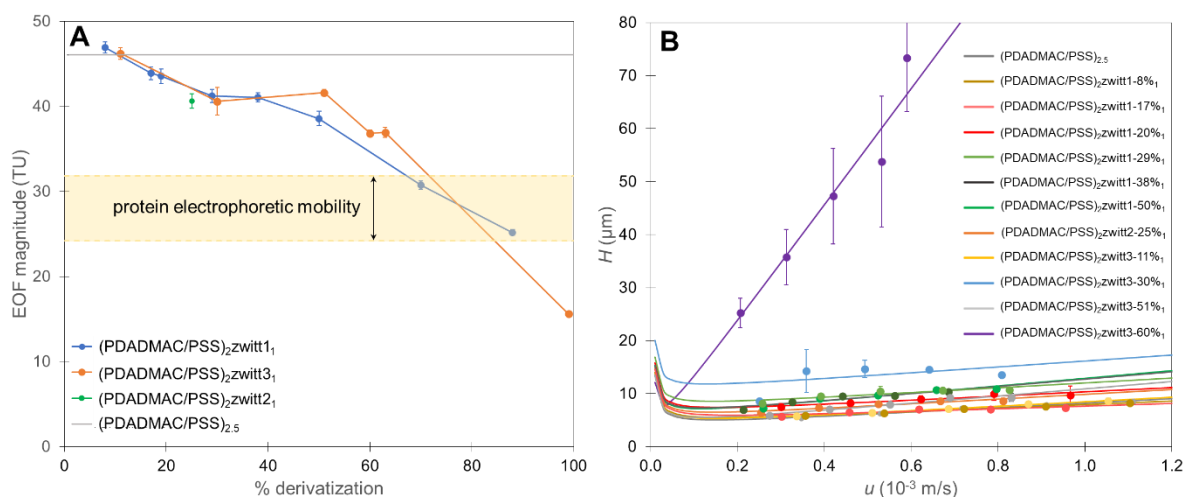
582



583

584 **Figure 6.** Influence of polyanion nature on the  $H$  vs  $u$  representations for the analysis of Lyz on  
 585 monolayer and 5-layer SMIL coatings, using PDADMAC MW  $4.5 \times 10^5$  g/mol (A) or PB (B) as the  
 586 polycation. PSS MW  $7 \times 10^4$  g/mol was used. Experimental conditions as in Fig. 2. Data for other  
 587 proteins is shown in Table S14.

588



589

590 **Figure 7.** Impact of zwitterionic last layer on EOF (A) and separation efficiency for CA (B). PDADMAC  
 591 MW  $4.5 \times 10^5$  g/mol and PSS MW  $7 \times 10^4$  g/mol were used. Each coating is made of  
 592 (PDADMAC/PSS)<sub>2</sub>(polyzwitterion)<sub>1</sub>. Experimental conditions: Capillary: 60 cm (51.5 cm to the  
 593 detector) x 50 μm I.D. BGE: 2 M acetic acid, pH 2.2. Flush before each run: BGE 1 bar, 5 min.  
 594 Hydrodynamic injection: 30 mbar, 6 s. Sample mixture: 0.2 g/L of each protein in BGE. Hydrodynamic  
 595 co-injection of 0.001% v/v DMF in BGE: 30 mbar, 3 s. Temperature: 25°C. Applied voltages: -30 kV, -  
 596 25 kV, -20 kV, -15 kV, -10 kV. For the coating procedure, see Section 2.4.

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598

## LIST OF CAPTIONS

599

600 **Figure 1.** Structure of PLL derivatized with zwitterionic moieties.  $i = 1$  to 3, designates the  
 601 type of zwitterion 1 to 3.  $j = 400 - k$  and  $k$  ranging from 32 - 396 indicate the composition of  
 602 the randomly derivatized PLL. The counterion chloride was exchanged against perchlorate.

