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

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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(α -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

49
50

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

66 Different coating stabilities and separation performances may be achieved
67 depending on the nature of the polyelectrolyte couple [5,8,9]. A systematic study on the
68 influence of polyelectrolyte nature has shown that both the polycation and polyanion have
69 an impact on separation efficiency and coating stability. Over 20 couples were studied for
70 the separation of model proteins in acetic acid MS compatible buffer, finding that
71 PDADMAC was the best choice of polycation and poly(L-lysine citramide) (PLC) for the
72 polyanion, with (PDADMAC/PLC)_{2.5} coatings resulting in up to 126×10^3 plates/m and
73 excellent repeatability over 100 runs (%RSD(t_m) = 0.1%) [9]. Moreover, physico-chemical
74 conditions such as construction buffer, background electrolyte (BGE) ionic strength and pH
75 have been shown to impact both electroosmotic flow (EOF) and separation efficiency
76 [9,11,14-16]. Adding salt to the construction buffer resulted in increased stability of the 5-
77 layer PDADMAC/PSS SMIL coating as well as thicker layers [11,16,17]. Thick coatings are
78 generally thought to be advantageous as they increase the distance between the solute and
79 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 $\geq 60\%$), β -lactoglobulin A from bovine milk (β -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(α -L-lysine) hydrochloride (PLL; average MW 66×10^3 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 4×10^5 – 5×10^5
128 g/mol, and low molar mass MW 1×10^5 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 5×10^3 g/mol; 29×10^3 g/mol; 7×10^4 g/mol; 148×10^3 g/mol; 330×10^3 g/mol) were
131 purchased from Acros Organics (Geel, Belgium). PB (MW 15×10^3 g/mol), PAA (MW 2×10^4
132 g/mol and 97×10^3 g/mol), PVS (MW 17×10^3 g/mol), and PEI (MW 75×10^4 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 ω -dimethylamino
138 carboxylic acids with systematically increasing chain length using propan-1,3-sultone. For
139 this, the methyl or ethyl ester protected ω -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 ¹H- und ¹³C-NMR spectroscopy as well as CE-MS analysis. ¹H- and ¹³C-
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 H₂O/MeOH 1:1 v/v at a concentration of 10 mg/mL. The
149 zwitterions were also dissolved in H₂O/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 ¹H 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⁻¹) 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⁻¹. 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)_i, 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⁻¹ 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_m*, 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)_{2.5} coated capillaries using
205 either PDADMAC MW 4.5×10^5 g/mol or MW 1×10^5 g/mol, both with PSS MW 7×10^4
