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1 Modulation of the electroosmotic mobility using polyelectrolyte
2 multilayer coatings for protein analysis by capillary electrophoresis

3
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11

12 **HIGHLIGHTS**

13 ● Electroosmotic mobility varies from -43 to -63 Tiselius units depending on polycation

14 ● Electroosmotic flow magnitude is in the order of $PB > \epsilon\text{-PLL} > \text{DEAEDq}$

15 ● Coatings with poly(L-lysine citramide) gave the best separation efficiencies

16 ● Average RSD on migration times (resp. separation efficiency) was 1.7% (resp. 9.6%)

17 ● CE-MS separation of positional isomers of the glycoprotein ribonuclease B

18

19 **ABSTRACT**

20

21 Successive multiple ionic-polymer layers (SMIL) coatings have been often used in capillary
22 electrophoresis due to their simplicity to implement and regenerate. However, the
23 performances of the separation are strongly dependent on the nature of the polyelectrolyte
24 partners used to build the SMIL coating. In this work, we investigate new couples of
25 polyelectrolytes that were tested before: namely, polybrene (PB), quaternized
26 diethylaminoethyl dextran (DEAEDq) and ϵ -poly(lysine) (ϵ -PLL), as polycations, in
27 combination with poly(acrylic acid), dextran sulfate, poly(styrenesulfonate),
28 poly(methacrylic acid) and poly(L-lysine citramide), as polyanions. Systematic study of intra-
29 and inter-capillaries repeatabilities / reproducibilities was performed based on the
30 determination of migration time, separation efficiency and electroosmotic mobility.
31 Interestingly, the electroosmotic flow was found to vary with the nature of the polycation
32 on a broad range of electroosmotic mobility decreasing in magnitude in the order of PB> ϵ -
33 PLL>DEAEDq, whatever the polyanion associated. Application of the coatings to the
34 separation of proteins is illustrated in a 0.5 M acetic acid BGE, including CE-MS separation of
35 ribonuclease B-glycoforms of the same mass (positional or structural isomers).

36

37

38 **Keywords:** capillary coatings, polyelectrolyte multilayers, protein separation, polymer
39 coatings

40

41 **1. Introduction**

42 Capillary coatings are frequently used to limit adsorption phenomena onto the capillary
43 wall in capillary electrophoresis (CE), and to increase repeatability / reproducibility of CE
44 separations, either for protein or small ions analysis [1]. Capillary coatings should thus
45 ensure high separation efficiency and good repeatability, which is particularly important for
46 CE coupled to mass spectrometry (CE-MS). Different approaches have already been
47 explored and reviewed [2-7], such as covalently bonded polymer coating, dynamic coating
48 or physically adsorbed polymers, including successive multiple ionic-polymer layers (SMIL).
49 Compared to the first category, SMIL coatings are very easy to prepare and to regenerate.

50 A 2-layers anionic terminating coating of polybrene (PB) and dextran sulfate (DS),
51 developed by Katayama *et al.*, was the first SMIL system applied for CE separation [8] and
52 permitted over 100 runs of acidic proteins separations in neutral phosphate buffer. This
53 SMIL system was next extended to a 3-layer cationic terminating coating for the separation
54 of basic proteins in acidic conditions (phosphate buffer, pH 3) and for CE/MS analysis of β -
55 blockers in formate buffer [9]. Catai *et al.* further investigated SMIL systems composed of
56 PB/poly(vinyl sulfate) (PVS) bilayers for the separation of proteins at pH 7 in phosphate-
57 based buffers of various concentrations [10]. They showed that the optimal separation
58 efficiency is obtained in 300 mM Tris-phosphate buffer. The same group also analyzed intact
59 proteins using a cationic 3-layers SMIL based on PB and DS in Tris-phosphate buffer by CE
60 and acetic acid buffer by CE/MS [11]. A CE/MS method was developed for the
61 characterization and integrity assessment of the antigens TB10.4 and Ag85B and their
62 chemically produced glycoconjugates, which are glycovaccine candidates against
63 tuberculosis [12]. In order to prevent protein adsorption to the inner capillary wall and to
64 achieve efficient separation of the antigen proteoforms, a 3-layers coating of PB-DS-PB was
65 used in combination with 1.5 M acetic acid as background electrolyte [12]. CE/MS analysis
66 revealed the presence of several closely related degradation products, including truncated,
67 oxidized and conformational variants. Both the affinity and composition of the therapeutic
68 protein EGa1, an antagonist of the epidermal growth factor receptor which is overexpressed
69 on the surface of tumor cells, were determined by CE and CE/MS, using PB/PVS and
70 PB/DS/PB capillary coatings and various background electrolytes, showing the heterogeneity
71 of EGa1 [13]. Efficient protein separations have been achieved by Schlenoff *et al.* using

72 multilayers of poly(diallyldimethylammonium) chloride (PDADMA) and poly(styrene
73 sulfonate) (PSS) [14]. Similar SMIL system was further studied by Nehmé *et al.* who
74 investigated the influence of various experimental parameters (polymer concentration,
75 number of layers, ionic strength, rinsing time, storage and regeneration conditions) on
76 separation efficiency and repeatability for the separation of peptides [15,16] and proteins
77 [16] in Tris-phosphate buffer at pH 2.5. The authors found that the best separations are
78 obtained using 1.5 M ionic strength coating solutions, 5-layers coatings and “sufficient”
79 polyelectrolyte (PE) concentrations (0.2% w/v) [15,16]. The influence of background
80 electrolyte (BGE) pH on EOF has been investigated on SMIL coatings and was found
81 substantial in the case of weak polyelectrolytes but not significant in the case of strong
82 polyelectrolytes [17]. Thickness and morphology of SMIL coatings has been investigated by
83 atomic force microscopy on PB, PB/DS and PB/DS/PB layer coatings [18]. Protein adsorption
84 was observed on fused silica capillary and on PB/DS but not on PB and on PB/DS/PB coatings
85 [18]. SMIL coatings based on PDADMA/DS have been used for the separation of
86 recombinant allergen variants in ammonium bicarbonate buffer at pH 6.7 with excellent
87 repeatability/reproducibility [19]. Recently, topographic and recognition imaging (TREC) was
88 performed by Leitner *et al.* [20] using atomic force microscopy on 4-layers SMIL coated
89 capillaries based on PDADMA and DS (or ending with a moderately charged statistical
90 poly(acrylamide-co-2-acrylamido-2-methyl-1-propane sulfonate), 55% PAMAMPS), showing
91 different topologies (roughness) and charge distributions (adhesion map). Due to lower
92 charge density, the 55% PAMAMPS coating decelerated the EOF compared to the DS in the
93 last layer, and displayed lower charge heterogeneity, while the topography was very similar
94 [20,21].

