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# Modifying last layer in polyelectrolyte multilayer coatings for capillary electrophoresis of proteins

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## Abstract:

Protein adsorption on the inner wall of the fused silica capillary wall is an important concern for capillary electrophoresis (CE) analysis since it is mainly responsible for separation efficiency reduction. Successive Multiple Ionic-polymer Layers (SMIL) are used as capillary coatings to limit protein adsorption, but even low residual adsorption strongly impacts the separation efficiency, especially at high separation voltages. In this work, the influence of the chemical nature and the PEGylation of the polyelectrolyte deposited in the last layer of the SMIL coating was investigated on the separation performances of a mixture of four model intact proteins (myoglobin (Myo), trypsin inhibitor (TI), ribonuclease a (RNase A) and lysozyme (Lyz)). Poly(allylamine hydrochloride) (PAH), polyethyleneimine (PEI),  $\epsilon$ -poly(L-lysine) ( $\epsilon$ PLL) and  $\alpha$ -poly(L-lysine) ( $\alpha$ PLL) were compared before and after chemical modification with polyethyleneglycol (PEG) of different chain lengths. The experimental results obtained by performing electrophoretic separations at different separation voltages allowed determining the residual retention factor of the proteins onto the capillary wall via the determination of the plate height at different solute velocities and demonstrated a strong impact of the polycationic last layer on the electroosmotic mobility, the separation efficiency and the overall resolution. Properties of SMIL coatings were also characterized by quartz microbalance and atomic force microscopy, demonstrating a glassy structure of the films.

**Keywords:** protein adsorption; capillary zone electrophoresis; successive multiple ionic-polymer layer; PEGylated polycation; polyelectrolyte multilayers

## Highlights:

- Impact of the last layer on SMIL capillary coating on analytical performances
- Impact of PEGylated last layer on electroosmotic flow and analytical performances
- Impact of the nature of the last polycation layer on SMIL thickness and hydration
- Comparable results between QCM-D and AFM experiment, in agreements with CE findings

## 1. Introduction

Theoretical separation efficiency in capillary electrophoresis (CE) is ideally controlled by solute axial diffusion [1]. For a globular protein of typical size about 1 nm (molecular diffusion coefficient  $D$  of about  $2 \times 10^{-10} \text{ m}^2\text{s}^{-1}$ , apparent electrophoretic mobility of 20 TU (Tiselius Units: 1 TU =  $10^{-9} \text{ m}^2\text{V}^{-1}\text{s}^{-1}$ ) and typical electric field strength of  $500 \text{ V cm}^{-1}$  corresponding to "typical" 20 kV per 40 cm total capillary length used in this study), about one million theoretical plates are expected in an ideal CE experiment. In practice, the plate number is generally much lower, with typically about a few tens of thousands plates [2-4]. Different factors can explain this reduction, among which radial gradient temperature inside the capillary [5] due to Joule effect and capillary cooling, electromigration dispersion [6,7], capillary coiling [8], or protein adsorption onto the capillary wall [3, 9-13]. Monolayer coatings and successive multiple ionic-polymer layer (SMIL) capillary coatings were developed to limit protein adsorption [14-28]. Pioneered in the late 1960 [29], multilayered polyelectrolyte coatings were rediscovered at the beginning of the 90s by Decher et al. [30, 31] and firstly applied to CE in 1998 [32]. SMIL coatings are built inside the capillary by successive adsorption of oppositely charged polyelectrolytes (PE) onto the capillary wall. SMIL coatings greatly improve the separation repeatability / reproducibility compared to bare fused silica capillary [4, 22, 33-35], but even very small residual protein adsorption can still reduce the separation efficiency [36], with a plate height  $H$  increasing with the power 2 of the retention factor  $k$ . For a fair comparison of the coating performances between different capillary coatings, and for a valuable quantitative estimation of their performances, it was thus crucial to quantify residual protein adsorption by the determination of the protein retention factor  $k$ . We recently demonstrated that this determination can be performed by quantifying protein separation efficiency at different separation voltages. Experimentally, the plate height  $H$  obtained for each protein at different separation voltages can be plotted as a function of the protein linear velocity  $u$  according to the following law [13, 37]:

$$H = \frac{2D}{u(1+k)} + \frac{d_c^2 u}{D} \frac{k^2}{16(1+k)} + \frac{2 d_f^2 u}{3 D_s} \frac{k}{1+k} + A \quad (1)$$

where  $d_c$  is the internal diameter of the capillary,  $u$  is the linear velocity of the analyte,  $d_f$  is the film thickness,  $D_s$  is the diffusion coefficient in the stationary phase and  $A$  is a constant related to extra column contributions. Knowing that  $d_c^2 \gg d_f^2$ , the third term related to the resistance in mass transfer in stationary phase can be neglected in equation (1) as a first approximation. Curve fitting of the plate height experimental data is an excellent way to determine  $k$  value for each protein on a given capillary coating. In equation (1),  $D$  was obtained experimentally by Taylor dispersion

68 analysis. The determination of  $k$  values allows a ranking of the capillary coating performances,  
69 whatever the electroosmotic mobility values, and thus, whatever the range of protein migration  
70 times which can greatly vary with the nature/surface charge of the coating.

71 Reducing protein adsorption, and thus the retention factor, is probably one of the most critical  
72 parameter to improve capillary coatings in CE enabling decreasing the dependency of the peak  
73 broadening with increasing separation voltage. The aim of this work was to study the impact of  
74 the chemical nature of the last polycationic layer deposited on a 4-layers  
75 poly(diallyldimethylammonium chloride) (PDADMAC) / poly(sodium styrene sulfonate) (PSS)  
76 sub-structure, on the CE separation performances of a mixture of 4 model intact proteins  
77 (myoglobin (Myo), Trypsin inhibitor (TI), ribonuclease A (RNase A) and lysozyme (Lyz)).  
78 PDADMAC, polyallylamine (PAH), polyethyleneimine (PEI),  $\epsilon$ -poly(L-lysine) ( $\epsilon$ PLL) and  $\alpha$ -  
79 poly(L-lysine) ( $\alpha$ PLL) were compared as a last layer, either used as it or after chemical grafting  
80 modification with PEG chains of two different lengths (1 kDa and 5 kDa) at various molar  
81 proportions. The different CE characteristic parameters and performances (electroosmotic  
82 mobility, protein retention factor  $k$ , constant  $A$ , separation efficiency, RSD of migration times)  
83 were systematically determined to compare all the tested capillaries.

84

85

## 86 **2. Experimental section**

### 87 *2.1. Chemicals and Materials*

88 **Reagents.** Copper sulfate (99.99% purity), sodium ascorbate (98% purity),  
89 tris(benzyltriazolylmethyl) amine (THPTA, 95% purity), sodium nitrate (99.0% purity), 4-(2-  
90 hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES, 99.5% purity) and sodium acetate  
91 anhydrous (99% purity) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).  
92 Sodium hydroxide (98 % purity) and sodium chloride (99.5% purity) were purchased from Fluka  
93 (Saint-Quentin-Fallavier, France). Triethylamine (99% purity) was obtained from Acros Organics  
94 (Geel, Belgium). Acetic acid (99.99% purity) was supplied by VWR Chemicals (Rosny-sous-  
95 Bois, France). Sodium azide (99.5% purity) was purchased from Merck (Darmstadt, Germany).  
96 Dimethylformamide (99.9% purity) was purchased from Carlo Erba Reagents (Val-de-Rueil,  
97 France). Trypsin Inhibitor (TI, from soybean, isoelectric point ( $pI$ ): 4.5, purity not indicated by  
98 the supplier), Myoglobin (Myo, from equine skeletal muscle,  $pI$ : 7.3, 95% purity), Ribonuclease  
99 A (RNase A from bovine pancreas,  $pI$ : 9.3, 60% purity) and Lysozyme (Lyz, from chicken egg  
100 white,  $pI$ : 11.1, 90% purity) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