603 **Figure 2.** Influence of PDADMAC MW  $4.5 \times 10^5$  g/mol (**A**) and MW  $1 \times 10^5$  g/mol (**B**) on the  
604 *H vs u* representations for the separation of 5 model proteins on a 5-layer PDADMAC/PSS  
605 SMIL coating, using PSS MW  $7 \times 10^4$  g/mol. Error bars are  $\pm$  one SD on  $n = 5$  repetitions.  
606 Experimental conditions: 5-layer SMIL coated capillary terminating with the polycation  
607 PDADMAC. Capillary: 40 cm (31.5 cm to the detector)  $\times$  50  $\mu$ m I.D. BGE: 2 M acetic acid, pH  
608 2.2. Flush before each run: BGE 1 bar, 5 min. Hydrodynamic injection: 30 mbar, 4 s. Sample  
609 mixture: 0.2 g/L of each protein in BGE. Hydrodynamic co-injection of 0.001% v/v DMF in  
610 BGE: 30 mbar, 3 s. Temperature: 25°C. Applied voltages: -25 kV, -20 kV, -15 kV, -12.5 kV, -10  
611 kV. For the coating procedure, see section 2.4.  $p$  and  $constant$  are calculated from average  
612 values of  $H$  and  $u$  over 5 runs for each voltage. Error is  $\pm$  one standard deviation of values  
613 determined with the jackknife resampling method. For PDADMAC MW  $4.5 \times 10^5$  g/mol, CA:  
614  $p(10^{-3}s) = 1.94 \pm 0.28$ ,  $constant (\mu m) = 2.32 \pm 0.22$ ; TI:  $p(10^{-3}s) = 2.35 \pm 0.13$ ,  $constant (\mu m)$   
615  $= 4.69 \pm 0.12$ ; Myo:  $p(10^{-3}s) = 1.97 \pm 0.27$ ,  $constant (\mu m) = 2.02 \pm 0.16$ ; RNase A:  $p(10^{-3}s) =$   
616  $1.62 \pm 0.07$ ,  $constant (\mu m) = 2.58 \pm 0.04$ ; Lyz:  $p(10^{-3}s) = 1.72 \pm 0.13$ ,  $constant (\mu m) = 1.74 \pm$   
617  $0.06$ . For PDADMAC MW  $1 \times 10^5$  g/mol, CA:  $p(10^{-3}s) = 17.45 \pm 0.14$ ,  $constant (\mu m) = 3.12 \pm$   
618  $0.13$ ; TI:  $p(10^{-3}s) = 13.32 \pm 0.16$ ,  $constant (\mu m) = 3.63 \pm 0.20$ ; Myo:  $p(10^{-3}s) = 17.89 \pm 0.64$ ,  
619  $constant (\mu m) = 2.63 \pm 0.47$ ; RNase A:  $p(10^{-3}s) = 16.71 \pm 0.56$ ,  $constant (\mu m) = 0.95 \pm 0.46$ ;  
620 Lyz:  $p(10^{-3}s) = 13.37 \pm 0.93$ ,  $constant (\mu m) = 2.10 \pm 0.81$ .

621 **Figure 3.** Influence of PSS molar mass on the *H vs u* representations for CA on a 5-layer  
622 PDADMAC/PSS SMIL coating, using PDADMAC MW  $4.5 \times 10^5$  g/mol. Experimental conditions  
623 as in Fig. 2.  $p$  and  $constant$  are calculated from average values of  $H$  and  $u$  over 5 runs for  
624 each voltage. Error is  $\pm$  one standard deviation of values determined with the jackknife  
625 resampling method. For PSS MW  $5 \times 10^3$  g/mol:  $p(10^{-3}s) = 2.40 \pm 0.61$ ,  $constant (\mu m) = 1.79$   
626  $\pm 0.55$ ; PSS MW  $29 \times 10^3$ :  $p(10^{-3}s) = 2.09 \pm 0.05$ ,  $constant (\mu m) = 2.92 \pm 0.04$ ; PSS MW  $70$   
627  $\times 10^3$  g/mol:  $p(10^{-3}s) = 1.94 \pm 0.28$ ,  $constant (\mu m) = 2.32 \pm 0.22$ ; PSS MW  $148 \times 10^3$  g/mol:  $p$   
628  $(10^{-3}s) = 3.25 \pm 0.79$ ,  $constant (\mu m) = 1.21 \pm 0.78$ ; PSS MW  $330 \times 10^3$  g/mol:  $p(10^{-3}s) = 3.81$   
629  $\pm 1.47$ ,  $constant (\mu m) = 0.53 \pm 1.07$ .