95 Twenty-three different 5-layers SMIL coatings were investigated, combining different
96 polyanions (poly(L-glutamic acid), poly(acrylic acid) (PAA), poly(acrylic acid-co-acrylamide),
97 hyaluronic acid (HA), dextran sulfate (DS), poly(styrene sulfonate) (PSS), poly(methacrylic
98 acid) (PMA), poly(L-lysine citramide) (PLC)) and different polycations (poly(L-lysine) (α -PLL)
99 and copolymers of lysine with serine and tyrosine, non quaternized diethylaminoethyl
100 dextran, polyallylamine (PAAM), PDADMA, polyethyleneimine (PEI), poly(dimethylamino
101 ethyl methacrylate) (PDMAEMA), and systematically compared regarding the separation
102 performances (separation efficiency, analysis time and repeatability/reproducibility on

103 migration times) of proteins in BGE conditions compatible with MS (0.5 M acetic acid) [22].
104 5-layers SMIL coating was chosen to get positively charged coating for separation in acidic
105 conditions and as good compromise between the number of layers and the performances of
106 the coating [22]. It was shown that not only the nature of the polycation constituting the
107 last layer, but also the nature of the polyanion in the intermediate layers of the SMIL, played
108 an important role in the separation performances. The best results were obtained with 5-
109 layer PDADMA/PLC SMIL with excellent run-to-run and capillary-to-capillary
110 repeatability/reproducibility on migration times (RSD = 0.1%) and peak efficiencies (RSD =
111 1.2%). However, we did not find any systematic correlation between PE characteristics such
112 as linear charge density (ξ), weak / strong acid-base moieties, PE backbone nature and the
113 separation performances. ξ is defined as the ratio of the Bjerrum length (0.71 nm in pure
114 water at room temperature) to the average distance b between two adjacent charges:

$$115 \quad \xi = l_b/b \quad (1)$$

116 The present work aims at studying 5-layers SMIL capillary coatings in acetic acid BGE
117 using new polycations that were not previously investigated: polybrene (PB), quaternized
118 diethylaminoethyl dextran (DEAEDq) and ϵ -poly(lysine) (ϵ -PLL), in combination with PAA, DS,
119 PSS, PMA and PLC as polyanions. The influence of the nature of the polyelectrolytes used in
120 the SMIL and of the ionic strength of the construction buffer on the performances of
121 separation of proteins is discussed. To identify the best polyelectrolyte couples, a test
122 mixture of 5 standard proteins was used to evaluate separation performances based on
123 separation efficiency and repeatability of the separations in 0.5 M acetic acid BGE. Intra and
124 inter capillary repeatability / reproducibility were investigated based on 7 repetitions per
125 capillary and 3 different capillaries for the most promising PE couples. The most promising
126 coatings were applied for the separation of glycoproteins by CE-MS.

127

128 **2. Materials and Methods**

129 *2.1. Chemicals*

130 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES) and acetic acid were
131 purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Lysozyme (Lys, purity \geq
132 90%), β -lactoglobulin (Lac, purity \geq 90%), Ribonuclease A (RNAse A, purity \geq 60%),

133 myoglobin (Myo, purity $\geq 95\%$) and trypsin inhibitor (TI, purity not indicated by the supplier)
134 were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Ultrapure water was
135 obtained using a MilliQ system from Millipore (Molsheim, France). Analytical grade NaCl was
136 purchased from Merck (Fontenay-sous-Bois, France).

137 Poly(acrylic acid) (PAA, M_w 30,000 g/mol, PDI = 2.5, pK_a = 3.9-4.5, ξ = 2.88), dextran
138 sulfate (DS, M_w 500,000 g/mol, ξ = 2.8) and hexadimethrine bromide (polybrene, PB, M_w
139 15,000 g/mol, purity $\geq 95\%$, pK_a = 10.5, ξ = 0.45) were purchased from Sigma-Aldrich (Saint-
140 Quentin Fallavier, France). Poly(styrene sulfonate) (PSS, M_w 70,000 g/mol, ξ = 2.88) was
141 purchased from Acros Organics (Geel, Belgium). Poly(L-lysine citramide) (PLC, M_w 39,000
142 g/mol, PDI = 2.0, pK_a = 4.3-4.6, ξ = 0.85) was kindly supplied by M. Boustta (IBMM,
143 University of Montpellier, France) [23]. PLC is a carboxylic polyacid composed of building
144 blocks based on alternating L-lysine and citric acid moieties. Poly(methacrylic acid) (PMA, M_n
145 50,000 g/mol, PDI = 2.5, pK_a = 4.2-4.8, ξ = 2.88) was synthesized as previously described [24].
146 Quaternized diethylaminoethyl dextran (DEAEDq, M_w 500,000 g/mol, ξ = 0.35) was obtained
147 from Pharmacia (Uppsala, Sweden). ϵ -poly(lysine) (ϵ -PLL, M_n 5,000 g/mol, pK_a = 9.0, ξ = 0.85)
148 was obtained from *Streptomyces Albulus* and was supplied by Chisso (Tokyo, Japan). It is an
149 homopolymeric compound of ~ 30 lysine monomers linked at the ϵ -amino groups [25]. All
150 the polyacids are under their sodium form and all the polybases are under their
151 hydrochloride form.

152 2.2. Capillary coatings

153 Capillaries were first conditioned with 1 M NaOH for 20 min, water for 5 min and 20 mM
154 HEPES + NaCl (varying from 0 to 1 M) buffer at pH 7.4 for 10 min. The polyelectrolyte
155 multilayer systems are built within the capillaries using successive flushes of polycation and
156 polyanion solutions at 3 g/L in the 20 mM HEPES buffer pH 7.4 (+ possibly NaCl as indicated
157 on the graphs). Each polyelectrolyte layer was deposited by a 7 min flush (starting with the
158 polycation) and with a 3 min rinsing with the HEPES/NaCl buffer between two
159 polyelectrolyte layers. Five layers were built finishing with a polycation layer. Before
160 separation, the capillaries were then flushed with the background electrolyte (0.5 M acetic
161 acid, pH 2.5) for 10 min.

162 2.3. CE separations

163 Bare fused silica capillaries were purchased from Composite Metal Services (Photonlines,
164 France). CE experiments were carried out using a 3D-CE Agilent technologies system
165 (Waldbronn, Germany) using 50 μm i.d. \times 40 cm (31.75 cm to the detector). The protein test
166 mixture was prepared by dissolving lysozyme, β -lactoglobulin, RNase A, myoglobin and
167 trypsin inhibitor at 1 g/L in pure water [26]. Protein mixture was injected hydrodynamically
168 at 30 mbar for 4 s. MS-compatible background electrolyte was 0.5 M acetic acid, pH 2.5.
169 0.01% DMF in the BGE was used as EOF marker and was co-injected hydrodynamically at 30
170 mbar for 4 s. The capillary was rinsed with buffer for 3 min between two successive runs.
171 The temperature of the capillary cartridge was set at 25°C. CE experiments were performed
172 by applying a negative polarity voltage of -30 kV. UV detection was performed at 214 nm.
173 All the separations were repeated seven times. Separation efficiency was determined using
174 the following equations:

$$175 \quad N = 5.54 \left(\frac{t_m}{\delta} \right)^2 \quad (1)$$

176 where t_m is the migration time and δ is the half-height peak width determined using CEval
177 0.6 software [27] available at the following address
178 [<https://echmet.natur.cuni.cz/software/ceval>].

179 2.4. CE-MS

180 CZE-ESI-MS was performed on an HP 3DCE electrophoresis instrument from Agilent
181 Technology (Waldbronn, Germany) coupled to a compact quadrupole – quadrupole time of
182 flight (QqTOF) – MS instrument from Bruker Daltonik (Bremen, Germany). The CZE was
183 performed using the same parameter (BGE, temperature, voltage) as given above. The CZE-
184 ESI-MS coupling was performed with a commercial triple-tube sheath liquid interface
185 (Agilent Technologies, Waldbronn, Germany). The sheath liquid (SL) was composed of
186 isopropanol (IPA) / H_2O (1:1) with 0.5% (v/v) of formic acid (FAc). The SL was delivered at a
187 flow rate of 4 $\mu\text{L}/\text{min}$ by a syringe pump (Cole-Parmer®, IL, USA) equipped with a 5-mL
188 syringe (5MDF-LL-GT, SGE Analytical Science, Melbourne, Australia). The nebulizer was set
189 to 0.3 bar. Electrospray ionization was performed in positive mode ($U=4500\text{V}$). Mass Spectra
190 were acquired at $m/z=500-2500$ using 1Hz data acquisition. Data processing was carried out

191 by Bruker Compass Data Analysis software (Version 4.3, Bruker Daltonik, Bremen,
192 Germany).