101 **Polymers.** Methoxy-(polyethylene glycol)-succinimidyl carboxyl methyl ester (mPEG-NHS-  
102 SCM) of 1 kDa (ref.: PLS-215) and 5 kDa (PLS-213) average molar masses; and methoxy-  
103 (polyethylene glycol)-alkyne (mPEG-Alkyne) of 1 kDa (PLS-2036) and 5 kDa (PLS-2034) molar  
104 masses, were purchased from Creative PEGWorks (Durham, NC, USA).  $\epsilon$ -poly(L-Lysine  
105 hydrochloride) ( $\epsilon$ PLL,  $M_w = 3.5$ -4.5 kDa) was purchased from Biosynth Carbosynth (Staad,  
106 Switzerland). Poly(allylamine hydrochloride) (PAH,  $M_w = 120$ -200 kDa) was supplied by Alfa  
107 Aesar (Kandel, Germany). Branched poly(ethyleneimine) (PEI,  $M_w = 750$  kDa) and  
108 poly(diallyldimethylammonium chloride) (PDADMAC,  $M_w = 450$  kDa) were purchased from  
109 Sigma Aldrich (Saint-Quentin-Fallavier, France).  $\alpha$ -poly(L-Lysine hydrobromide)-graft-  
110 (azidoalkylamide) 10% ( $\alpha$ PLL-10%,  $M_w = 57$  kDa) and 20% ( $\alpha$ PLL-20%,  $M_w = 62$  kDa) were  
111 purchased from Alamanda Polymers (Huntsville, AL, USA). Poly(sodium styrenesulfonate)  
112 (PSS,  $M_w = 70$  kDa) was purchased from Acros Organics (Geel, Belgium). Except for PAH and  
113 PEI, all reagents and polymers were used as received without any further purification. All aqueous  
114 solutions were prepared using deionized water ( $18 \text{ M}\Omega\text{cm}^{-1}$ ) delivered by a Synergy UV water  
115 purification system (Millipore, Fontenay-sous-Bois, France).

116 0.1  $\mu\text{m}$  cut-off Durapore membrane filters were purchased from Millipore (Molsheim, France).  
117 Dialysis tubings (cut-off: 1, 3.5, 8 and 12–14 kDa) were purchased from Spectrum Labs (San  
118 Francisco, CA, USA).

119

## 120 2.2. Proton NMR

121  $^1\text{H}$  NMR spectra were recorded on a Varian Unity Inova 400 MHz spectrometer in a  $\text{D}_2\text{O}$  solution,  
122 using the resonance of water (4.8 ppm) as chemical shift internal reference. Samples of freeze-  
123 dried polymers were dissolved at ca.  $1 \text{ gL}^{-1}$  in 99.9%  $\text{D}_2\text{O}$ . The dissolution of PEGylated polymers  
124 (with 5 kDa PEG chains) in  $\text{D}_2\text{O}$  was performed at  $0^\circ\text{C}$ . All the  $^1\text{H}$  NMR spectra are provided in  
125 Supporting Information, Section 1.C (Figure SI 8 to Figure SI 12).  $^1\text{H}$  NMR integrations were  
126 used to estimate the PEGylation ratio achieved at the end of the synthesis (see Table SI1)  
127 according to the calculation method described in Figure SI 8.

128

## 129 2.3. Preparative Size Exclusion Chromatography (SEC)

130 Automated preparative SEC experiments were performed on a General Electric Healthcare Äkta  
131 Purifier 100 (Chicago, IL, USA) including a P-900 pump unit and a UV-900 multi wavelength  
132 UV-visible detector unit. Separations were carried out on an OHPak SBG Shodex column guard  
133 ( $50 \times 6 \text{ mm}$ ) followed by a  $300 \times 8 \text{ mm}$  SB-805M-HQ Shodex column. The eluent was an aqueous  
134 solution of acetic acid (50 mM), sodium acetate (50 mM), sodium chloride (500 mM) and sodium

135 azide ( $300 \text{ mgL}^{-1}$ ), pH 4.8. Prior to use, the eluent was filtered through a  $0.1 \text{ }\mu\text{m}$  cut-off Durapore  
136 membrane. Polymers samples dissolved at  $4 \text{ gL}^{-1}$  in the eluent were injected by  $500\text{-}\mu\text{L}$  successive  
137 batches and eluted at  $1.0 \text{ mLmin}^{-1}$  flow rate. The collected polymer fractions were dialyzed  
138 against deionized water, concentrated on a rotatory evaporator, and finally freeze-dried. The  
139 resulting powder was stored under inert atmosphere at  $-20^\circ\text{C}$ .