630 **Figure 4.** Superposition of five successive series of separations of five model proteins on a 5-  
631 layer PDADMAC/PSS SMIL coating (**A**) and a single layer PDADMAC coating (**B**). PDADMAC  
632 MW  $4.5 \times 10^5$  g/mol and PSS MW  $7 \times 10^4$  g/mol were used. *H vs u* curves shown for Myo.  
633 Error bars are  $\pm$  one SD on  $n = 5$  repetitions. Experimental conditions as in Fig. 2.  $p$  and  
634  $constant$  are calculated from average values of  $H$  and  $u$  over 5 runs for each voltage. Error is  
635  $\pm$  one standard deviation of values determined with the jackknife resampling method. For  
636 (PDADMAC/PSS)<sub>1</sub>, CA:  $p(10^{-3}s) = 2.38 \pm 0.56$ ,  $constant (\mu m) = 4.02 \pm 0.42$ ; TI:  $p(10^{-3}s) =$   
637  $2.85 \pm 0.28$ ,  $constant (\mu m) = 6.35 \pm 0.19$ ; Myo:  $p(10^{-3}s) = 1.55 \pm 0.38$ ,  $constant (\mu m) = 3.95 \pm$   
638  $0.21$ ; RNase A:  $p(10^{-3}s) = 0.79 \pm 0.14$ ,  $constant (\mu m) = 4.67 \pm 0.14$ ; Lyz:  $p(10^{-3}s) = 1.09 \pm$   
639  $0.18$ ,  $constant (\mu m) = 3.13 \pm 0.12$ . For (PDADMAC/PSS)<sub>2.5</sub>, CA:  $p(10^{-3}s) = 1.87 \pm 0.32$ ,  
640  $constant (\mu m) = 2.53 \pm 0.26$ ; TI:  $p(10^{-3}s) = 2.35 \pm 0.13$ ,  $constant (\mu m) = 4.69 \pm 0.12$ ; Myo:  $p$   
641  $(10^{-3}s) = 1.97 \pm 0.27$ ,  $constant (\mu m) = 2.02 \pm 0.16$ ; RNase A:  $p(10^{-3}s) = 1.33 \pm 0.67$ ,  $constant$   
642  $(\mu m) = 2.83 \pm 0.57$ ; Lyz:  $p(10^{-3}s) = 1.72 \pm 0.13$ ,  $constant (\mu m) = 1.74 \pm 0.06$ .

643 **Figure 5.** Influence of the number of layers and the use of PEI as the first layer on the *H vs u*  
644 representations for the separation of 5 model proteins on PDADMAC/PSS SMIL coatings,  
645 shown for CA. PDADMAC MW  $4.5 \times 10^5$  g/mol and PSS MW  $7 \times 10^4$  g/mol were used.  
646 Experimental conditions as in Fig. 2.



647 **Figure 6.** Influence of polyanion nature on the  $H$  vs  $u$  representations for the analysis of Lyz  
648 on monolayer and 5-layer SMIL coatings, using PDADMAC MW  $4.5 \times 10^5$  (A) or PB (B) as the  
649 polycation. PSS MW  $7 \times 10^4$  g/mol was used. Experimental conditions as in Fig. 2. Data for  
650 other proteins is shown in Table SI4.

651 **Figure 7.** Impact of zwitterionic last layer on EOF (A) and separation efficiency for CA (B).  
652 PDADMAC MW  $4.5 \times 10^5$  g/mol and PSS MW  $7 \times 10^4$  g/mol were used. Each coating is made  
653 of (PDADMAC/PSS)<sub>2</sub>(polyzwitterion)<sub>1</sub>. Experimental conditions: Capillary: 60 cm (51.5 cm to  
654 the detector) x 50  $\mu$ m I.D. BGE: 2 M acetic acid, pH 2.2. Flush before each run: BGE 1 bar, 5  
655 min. Hydrodynamic injection: 30 mbar, 6 s. Sample mixture: 0.2 g/L of each protein in BGE.  
656 Hydrodynamic co-injection of 0.001% v/v DMF in BGE: 30 mbar, 3 s. Temperature: 25°C.  
657 Applied voltages: -30 kV, -25 kV, -20 kV, -15 kV, -10 kV. For the coating procedure, see  
658 Section 2.4.