193

194 **3. Results and discussion**

195 Three polycations, namely polybrene (PB), quaternized diethylaminoethyl dextran
196 (DEAEDq) and ϵ -poly(lysine) (ϵ -PLL), were selected for the present study to complete the
197 previous study based on α -PLL, copolymers of lysine with serine and tyrosine,
198 diethylaminoethyl dextran, PAAM, PDADMA, PDMAEMA and PEI [22]. DEAEDq is a strong
199 polybase as PDADMA, the latter of which was previously found to be the best polycation in
200 terms of separation performances [22]. ϵ -PLL has low charge density (compared to α -PLL)
201 similarly to PLC polyanion which was previously found the best polyanion in terms of
202 separation performances [22]. PB is often used in the literature and should thus be
203 compared to other polycations [8-13, 18]. The five polyanions, namely PAA, PMA, PSS
204 (acrylic polymers), PLC (polyamide) and DS (polysaccharide), were selected to be associated
205 with the three polycations. **This work investigates 5-layers SMIL coatings for two reasons: (i)**
206 **an odd number of layers was chosen to get positively charged coatings for separation in**
207 **acidic conditions; and (ii) 5-layers was found to be a good compromise in terms of protein**
208 **separation efficiency and coating stability [22].** As coating solutions, PE were dissolved at 3
209 g/L in HEPES buffer at pH 7.4 to ensure the ionization of all PE, including weak polybases /
210 polyacids.

211 *3.1. Influence of the nature of the PE and intra-capillary repeatability*

212 Figure 1A (resp. Figure 1B) displays the electropherograms obtained for 5-layers SMIL
213 coatings using PB and ϵ -PLL (resp. DEAEDq) polycations. Surprisingly, the range of migration
214 times are very different for PB (total analysis time as low as 3 min), ϵ -PLL (total analysis time
215 up to 6 min), or DEAEDq (up to 20 min and even more when combined to PAA or PSS). This
216 is due to the broad range of electroosmotic mobility (from -39.5 Tiselius units (TU) in the
217 case of the DEAEDq/PAA to -62.6 TU for PB/PSS). Electroosmotic mobility values are given
218 by decreasing order in Table 1 together with the separation efficiency (N/l) and RSD on the
219 migration times of proteins and of the electroosmotic marker. EOF magnitude (absolute

220 value of the electroosmotic mobility) was found to decrease in the order order PB> ϵ -
221 PLL>DEAEDq, whatever the polyanion associated. The modulation of the EOF according the
222 nature of the polycation (and polyanion) on a wide range of mobilities (about 23 TU) is of
223 great practical interest for applications, either to speed up the analysis, or to increase the
224 resolution by decreasing the EOF magnitude. In the case of DEAEDq, the EOF was so slow
225 with PAA, PSS or PMA that all the proteins were not detected in about 20 min. RSD on
226 migration times are usually lower than 3-4%, with excellent results for PLC polyanion. The
227 lowest repeatability was obtained with PAA polyanion.

228 In agreement with the previous study [22], coatings with PLC gave the best results in
229 terms of separation efficiencies (around 300,000 plates/m for PB/PLC and ϵ -PLL/PLC
230 couples). Such high separation efficiency can be also directly observed on the
231 electropherograms in Figure 1A when looking at the fine resolution of small protein peaks
232 (close to the main proteins). Surprisingly, PLC, which is the polyanion with the lowest linear
233 charge density among those studied in this work, can lead to the lowest EOF magnitude
234 (when combined with highly charged PB) or to the highest (when combined with lower
235 charged ϵ -PLL or with DEAEDq), showing the complexity of the physicochemical parameters
236 involved in the regulation of the EOF magnitude (thickness and compactness of the SMIL
237 layers, charge repartition [20,21], penetration of the electrostatic double layer in the SMIL
238 [28], charge stoichiometry of complexation between oppositely charged PE layers [29,30]).
239 As already reported in [22], we confirm here that the nature of the polyanion is of primary
240 importance for the separation efficiency, even if the polyanion is not the last layer of the
241 SMIL coating, and thus not in direct contact with the proteins. Regarding the choice of
242 polycation leading to the best performances, the best polycations are ϵ -PLL, PB, DEAEDq
243 and PDADMAC [22]; the worth polycations are α -PLL, PAAM, PDMAEMA, PEI and non-
244 quaternized DEAED. From this study and from [22], we can conclude that the best SMIL
245 coatings were obtained with: (i) polycations having low to moderate linear charge densities
246 (ξ between 0.35 and 1.44) and (ii) with PLC polyanion (low charge density and amide
247 structure) ahead from vinylic polyanions (in the decreasing order of PMA, PSS and PAA), and
248 far beyond polysaccharides (DS et HA). It is however difficult to find a general rational
249 explanation for the better performances of PLC, since its main chemical characteristics (low

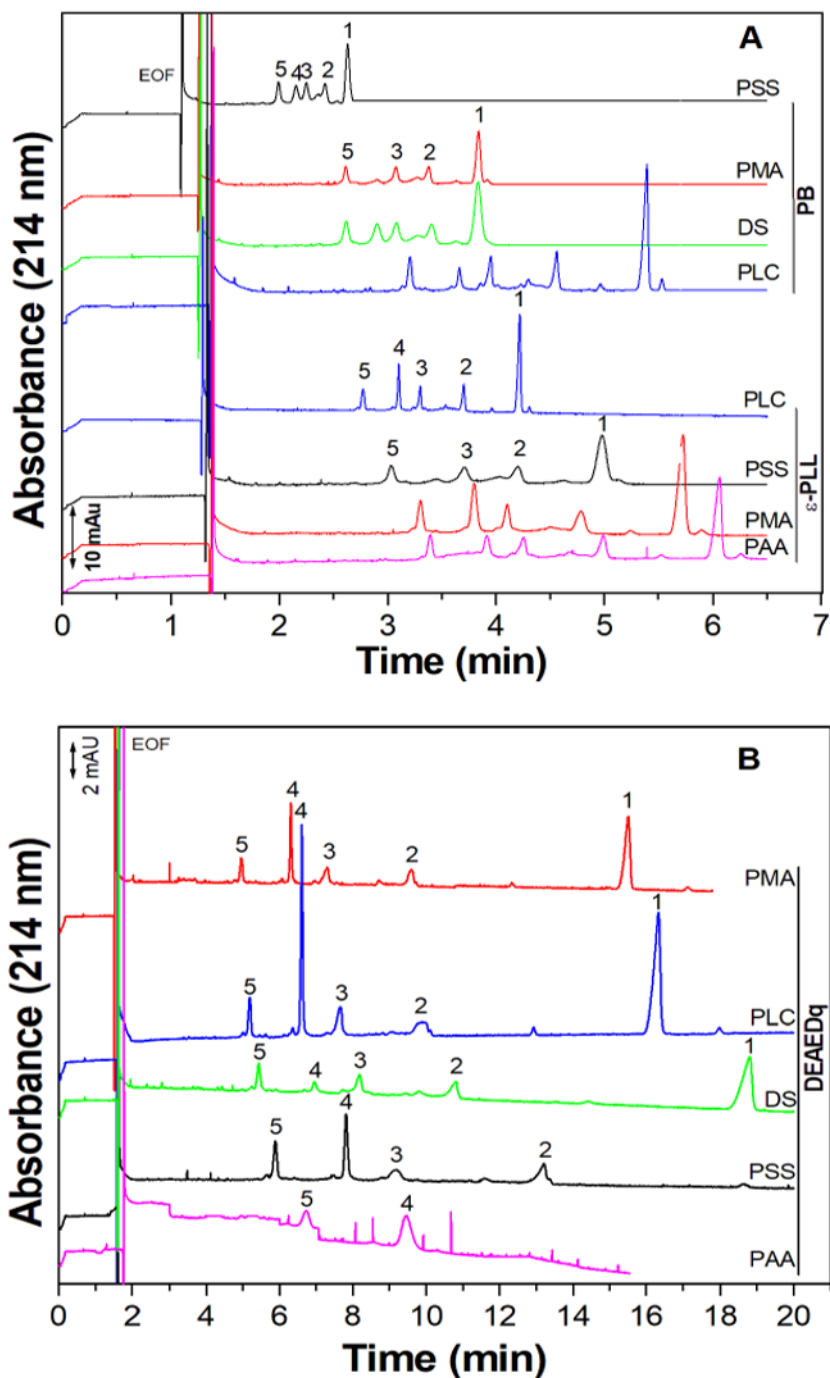
250 charge density and high hydroxyl groups content) are not present in the second best
251 polyanions (PMA and PSS [22]), or are present in one of the worst (HA).