140 Prior to use for PEG grafting, commercial PAH and PEI (of polydispersity indexes  $\text{PDI} = 3.5$  and  
141  $6.0$ , respectively) were fractionated by preparative SEC, affording samples with final  $\text{PDI} = 1.5$   
142 and  $1.9$ , respectively (see Table SI 1, Figure SI 1 to SI 7 for more information).

#### 144 *2.4. Size Exclusion Chromatography coupled to Multi-Angle Laser Light Scattering (SEC-* 145 *MALLS)*

146 SEC-MALLS analyses were carried out on a Thermo Scientific Dionex Ultimate 3000 instrument  
147 (Thermo Fisher Scientific, Waltham, MA, USA) including an auto-sampler & column  
148 compartment unit, connected to a Wyatt MiniDawn Treos three-angle laser light scattering  
149 detector ( $41.5^\circ$ ,  $90^\circ$ , and  $138.5^\circ$ ) operating at  $658 \text{ nm}$  (Wyatt Technology Corp., Santa Barbara,  
150 CA, USA) and to a Shimadzu RID-20A refractometer (Shimadzu Corp., Kyoto, Japan).  
151 Separations were carried out on a  $50 \times 6 \text{ mm}$  OHPak SBG Shodex column guard followed by a  
152  $300 \times 8 \text{ mm}$  SB-805M-HQ Shodex column. The eluent was composed of a mixture of  $50 \text{ mM}$   
153 acetic acid,  $300 \text{ mM}$  sodium nitrate and  $300 \text{ mgL}^{-1}$  sodium azide, pH 3.6. Prior to use, the eluent  
154 was filtered through a  $0.1 \text{ }\mu\text{m}$  cut-off Durapore membrane.  $100 \text{ }\mu\text{L}$  polymer samples (dissolved  
155 at  $1 \text{ gL}^{-1}$  in the eluent) were injected and eluted at a  $1.0 \text{ mLmin}^{-1}$  flow rate. Raw instrument data  
156 were processed using the Astra software (v6.1.1.17, Wyatt Technology Corp.), analyzing the 5–  
157 17 mL elution volume range in all cases.

158 SEC-MALLS analytic results were used to assess PEG/polycation ratios and to quantify the  
159 amount of residual ungrafted mPEG in the grafted polymers (see Sections I.D and I.E in  
160 Supporting Information).

#### 162 *2.5. Incremental refractive index $(dn/dc)_\mu$ determination*

163 Incremental refractive indexes  $(dn/dc)_\mu$  of polymers were measured at constant chemical potential  
164 ( $\mu$ ) on the RID-20A refractometer unit. Mother polymer solutions at  $2.0 \text{ gL}^{-1}$  in SEC eluent were  
165 prepared by weighing the dry polymer and the SEC eluent. Various dilutions of mother polymer  
166 solutions in SEC eluent (final polymer concentrations:  $0.25$ ,  $0.5$ ,  $0.75$ ,  $1.0$ ,  $1.5$ , and  $2.0 \text{ gL}^{-1}$ ) were  
167 then submitted to RID measurement. All molar masses and concentrations used for  $dn/dc$   
168 measurements included the polymer counter-ions (see Figure SI3 to SI7). The values of  $(dn/dc)_\mu$

169 were 0.1887 for  $\epsilon$ PLL, 0.1731 for  $\alpha$ PLL 10%, 0.1837 for  $\alpha$ PLL 20%, 0.2125 for PAH and 0.3004  
170 for PEI.

171

## 172 2.6. PEGylation of PAH by NHS activation

173 HEPES-NaOH buffer pH 7.52 was prepared by mixing HEPES at 30 mM (1.786 g in 250 mL  
174 deionized water) with sodium hydroxide at 15 mM (0.1501 g in 250 mL deionized water). In all  
175 weighing and transfer operations, the intermediate vessel was rinsed with 5 mL buffer to  
176 maximize substrate/reagent recovery into the reaction vessel. In a typical experiment, 32.5 mg  
177 (348.6  $\mu$ mol monomer units) of PAH were dissolved in 5 mL HEPES-NaOH buffer, then the  
178 resulting solution was transferred into a 50 mL flask under vigorous stirring for 5 min (the vial  
179 used for weighing was subsequently rinsed with 5 mL buffer). 40.1 mg (41.8  $\mu$ mol endgroups) of  
180 mPEG-SCM were added portionwise into the PAH solution. The intermediate vial was rinsed  
181 with 5 mL of buffer to transfer the leftover reagent. The flask was closed, and the solution was  
182 stirred overnight. The reaction medium was then dialyzed over 48 h against water (1 kDa cutoff  
183 tubing) to remove ungrafted mPEG, meanwhile the outer water was renewed 7 times. The solution  
184 was then concentrated on a rotatory evaporator and finally freeze-dried overnight. The resulting  
185 solid was then characterized by 400 MHz  $^1$ H NMR (see Figure SI 8 to SI 12) and by SEC-MALLS  
186 (see Figure SI 13 to SI 20). Quantitative data for all examples are gathered in Table SI1.