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 1×10^5 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 125×10^3 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 4.28×10^5 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 3×10^4 g/mol PDADMAC at $I = 0.005$ M [39]. The EOF
235 magnitude was much lower when MW 1×10^4 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, 75×10^3 g/mol and 85×10^2 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)_{2,5} SMILs with MW 4.5×10^5 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)_{2.5} 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)_{10.5} 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)_{2.5} 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 1×10^5 g/mol shown in
301 Fig. 2B, even though the molar mass of PB is much lower (MW 1.5×10^4 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 2×10^4 g/mol for PAA to over MW 17×10^4 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 ϵ -
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 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 (PDADMAC/PSS)_{2.5} coating except for the higher percentages of
329 derivatization. Zwitt3-63%, Zwitt1-88%, and Zwitt3-99% led to much higher plate heights
330 (see Fig. SI2), while Zwitt1-70% 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, zwitteration 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, PDADMAC(PSS/zwitt)₂ led to similar
342 results to the (PSS/zwitt)_{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)₂ coatings, effectively
346 reducing the EOF to as low as 2 TU with 11% derivatized MW 5×10^3 g/mol poly(allylamine
347 hydrochloride) (PAH) [37]. The modified polymers led to similar separation efficiency
348 compared to the unmodified ones (200×10^3 plates/m and 263×10^3 plates/m, respectively,
349 for Lyz) [37]. The difference is even lower between (PDADMAC/PSS)₂zwitter3-11%_{0.5} and
350 (PDADMAC/PSS)_{2.5} for the same protein (316×10^3 plates/m and 311×10^3 plates/m
351 respectively). The best zwitterionic coating in the current work, (PDADMAC/PSS)₂zwitter1-
352 17%, led to plate heights as low as 2.7 μm , which is equivalent to 375×10^3 plates/m. Hence,
353 using polymers with up to 20% of zwitterions as the last layer of (PDADMAC/PSS)_{2.5} 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)_{2.5}
369 which was notably worse. Overall, (PDADMAC/PSS)_{2.5} appears to be the best choice in terms
370 of separation efficiency and preparation time, reaching 326×10^3 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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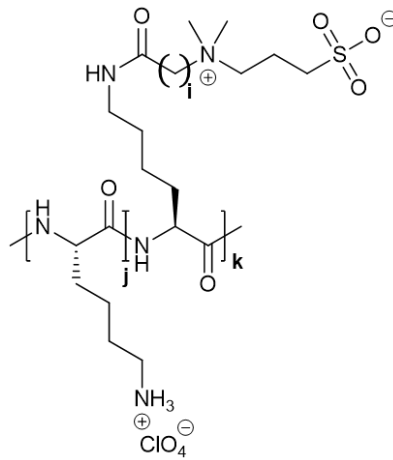
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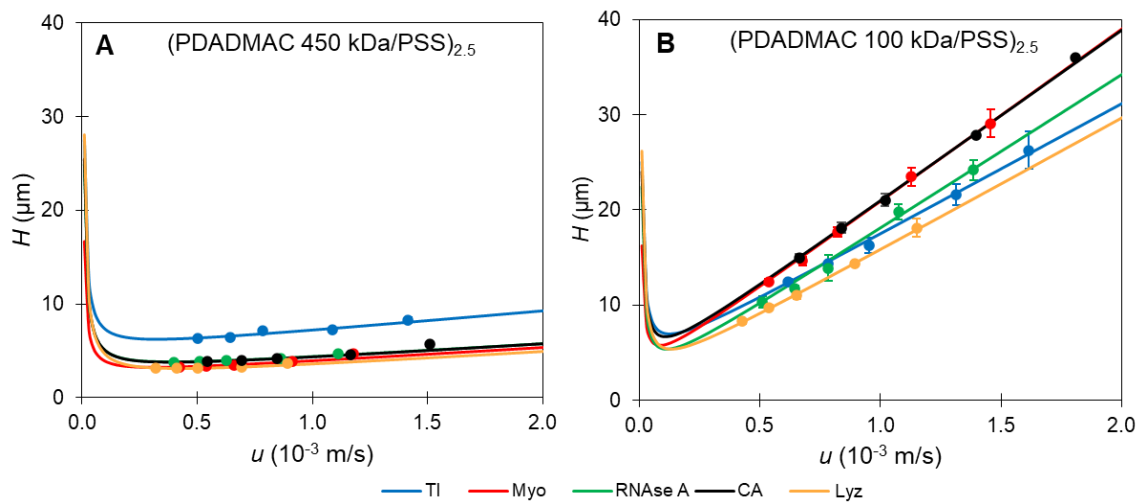
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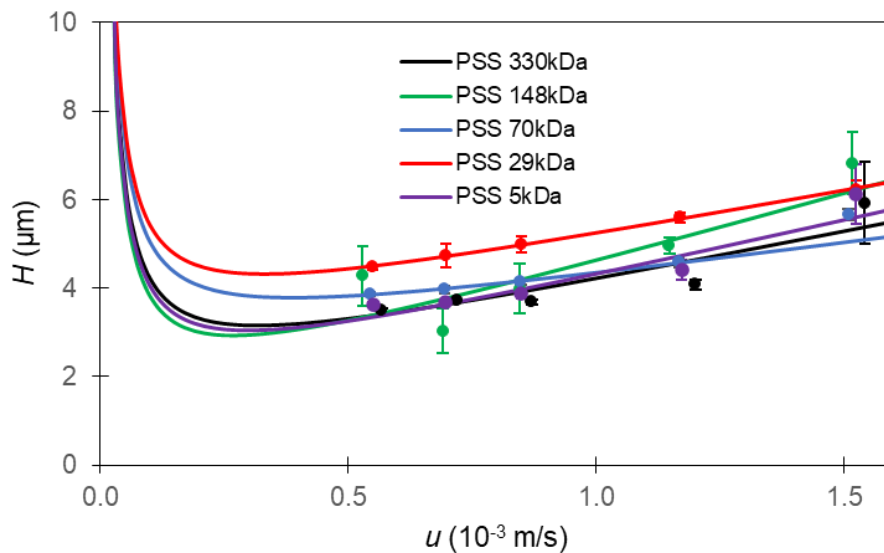
532 **Figure 1.** Structure of PLL derivatized with zwitterionic moieties. $i = 1$ to 3, designates the type of
 533 zwitterion 1 to 3. $j = 400 - k$ and k ranging from 32 - 396 indicate the composition of the randomly
 534 derivatized PLL. The counterion chloride was exchanged against perchlorate.