252 Among the 13 new SMIL coatings studied in this work and presented in Figures 1A and
253 1B, six coatings with the highest separation performances were selected for the inter-
254 capillary reproducibility study (see section 3.2): namely PB/PSS, PB/PMA, PB/PLC, ϵ -PLL/PLC,
255 DEAEDq/PMA and DEAEDq/PLC. The six polyelectrolyte couples were selected to get both
256 the best separation efficiencies and to cover a wide range of electroosmotic mobilities.

257 As a matter of comparison, Table 2 gathers some figures of merit about the separation
258 efficiency, and the repeatability on migration times, using SMIL coatings and volatile
259 electrolytes (mostly in acidic conditions), taken in the literature and the 6 best coatings
260 studied in this work. Compared to Table 1, Table 2 also gives the detailed performances for
261 each protein, and the N/l values averaged on n repetitions when available ($n=7$ in this work).
262 The electrophoretic conditions (capillary dimensions, voltage, electroosmotic mobility) and
263 the solutes (different proteins and one peptide (oxytocine)) reported in Table 2 are
264 generally different, which makes the comparison difficult. Nevertheless, we can emphasize
265 that the average N/l values on all data presented in Table 2 is 160,000 plates/m; with
266 significantly higher performances for ϵ PLL-PLC, PB-PLC and PDGMI [31]. It should be also
267 noted that separation efficiency is generally higher for the highly basic lysozyme, probably
268 because of strong electrostatic repulsion between the positively charged coating and the
269 protein.

270

271



272

273 **Figure 1.** Electrophoretic separation of 5 test proteins in acetic acid BGE on 5-layers SMIL coatings
 274 based on ϵ -PLL or PB polycations (A) or on DEAEDq polycation (B) (polyanion as indicated on the
 275 graph). Experimental conditions: 5-layers SMIL coated capillary terminating with the polycation.
 276 Capillary: 40 cm (31.75 cm to the detector) x 50 μ m I.D. Electrolyte: 0.5 M acetic acid, pH 2.5.
 277 Applied voltage: -30 kV. Hydrodynamic injection: 30 mbar, 4 s. Sample mixture: 1 g/L of each protein
 278 in water. Peak identification: Lyz (1), Lac (2), RNase A (3), Myo (4), TI (5) at 1 g/L each in water.
 279 Hydrodynamic co-injection of 0.01 % DMF: 30 mbar, 4 s. Temperature: 25 $^{\circ}$ C. For the coating
 280 procedure, see the experimental section.

281 **Table 1.** Electroosmotic mobility and separation performances (separation efficiency and
 282 repeatability) obtained for the 5-layers polyelectrolyte coatings studied in the present work.
 283 Experimental conditions as in Figure 1.

Polycation	Polyanion	μ_{eof} ($10^{-9}m^{-2}V^{-1}s^{-1}$) ^a	N/l (10^3 plates/m) ^b	RSD t_m (%) ^c	RSD t_{eof} (%) ^c
PB	PSS	-62.6 +/- 1.1	103	2.5	1.1
	PMA	-55.3 +/- 0.5	145	1.1	0.5
	DS	-54.3 +/- 1.5	67	4.5	1.5
	PLC	-50.5 +/- 0.8	293	2.3	0.8
ϵ -PLL	PLC	-53.9 +/- 0.4	336	1.0	0.4
	PSS	-51.2 +/- 1.4	58	4.9	1.3
	PMA	-50.3 +/- 1.0	102	3.4	1.0
	PAA	-48.0 +/- 2.3	115	8.1	2.2
DEAEDq	PMA	-45.6 +/- 0.8	139	3.4	0.8
	PLC	-43.3 +/- 0.3	136	0.5	0.3
	DS	-43.0 +/- 0.8	87	4.4	0.8
	PSS	-42.8 +/- 0.4	125	3.3	0.4
	PAA	-39.5 +/- 1.2	94	5.6	1.2

284 ^a +/- one SD based on $n=7$ repetitions

285 ^b average on all detected proteins for the first run

286 ^c RSD (%) on migration times are calculated by the average of the RSD obtained for the five proteins
 287 on $n=7$ repetitions.

288

289 **Table 2.** Figures of merit of different SMIL coatings reported in the literature for the separation of
 290 proteins and one peptide (oxytocine) in volatile electrolytes and comparison with the experimental
 291 data obtained in this work.

Nature of the SMIL coating	Solute (injected concentration)	Experimental conditions ^a	μ_{eof} ($10^{-9}m^{-2}V^{-1}s^{-1}$), RSD on t_m	N/l (10^3 plates/m) ^b	n ^b	Ref
PB-PVS	rhGH (1.5 g/L)	75 mM ammonium formate, pH 8.5 ; 80 cm \times 70 cm \times 50 μ m ; + 30 kV ; 20 $^{\circ}$ C	<i>Not mentioned</i>	150	5	[31]
PB-DS-PB	Oxytocin (50 mg/L)	50 mM acetic acid, pH 3 ; 80 cm \times 70 cm \times 50 μ m ; - 30 kV ; 20 $^{\circ}$ C	<i>Not mentioned</i>	143	5	[31]
PB-DS-PB	Oxytocin (50 mg/L)	525 mM acetic acid, pH 3 ; 80 cm \times 70 cm \times 50 μ m ; - 30 kV ; 20 $^{\circ}$ C	1.4 %	429	5	[31]
PDADMA-PLC-	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm \times 30 cm \times 50 μ m ; - 30 kV ; 25 $^{\circ}$ C	-54.6	135	4	[22]
PDADMA-PLC-	Lac		0.8 %	170		
PDADMA-PLC-	RNAse A			62		
PDADMA-PLC-	Myo			122		
PDADMA	TI ^c			65		