187

## 188 2.7. Capillary coating procedure

189 Bare fused silica capillary (purchased from Polymicro Technologies (Phoenix, AZ, USA)) was  
190 first activated according to the following procedure: successive flushes (at 96.5 kPa (14.5 psi)) of  
191 NaOH (1 M, 10 min, 1 mL in a 1.5 mL glass vial), pure water (5 min, 1 mL in a 1.5 mL glass  
192 vial) and construction buffer (HEPES 20 mM, NaOH 10 mM, pH 7.4, 10 min, 1 mL in a 1.5 mL  
193 glass vial). A 4-layer SMIL capillary coating, used as a substructure, was obtained by flushing (at  
194 96.5 kPa) with polycation solution (PDADMAC, 3 gL<sup>-1</sup> in HEPES buffer, 7 min, 200  $\mu$ L in  
195 polypropylene vial), HEPES buffer (3 min, 1 mL in a 1.5 mL glass vial) to remove the excess of  
196 polycation, polyanion solution (PSS, 3 gL<sup>-1</sup> in HEPES buffer, 7 min, 200  $\mu$ L in polypropylene  
197 vial), HEPES buffer (3 min, 1 mL in a 1.5 mL glass vial) to remove the excess of polyanion. To  
198 avoid polyelectrolyte contamination, HEPES solutions for PDADMAC and PSS rinsing flushes  
199 were stored in different vials. This procedure was repeated once more to obtain the 4-layer SMIL  
200 substructure. The fifth layer was deposited by flushing (at 96.5 kPa, 7 min, 200  $\mu$ L in  
201 polypropylene vial) with either PDADMAC, PAH, PEI,  $\epsilon$ PLL,  $\alpha$ PLL (either native or PEGylated),  
202 followed by a rinsing step with HEPES buffer (3 min, 1 mL in another 1.5 mL glass vial) and 5

203 min wait. Finally, the coated capillary was flushed (at 96.5 kPa) with pure water (3 min) and  
204 background electrolyte (BGE) (2 M acetic acid, pH 2.2, 10 min) followed by another 5 min wait  
205 before analysis.

206

### 207 *2.8. Separation of intact proteins by capillary zone electrophoresis*

208 Capillary zone electrophoresis experiments were performed on a P/ACE MDQ Beckman Coulter  
209 (Sciex, Villebon-sur-Yvette, France) piloted by 32 Karat software. Analyses were performed on  
210 a 40 cm total length capillary (30 cm effective length), with inner (i.d.) and outer (o.d.) diameters  
211 of 50  $\mu\text{m}$  ( $\pm 0.3 \mu\text{m}$ ) and 363  $\mu\text{m}$  ( $\pm 15 \mu\text{m}$ ), respectively. Electrophoretic separations were  
212 performed in a 2 M acetic acid BGE, pH 2.2 (6.4 mM ionic strength). BGE was flushed (at 96.5  
213 kPa) for 3 min between each run. Separations were performed at different voltages between  $\pm 10$   
214 and  $\pm 20$  kV, with a polarity depending on the capillary surface charge (as indicated on the Figure  
215 caption). DMF (0.001% m/v in the BGE) was hydrodynamically injected as EOF marker at 2.7  
216 kPa (0.4 psi) for 2 s, followed by a protein mixture (0.25  $\text{gL}^{-1}$  each in the BGE) at 2.7 kPa for 4 s.  
217 RNase A, Lyz, Myo and TI were kept separately in pure water (at 2  $\text{gL}^{-1}$ ) in the freezer before  
218 dilution in the BGE for the preparation of the sample mixture. The temperature of the cartridge  
219 was set at 25°C. Detection wavelength was 214 nm.