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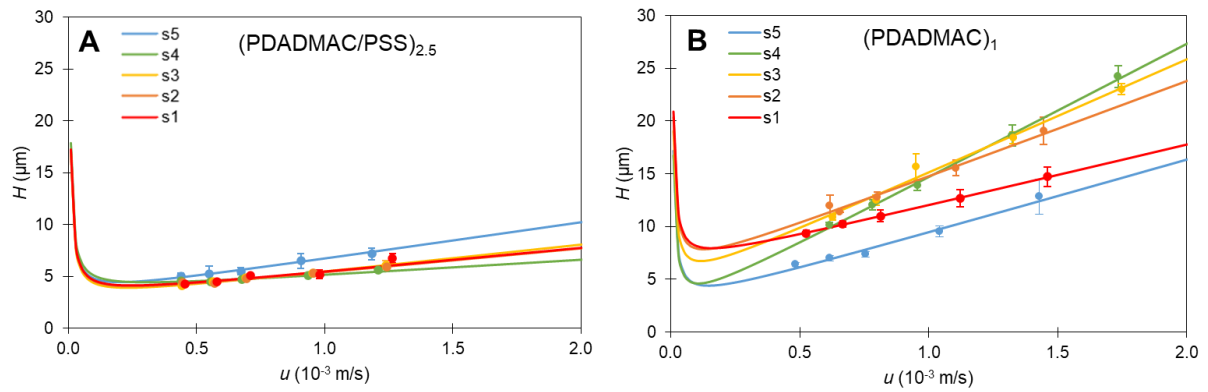
536

537 **Figure 2.** Influence of PDADMAC MW 4.5×10^5 g/mol (A) and MW 1×10^5 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 7×10^4 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 μ 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 4.5×10^5
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 ± 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 1×10^5 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 ± 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 $(\mu m) = 2.10 \pm 0.81$.