Plys-PLC- Plys-PLC-Plys	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-54.6	111	4	[22]
	Lac		1.7 %	73		
	RNase A			46		
	Myo TI ^c			96 78		
PDADMA- PMA- PDADMA- PMA- PDADMA	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-55	84	4	[22]
	Lac		0.2 %	57		
	RNase A			78		
	Myo TI ^c			68 55		
PDGMI	Lys	0.1 M ammonium acetate, pH3 ; 54 cm × 44 cm × 50 μm ; + 30 kV ; 25 °C	<i>Not</i>	515	20	[32]
	Cyt C		<i>mentionned</i>	110		
	RNase A ^c			685		
PDGMI2	Lys	25 mM phosphoric acid, pH 2.5 ; 54 cm × 44 cm × 50 μm ; + 30 kV ; 25 °C	<i>Not</i>	236	20	[32]
	Cyt C		<i>mentionned</i>	140		
	RNase A ^c			231		
DEAEDq- PLC- DEAEDq- PLC-DEAEDq	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-43.3	179	7	This work
	Lac		0.3 %	85		
	RNase A			99		
	Myo TI ^c			251 98		
DEAEDq- PMA- DEAEDq- PMA- DEAEDq	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-45.6	147	7	This work
	Lac		0.8 %	66		
	RNase A			67		
	Myo TI ^c			228 82		
εPLL-PLC- εPLL-PLC- εPLL	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-53.9	209	7	This work
	Lac		0.4 %	208		
	RNase A			224		
	Myo TI ^c			209 128		
PB-PLC-PB- PLC-PB	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-50.5	281	7	This work
	Lac		0.8 %	280		
	RNase A			139		
	Myo TI ^c			270 147		
PB-PMA-PB- PMA-PB	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-55.3	235	7	This work
	Lac		0.5 %	110		
	RNase A			240		
	TI ^c			142		
PB-PSS-PB- PSS-PB	Lys	0.5 M acetic acid, pH 2.5 ; 40 cm × 30 cm × 50 μm ; - 30 kV ; 25 °C	-62.6	128	7	This work
	Lac		1.1 %	123		
	RNase A			120		
	Myo TI ^c			84 108		

292 ^a BGE, capillary dimensions, voltage, temperature

293 ^b *n* is the number of repetitions for the calculation of *N//*

294 ^c Each protein at 1 g/L

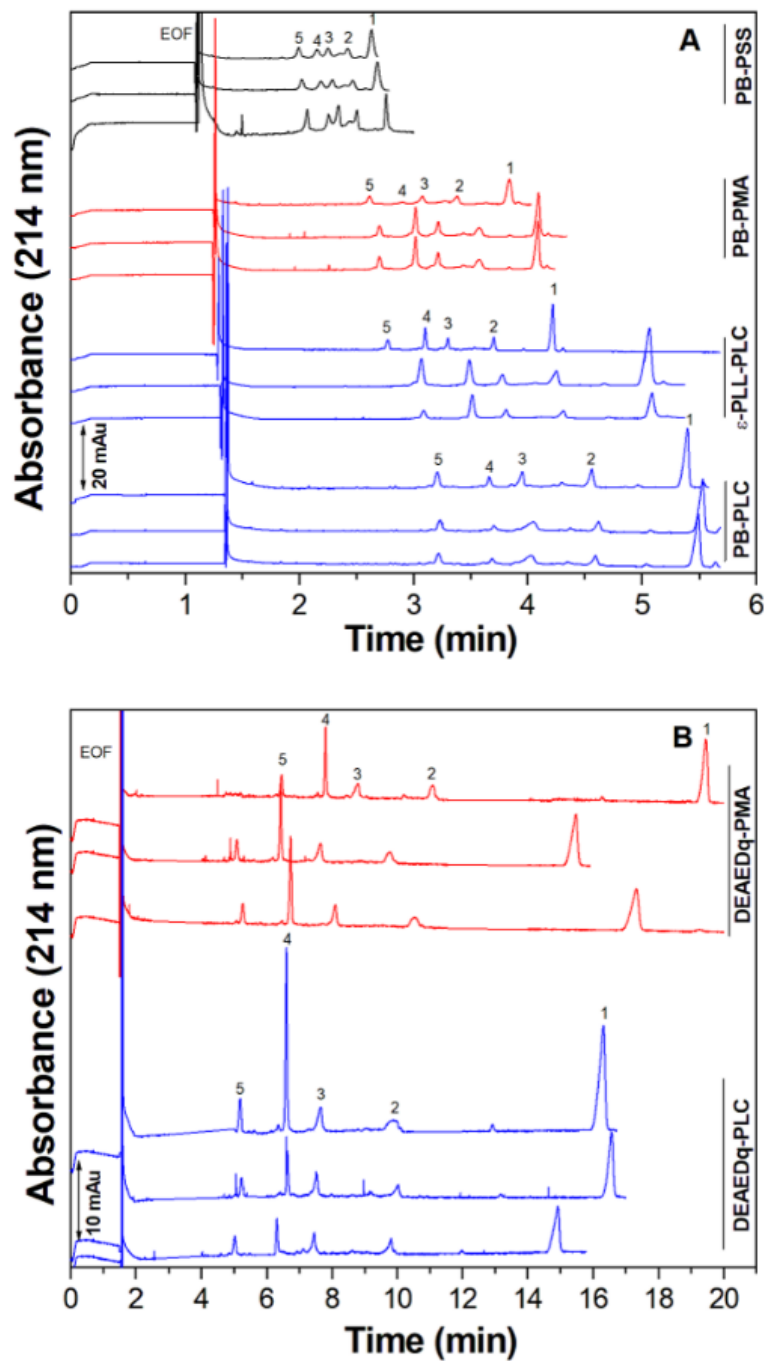
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296 3.2. Study of intra-capillary and inter-capillary reproducibility on the best capillary coatings

297 Inter-capillary reproducibility was tested on three different capillaries for the 6 previously
298 selected SMIL coatings. Such studies are important in practice to increase the
299 reproducibility of CE in terms of migration times and separation efficiency. However, the
300 inter-capillary reproducibility is rarely investigated in the literature, most likely because such
301 studies are time consuming. Examples of electropherograms obtained on three different
302 capillaries are presented in Figure 2. All the numerical values relative to EOF mobility,
303 separation efficiency and RSD on migration times are gathered in Table 3, for each of the 18
304 tested capillaries, showing the reproducibility for each polyelectrolyte system. Basically, the
305 intra-capillary RSD (calculated on 7 runs and averaged for the 5 test proteins) on protein
306 migration times are very low: typically lower than 2.5%, with an average value on the 18
307 studied capillaries of 1.3%. The same was observed for the migration time of the EOF
308 marker: RSD lower than 1.1% with an average of 0.6% for all the capillaries. For the 6
309 selected couples of polyelectrolytes, the intra-capillary average $\langle N/l_{5P} \rangle_{7 \text{ runs}}$ calculated on 7
310 runs and averaged on the 5 test proteins was systematically found higher than 100,000
311 plates/m (except for one PB-PSS capillary). Average RSD on N/l values was obviously higher
312 than for migration times, but still reasonable for such experimental parameter: RSD \sim 9.6%
313 on the 18 tested capillaries. The detailed figures of merit obtained for the separation
314 efficiency of each protein are given in Table SI1 (in Supporting Information).

315 From this study, we can conclude that the 6 investigated PE couples can be used for
316 repeatable and reasonably reproducible separations with a broad range of electroosmotic
317 mobility (from to -43.7 to -62.9 TU) and separation efficiency higher than 10^5 plates/meter.
318 Nevertheless, there is still place for some possible improvement in the inter-capillaries
319 reproducibility. ANOVA statistical tests performed on μ_{eof} , $\langle N/l_{5P} \rangle$ and the migration times
320 of proteins (not shown) clearly demonstrate that the inter-capillary dispersions on these
321 parameters are significantly higher than the intra-capillary dispersions. This can be for
322 instance illustrated by the third PB-PSS capillary (third trace from the top in Figure 2A) which
323 provided significantly higher separation efficiencies than the two others. For PB-PMA, the
324 first capillary (first trace from the top in Figure 2A) lead to lower separation efficiencies. By
325 the way, this study already gives important hints about the nature of the PE couples that are
326 the most adequate to improve coating performances and reproducibility. One possible

327 important parameter to optimize SMIL coating performances is the ionic strength of the
328 coating solutions.



329

330 **Figure 2.** Inter-capillary reproducibility of 5-layers SMIL coatings (PB-PLC, ε-PLL-PLC, PB-PMA, PB-PSS
331 couples (A) or DEAEq-PLC, DEAEq-PMA couples (B)) for the electrophoretic separation of 5 test
332 proteins in 0.5 M acetic acid. 3 different capillaries were tested for each coating ($n=7$ for each
333 capillary). Only the first separation on each capillary is shown. Experimental conditions as in Figure
334 1.

335

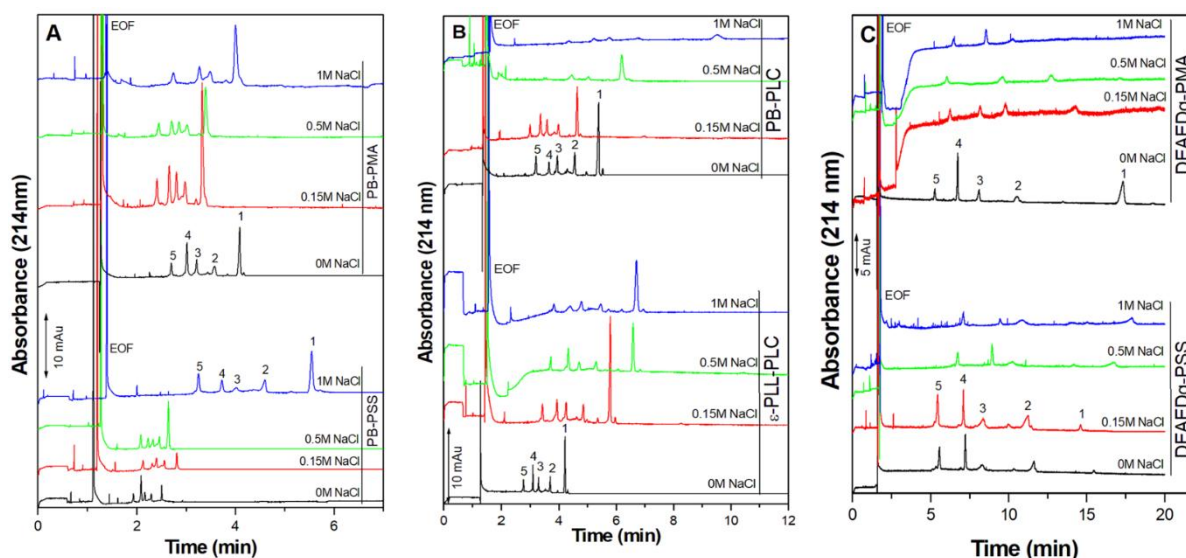
336 **Table 3.** Inter-capillary study on PB-PLC, ϵ -PLL-PLC, PB-PMA, PB-PSS, DEAEDq-PLC and DEAEDq/PMA
 337 5-layers SMIL coatings. Average electroosmotic mobility, average separation efficiency $\langle N/l_{5P} \rangle_{7 \text{ runs}}$
 338 and relative standard deviations on separation efficiency and migration times obtained for 3
 339 different capillaries of each coating. $\langle N/l_{5P} \rangle_{7 \text{ runs}}$ and RSD% on migration times are calculated on the
 340 average of the five proteins and on 7 repetitions. Experimental conditions as in Figure 1.

Couples	μ_{eof} ($10^{-9} \text{ m}^{-2} \text{ V}^{-1} \text{ s}^{-1}$)		$\langle N/l_{5P} \rangle_{7 \text{ runs}}$ (10^3 plates/m)		RSD% $\langle N/l_{5P} \rangle_{7 \text{ runs}}$		RSD% t_m		RSD% t_{eof}	
	intra	av.	intra	av.	intra	av.	intra	av.	intra	av.
PB/PSS	-62.6		87		11.3		2.5	1.3	1.1	
	-64.0		118	115	8.0	7.8	0.9	±	0.5	0.6
	-61.4	± 1.3	141	± 27	3.9	± 3.7	0.5	1.0	0.2	± 0.5
PB/PMA	-55.3		130		8.8		1.1	1.3	0.5	
	-56.1		226	188	4.1	5.3	2.7	±	1.1	0.6
	-52.3	± 0.5	209	± 51	2.9	± 3.1	0.1	1.3	0.1	± 0.5
ϵ -PLL/PLC	-53.9		297		7.5		1.0	0.8	0.4	
	-52.1		156	200	11.1	8.6	0.8	±	0.4	0.4
	-52.3	± 0.8	148	± 84	7.2	± 2.2	0.6	0.2	0.3	± 0.1
PB/PLC	-50.5		266		9.8		2.3	1.9	0.8	
	-51.5		227	236	9.8	7.8	2.8	±	0.8	0.6
	-51.4	± 0.5	214	± 27	3.9	± 3.4	0.7	1.1	0.1	± 0.4
DEAEDq /PMA	-44.8		120		11.3		3.4	2.3	0.8	
	-43.9		120	115	4.3	6.3	2.1	±	0.5	0.6
	-43.3	± 0.8	105	± 9	3.2	± 4.4	1.4	1.0	0.4	± 0.2
DEAEDq /PLC	-43.2		128		4.6		0.5	1.9	0.3	
	-43.3		136	146	10.8	8.3	0.9	±	0.2	0.4
	-44.7	± 0.8	174	± 25	9.4	± 3.3	4.1	2.0	0.8	± 0.3

341

342 3.3 Influence of the ionic strength of the SMIL construction electrolyte

343 The impact of the ionic strength of the coating solution is known to be a crucial
 344 parameter in the building of SMIL coating [15, 16, 33] and polyelectrolyte complexes [34]. It
 345 notably set the thickness of the PE layer. Higher ionic strength leads to thicker deposited PE
 346 layer with the formation of loops [33]. On the contrary, low ionic strength leads to thin
 347 layers due to electrostatic repulsions [33]. In this work, different concentrations (0, 0.15 M,
 348 0.5 M and 1 M) of NaCl were added into the 20 mM HEPES solution buffer pH 7.4 used as a
 349 background electrolyte for the SMIL construction. The corresponding electrophoregrams are
 350 given in Figure 3A to 3C for the 6 couples of PE (PB/PSS, PB/PMA, PB/PLC, ϵ -PLL/PLC,
 351 DEAEDq/PMA and DEAEDq/PLC). Three different Figures are used because the migration
 352 times are very different from one coating to the other due to differences in EOF. Numerical
 353 data about the repeatability of the separation performances are given in Table 4.