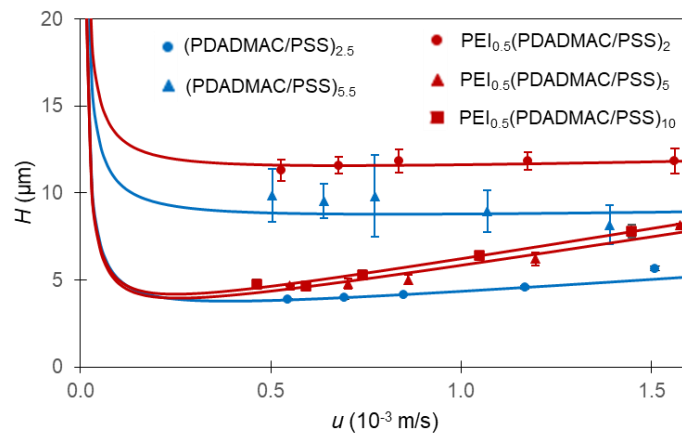
554
555 **Figure 3.** Influence of PSS molar mass on the H vs u representations for CA on a 5-layer
556 PDADMAC/PSS SMIL coating, using PDADMAC MW 4.5×10^5 g/mol. Experimental conditions as in
557 Fig. 2. p and $constant$ are calculated from average values of H and u over 5 runs for each voltage.
558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

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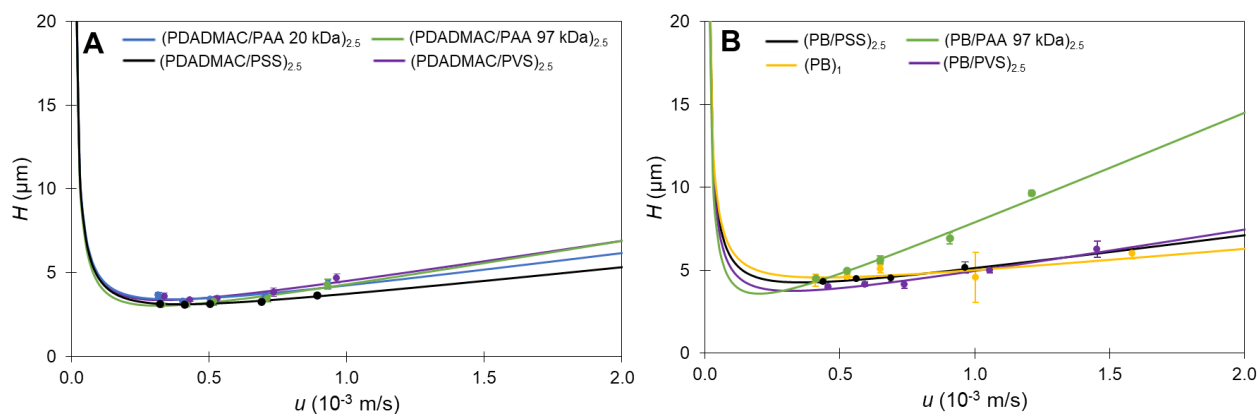
565 **Figure 4.** Superposition of five successive series of separations of five model proteins on a 5-layer
 566 PDADMAC/PSS SMIL coating (A) and a single layer PDADMAC coating (B). PDADMAC MW 4.5×10^5
 567 g/mol and PSS MW 7×10^4 g/mol were used. H vs u curves shown for Myo. Error bars are \pm one 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)₁, CA: p (10^{-3} s) = 2.38 ± 0.56 ,
 571 $constant$ (μm) = 4.02 ± 0.42 ; TI: p (10^{-3} s) = 2.85 ± 0.28 , $constant$ (μm) = 6.35 ± 0.19 ; Myo: p (10^{-3} s) =
 572 1.55 ± 0.38 , $constant$ (μm) = 3.95 ± 0.21 ; RNase A: p (10^{-3} s) = 0.79 ± 0.14 , $constant$ (μm) = $4.67 \pm$
 573 0.14 ; Lyz: p (10^{-3} s) = 1.09 ± 0.18 , $constant$ (μm) = 3.13 ± 0.12 . For (PDADMAC/PSS)_{2.5}, CA: p (10^{-3} s) =
 574 1.87 ± 0.32 , $constant$ (μm) = 2.53 ± 0.26 ; TI: p (10^{-3} s) = 2.35 ± 0.13 , $constant$ (μm) = 4.69 ± 0.12 ;
 575 Myo: p (10^{-3} s) = 1.97 ± 0.27 , $constant$ (μm) = 2.02 ± 0.16 ; RNase A: p (10^{-3} s) = 1.33 ± 0.67 , $constant$
 576 (μm) = 2.83 ± 0.57 ; Lyz: p (10^{-3} s) = 1.72 ± 0.13 , $constant$ (μm) = 1.74 ± 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 4.5×10^5 g/mol and PSS MW 7×10^4 g/mol were used. Experimental conditions as
 581 in Fig. 2.