354

355 **Figure 3.** Influence of the NaCl concentration added to the construction electrolyte on the
 356 electrophoretic separation of 5 test proteins in 0.5 M acetic acid. Experimental conditions: 5-layers
 357 SMIL coatings (PB/PSS, PB/PMA, PB/PLC, ϵ -PLL/PLC, DEAEq/PMA and DEAEq/PLC, as indicated on
 358 the graphs), 40 cm (31.75 cm to detector) x 50 μ m I.D. capillary. Electrolyte: 0.5 M acetic acid, pH
 359 2.5. Applied voltage: -30 kV. Hydrodynamic injection: 30 mbar, 4 s (0.74% of the total capillary
 360 volume). Sample mixture: 1 g/L of each protein in water. Peak identification: Lyz (1), Lac (2), RNase
 361 (3), Myo (4), TI (5). Hydrodynamic co-injection of 0.01 % DMF: 30 mbar, 4 s. Temperature: 25 $^{\circ}$ C.
 362 Coating solutions: 3g/L PE in 20 mM HEPES pH 7.4 containing NaCl at a concentration indicated on
 363 the graphs. See the experimental section for other experimental conditions.

364

365 From Figures 3A to 3C and Table 4, it can be concluded that increasing the ionic strength
 366 of the coating tends to decrease the coating performances (lower N/l values) and stability
 367 (higher RSD on migration times). This conclusion is different to what was obtained for the
 368 separation of peptides and proteins in acidic phosphate buffer on a PSS/PDADMA SMIL
 369 coating [15, 16], where the optimum ionic strength was found at 1 M for the coating buffer.
 370 One explanation is that the polyelectrolyte couples selected in the present work are
 371 composed of two weak polyelectrolytes or one weak and one strong polyelectrolyte,
 372 whereas PSS and PDADMA used in [15-16] are both strong polyelectrolytes, and thus lead to
 373 very stable polyelectrolyte complexes towards salt addition even at high concentration. The
 374 low stability of the coating and poor separation efficiency can be due to a lower
 375 homogeneity of the layer exposed to the solution in the capillary due to the destabilization
 376 of the coating with increasing ionic strength of the construction electrolyte. As a general
 377 trend, the electroosmotic mobility decreased (in absolute value) with increasing ionic
 378 strength of the coating solution from typically about 5 to 10 TU when passing from 0 to 1M

379 added NaCl. The loss in separation efficiency with increasing ionic strength is not similar for
 380 all coatings: PB-PSS seems more robust than those with PLC or DEAEDq.

381 **Table 4.** Electroosmotic mobility and separation performances (separation efficiency and RSD on
 382 migration times) obtained for 5-layers polyelectrolyte coating at different concentrations of NaCl in
 383 the construction electrolyte. μ_{eof} and $\langle N/l \rangle_{7 \text{ runs}}$ are calculated on the average of 7 repetitions. RSD%
 384 on migration times are calculated by the average of the RSD on all detected proteins. See
 385 experimental section for experimental conditions.

NaCl (M)	Polycation	Polyanion	μ_{eof} ($10^{-9} \text{ m}^{-2} \text{ V}^{-1} \text{ s}^{-1}$)	$\langle N/l \rangle_{7 \text{ runs}}$ (10^3 plates/m)			RSD% t_m	RSD% t_{eof}
				5 p ^a	4 p ^b	3 p ^c		
0	PB	PSS	-62.0	321			3.4	1.3
0.15			-57.9	216			1.9	0.4
0.5			-54.9	193			2.3	0.5
1			-49.7	123			7.7	1.9
0	PB	PMA	-55.3	209	204		0.1	0.1
0.15			-54.4	128	132		6.7	3.1
0.5			-53.1	122	110		2.9	0.6
1			-50.1	-	86		3.8	0.8
0	PB	PLC	-50.5	266	250		2.3	0.8
0.15			-50.8	191	196		3.7	0.4
0.5			-47.3	-	61		5.2	1.4
1			-42.0	45	46		6.7	0.8
0	ϵ -PLL	PLC	-53.9	297			1.0	0.4
0.15			-47.2	264			5.8	1.0
0.5			-45.1	174			5.6	0.7
1			-43.2	82			4.6	1.0
0	DEAEDq	PMA	-43.3	105		115	1.4	0.5
0.15			-39.9	-	-	65	6.8	1.2
0.5			-39.2	-	-	45	8.6	0.1
1			-35.8	-	-	82	5.3	0.7
0	DEAEDq	PSS	-43.1	-	107		3.3	0.4
0.15			-41.4	-	96		8.6	1.0
0.5			-39.4	-	85		8.0	0.8
1			-39.2	-	76		9.0	0.8

386 ^a 5 proteins: Lyz (1), Lac (2), RNase (3), Myo (4), TI (5)

387 ^b 4 proteins: Lac (2), RNase (3), Myo (4), TI (5). Protein (1) was not detected (EOF was too slow).

388 ^c 3 proteins: RNase (3), Myo (4), TI (5). Protein (1) and (2) were not detected (EOF was too slow).

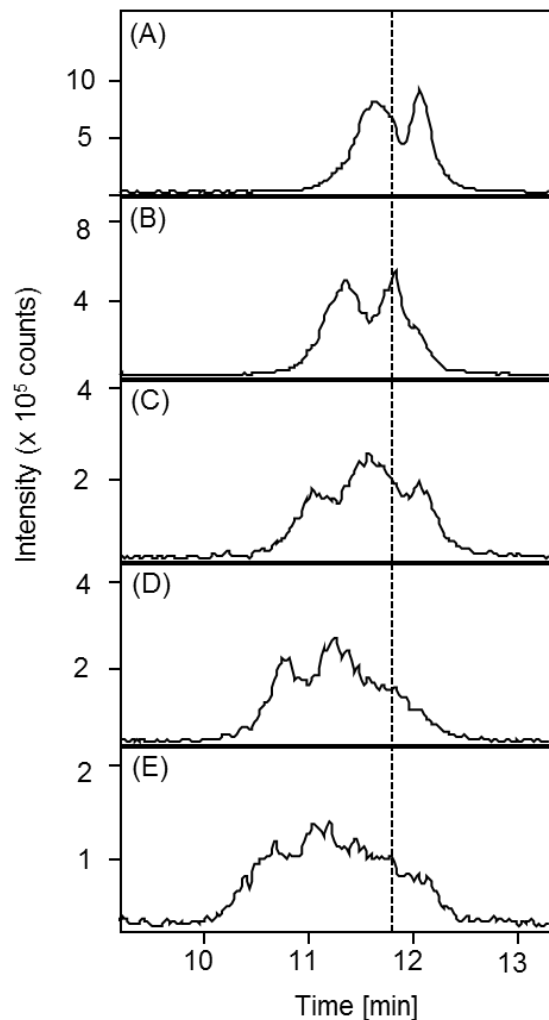
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390

391 3.4. CE-MS experiments

392 Various CE-MS experiments have been performed applying several of the SMIL coatings to
 393 both the standard proteins and some glycoproteins. It turns out that the coated capillaries
 394 can be used for CE-MS experiments without any interferences in the ionization or in the

395 resulting mass spectra. Based on the results for the standard proteins, it can be concluded
396 that the separation efficiency can be maintained beside some minor impact of the interface
397 (open capillary rather than a pressure balanced system as in the CZE-UV experiments). To
398 illustrate the possibilities, the basic glycoprotein ribonuclease B was measured with
399 DEAEDq-PLC coating. Extracted ion electropherograms of the four most abundant charge
400 states of the various glycoforms (5-9 mannose from the N-glycosylation of the high-
401 mannose type) are shown in Figure 4.