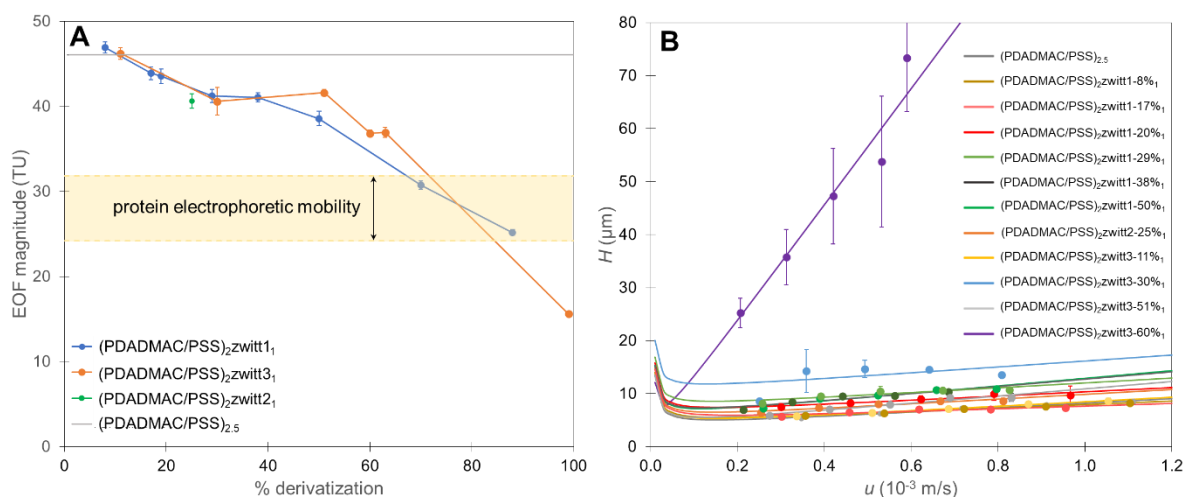
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583

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 4.5×10^5 g/mol (A) or PB (B) as the
 586 polycation. PSS MW 7×10^4 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 4.5×10^5 g/mol and PSS MW 7×10^4 g/mol were used. Each coating is made of
 592 (PDADMAC/PSS)₂(polyzwitterion)₁. 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.

597

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 4.5×10^5 g/mol (**A**) and MW 1×10^5 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 7×10^4 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) x $50 \mu\text{m}$ 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
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614 $p(10^{-3}\text{s}) = 1.94 \pm 0.28$, $constant (\mu\text{m}) = 2.32 \pm 0.22$; TI: $p(10^{-3}\text{s}) = 2.35 \pm 0.13$, $constant (\mu\text{m})$
615 $= 4.69 \pm 0.12$; Myo: $p(10^{-3}\text{s}) = 1.97 \pm 0.27$, $constant (\mu\text{m}) = 2.02 \pm 0.16$; RNase A: $p(10^{-3}\text{s}) =$
616 1.62 ± 0.07 , $constant (\mu\text{m}) = 2.58 \pm 0.04$; Lyz: $p(10^{-3}\text{s}) = 1.72 \pm 0.13$, $constant (\mu\text{m}) = 1.74 \pm$
617 0.06 . For PDADMAC MW 1×10^5 g/mol, CA: $p(10^{-3}\text{s}) = 17.45 \pm 0.14$, $constant (\mu\text{m}) = 3.12 \pm$
618 0.13 ; TI: $p(10^{-3}\text{s}) = 13.32 \pm 0.16$, $constant (\mu\text{m}) = 3.63 \pm 0.20$; Myo: $p(10^{-3}\text{s}) = 17.89 \pm 0.64$,
619 $constant (\mu\text{m}) = 2.63 \pm 0.47$; RNase A: $p(10^{-3}\text{s}) = 16.71 \pm 0.56$, $constant (\mu\text{m}) = 0.95 \pm 0.46$;
620 Lyz: $p(10^{-3}\text{s}) = 13.37 \pm 0.93$, $constant (\mu\text{m}) = 2.10 \pm 0.81$.

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 4.5×10^5 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 5×10^3 g/mol: $p(10^{-3}\text{s}) = 2.40 \pm 0.61$, $constant (\mu\text{m}) = 1.79$
626 ± 0.55 ; PSS MW 29×10^3 : $p(10^{-3}\text{s}) = 2.09 \pm 0.05$, $constant (\mu\text{m}) = 2.92 \pm 0.04$; PSS MW 70
627 $\times 10^3$ g/mol: $p(10^{-3}\text{s}) = 1.94 \pm 0.28$, $constant (\mu\text{m}) = 2.32 \pm 0.22$; PSS MW 148×10^3 g/mol: p
628 $(10^{-3}\text{s}) = 3.25 \pm 0.79$, $constant (\mu\text{m}) = 1.21 \pm 0.78$; PSS MW 330×10^3 g/mol: $p(10^{-3}\text{s}) = 3.81$
629 ± 1.47 , $constant (\mu\text{m}) = 0.53 \pm 1.07$.

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 4.5×10^5 g/mol and PSS MW 7×10^4 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)₁, CA: $p(10^{-3}\text{s}) = 2.38 \pm 0.56$, $constant (\mu\text{m}) = 4.02 \pm 0.42$; TI: $p(10^{-3}\text{s}) =$
637 2.85 ± 0.28 , $constant (\mu\text{m}) = 6.35 \pm 0.19$; Myo: $p(10^{-3}\text{s}) = 1.55 \pm 0.38$, $constant (\mu\text{m}) = 3.95 \pm$
638 0.21 ; RNase A: $p(10^{-3}\text{s}) = 0.79 \pm 0.14$, $constant (\mu\text{m}) = 4.67 \pm 0.14$; Lyz: $p(10^{-3}\text{s}) = 1.09 \pm$
639 0.18 , $constant (\mu\text{m}) = 3.13 \pm 0.12$. For (PDADMAC/PSS)_{2.5}, CA: $p(10^{-3}\text{s}) = 1.87 \pm 0.32$,
640 $constant (\mu\text{m}) = 2.53 \pm 0.26$; TI: $p(10^{-3}\text{s}) = 2.35 \pm 0.13$, $constant (\mu\text{m}) = 4.69 \pm 0.12$; Myo: p
641 $(10^{-3}\text{s}) = 1.97 \pm 0.27$, $constant (\mu\text{m}) = 2.02 \pm 0.16$; RNase A: $p(10^{-3}\text{s}) = 1.33 \pm 0.67$, $constant$
642 $(\mu\text{m}) = 2.83 \pm 0.57$; Lyz: $p(10^{-3}\text{s}) = 1.72 \pm 0.13$, $constant (\mu\text{m}) = 1.74 \pm 0.06$.

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 4.5×10^5 g/mol and PSS MW 7×10^4 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 4.5×10^5 (A) or PB (B) as the
649 polycation. PSS MW 7×10^4 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 4.5×10^5 g/mol and PSS MW 7×10^4 g/mol were used. Each coating is made
653 of (PDADMAC/PSS)₂(polyzwitterion)₁. Experimental conditions: Capillary: 60 cm (51.5 cm to
654 the detector) x 50 μ 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.