402

403 **Figure 4.** CZE-MS of ribonuclease B applying a DEAEDq-PLC coating. Extracted Ion
404 Electropherogramm of glycoforms with 5 mannose ($m/z=1355.5$; 1490.9 ; 1656.5 ; 1863.4
405 corresponding to $14899.3u$) (A), 6 mannose (1370.2 ; 1507.1 ; 1674.5 ; 1883.7 corresponding to
406 $15061.5u$) (B), 7 mannose (1385.0 ; 1523.4 ; 1692.5 ; 1904.0 corresponding to $15223.6u$) (C), 8
407 mannose (1399.7 ; 1539.6 ; 1710.5 ; 1924.2 corresponding to $15385.8u$) (D), 9 mannose (1414.4 ;
408 1555.8 ; 1728.5 ; 1944.5 corresponding to $15547.9u$) (E). The vertical line through the maximum of (B)
409 is drawn to illustrate the separation.

410

411 Interestingly it is possible not only to separate according to the number of mannose, but
412 also to separate several positional isomers of the same mass, which was not possible
413 applying a neutral coating (data not shown). To the best of our knowledge this is the first
414 time that such a separation is shown for intact glycoproteins. For the 5-mannose variant of
415 ribonuclease up to 4 positional isomers have been determined on the glycan level [35]. Here
416 one wider and one more narrow peaks are observed. Similarly, for the 6 mannose
417 glycoform, two peaks and for the 7 mannose glycoform, three peaks can be distinguished.
418 For the higher masses, the situation is not clear due to weak intensity and potentially due to
419 the high number of possible isomers.

420

421 **Conclusions**

422 In this work, up to 43 different 5-layers SMIL coated capillaries were evaluated for CE
423 separation of proteins in a 0.5M acetic acid BGE. It has been shown that the nature of the
424 polycation used in the SMIL coating allows modulating the electroosmotic mobility on a
425 wide range of mobilities (about 23 TU interval): from -39.5 TU in the case of the
426 DEAEDq/PAA, up to -62.6 TU for PB/PSS. EOF magnitude (absolute value of the
427 electroosmotic mobility) was found to decrease in the order $PB > \epsilon\text{-PLL} > \text{DEAEDq}$, whatever
428 the polyanion associated. Coatings with PLC gave the best results in terms of separation
429 efficiencies (around 300,000 plates/m for PB/PLC and $\epsilon\text{-PLL/PLC}$ couples). The second best
430 polyanion after PLC was PMA, closely followed by PSS. From this study and from [22], we
431 can conclude that the best SMIL coatings were obtained with: (i) polycations having low to
432 moderate linear charge densities (ξ between 0.35 and 1.44) and (ii) with PLC polyanion (low
433 charge density and amide structure) ahead from vinylic polyanions (in the decreasing order
434 of PMA, PSS and PAA), and far beyond polysaccharides (DS et HA). Excellent average intra-
435 capillary RSD on protein migration times was obtained (1.3%). The time of preparation of
436 the 5-layers SMIL coating is about 1.5 h, including the activation of the capillary (35 min),
437 but the coating is robust and doesn't require regeneration between runs. The whole
438 procedure is simple and easy to implement and is fully automatized using commercial CE
439 equipment. Surprisingly, increasing the ionic strength of the coating solution tends to

440 decrease the separation efficiency of the proteins, at least in the 0.5 M acetic acid BGE. The
441 presented novel coatings have been shown to be compatible with CE-MS (no interferences
442 have been observed). The separation of glycoforms of the same mass (positional or
443 structural isomers) of ribonuclease B shows the potential of the set-up.

444

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LIST OF CAPTIONS

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542 **Figure 2.** Electrophoretic separation of 5 test proteins in acetic acid BGE on 5-layers SMIL
543 coatings based on ϵ -PLL or PB polycations (A) or on DEAEDq polycation (B) (polyanion as
544 indicated on the graph). Experimental conditions: 5-layers SMIL coated capillary terminating
545 with the polycation. Capillary: 40 cm (31.75 cm to the detector) x 50 μ m I.D. Electrolyte: 0.5
546 M acetic acid, pH 2.5. Applied voltage: -30 kV. Hydrodynamic injection: 30 mbar, 4 s. Sample
547 mixture: 1 g/L of each protein in water. Peak identification: Lyz (1), Lac (2), RNase A (3), Myo
548 (4), TI (5) at 1 g/L each in water. Hydrodynamic co-injection of 0.01 % DMF: 30 mbar, 4 s.
549 Temperature: 25 °C. For the coating procedure, see the experimental section.

550

551 **Figure 2.** Inter-capillary reproducibility of 5-layers SMIL coatings (PB-PLC, ϵ -PLL-PLC, PB-
552 PMA, PB-PSS couples (A) or DEAEDq-PLC, DEAEDq-PMA couples (B)) for the electrophoretic
553 separation of 5 test proteins in 0.5 M acetic acid. 3 different capillaries were tested for each
554 coating ($n=7$ for each capillary). Only the first separation on each capillary is shown.
555 Experimental conditions as in Figure 1.

556

557 **Figure 3.** Influence of the NaCl concentration added to the construction electrolyte on the
558 electrophoretic separation of 5 test proteins in 0.5 M acetic acid. Experimental conditions:
559 5-layers SMIL coatings (PB/PSS, PB/PMA, PB/PLC, ϵ -PLL/PLC, DEAEDq/PMA and
560 DEAEDq/PLC, as indicated on the graphs), 40 cm (31.75 cm to detector) x 50 μ m I.D.
561 capillary. Electrolyte: 0.5 M acetic acid, pH 2.5. Applied voltage: -30 kV. Hydrodynamic
562 injection: 30 mbar, 4 s (0.74% of the total capillary volume). Sample mixture: 1 g/L of each
563 protein in water. Peak identification: Lyz (1), Lac (2), RNase (3), Myo (4), TI (5).
564 Hydrodynamic co-injection of 0.01 % DMF: 30 mbar, 4 s. Temperature: 25 °C. Coating
565 solutions: 3g/L PE in 20 mM HEPES pH 7.4 containing NaCl at a concentration indicated on
566 the graphs. See the experimental section for other experimental conditions.

567

568 **Figure 4.** CZE-MS of ribonuclease B applying a DEAEDq-PLC coating. Extracted Ion
569 Electropherogram of glycoforms with 5 mannose ($m/z=1355.5$; 1490.9; 1656.5; 1863.4
570 corresponding to 14899.3u) (A), 6 mannose (1370.2; 1507.1; 1674.5; 1883.7 corresponding
571 to 15061.5u) (B), 7 mannose (1385.0; 1523.4; 1692.5; 1904.0 corresponding to 15223.6u)
572 (C), 8 mannose (1399.7; 1539.6; 1710.5; 1924.2 corresponding to 15385.8u) (D), 9 mannose
573 (1414.4; 1555.8; 1728.5; 1944.5 corresponding to 15547.9u) (E). The vertical line through
574 the maximum of (B) is drawn to illustrate the separation.

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