220

### 221 *2.9. Single channel Quartz Crystal Microbalance with Dissipation (QCM-D)*

222 QCM-D experiments were performed on a X1 Single Channel device (from AWSensors,  
223 Valencia, Spain), using an “in-flow” QCM cell at 23°C, equipped with a 14 mm diameter quartz  
224 sensor coated by  $\text{SiO}_2$  and with a fundamental resonance frequency of 5 MHz. The coating  
225 procedure was performed on bare sensors first activated by  $\text{O}_2$  plasma (5 min,  $P(\text{O}_2) = 0.5$  mbar,  
226 80W). Normalized frequency shifts ( $\Delta f_n/n$  in Hz) and corresponding normalized dissipation shifts  
227 ( $\Delta D_n/n$ ) for the 3<sup>rd</sup>, 5<sup>th</sup>, 7<sup>th</sup> and 9<sup>th</sup> resonance harmonics ( $n$ ) were first measured in air, then  
228 monitored throughout the experiment. Before starting the SMIL buildup, pure water was flushed  
229 at 0.05  $\text{mLmin}^{-1}$  until stabilization of QCM signals, followed by flushing with the construction  
230 buffer (HEPES 20 mM, NaOH 10 mM, pH 7.4) for 20 to 30 min. The film was assembled by  
231 sequential injection of PDADMAC and PSS solutions (at 3  $\text{gL}^{-1}$  in construction buffer) for 30 min  
232 at 0.05  $\text{mLmin}^{-1}$ , starting with PDADMAC as first layer. Rinsing with the construction buffer was  
233 performed for 30 min between each polyelectrolyte adsorption step. Once a 4-layer SMIL  
234 (PDADMAC/PSS)<sub>2</sub> was obtained, a differentiated capping layer based either on  $\epsilon\text{PLL}$  or PAH (at  
235 3  $\text{gL}^{-1}$  in construction buffer) with PEG grafting ratios of 0, 11 and 20 % was deposited for 30  
236 min at 0.05  $\text{mLmin}^{-1}$ . After rinsing with the construction buffer, the SMIL-coated QCM sensor



237 was stabilized in pure water with a 0.05 mLmin<sup>-1</sup> flow rate. Both the normalized resonance  
238 frequency shift ( $\Delta f_n/n$  in Hz; related to the mass deposited on the sensor), and the normalized  
239 dissipation shift ( $\Delta D_n/n$ ; related to the energy dissipated by the resonator in its direct surrounding),  
240 were discussed. High  $\Delta D_n/n$  values and  $\Delta f_n/n$  spreading are characteristics of a viscoelastic  
241 coating, such as hydrated SMIL film structures. Overtones frequency shifts overlap and low  
242 dissipation shift values (i.e., when the final  $\Delta D_n/n$  divided by the final  $\Delta f_n/n$  is equal or lower to  $4$   
243  $\times 10^{-7}$ ) [38] are typical of rigid glassy film structures. In the case of thin rigid films, the mass  
244 deposited on the crystal can be calculated from the Sauerbrey equation [39]:

$$\Delta m = -C \times \Delta f_n/n \quad (2)$$

246 where  $\Delta m$  (in ngcm<sup>-2</sup>) corresponds to the variation of areal mass,  $C$  (in ngcm<sup>-2</sup>Hz<sup>-1</sup>) is the mass-  
247 sensitivity constant that depends on crystal property,  $n$  is the overtone number and  $\Delta f$  is the  
248 frequency shift (in Hz). A typical mass sensitivity constant  $C = -17.7$  ngcm<sup>-2</sup>Hz<sup>-1</sup> value can be  
249 used for a 5 MHz quartz crystal resonator at room temperature.

250 The film buildup kinetics was evaluated by plotting the values of  $\Delta f_3/3$  and  $\Delta D_3/3$  for each layer  
251 after HEPES rinsing, as well as the corresponding calculated areal masses deposited on the crystal  
252 according to Saurebrey's model. Determination of the film thickness was performed using the  
253 QTM software, taking account of all frequency overtones and approximating the SMIL mass  
254 density as  $\rho = 1$  gcm<sup>-3</sup> [40].

### 256 2.10. Atomic Force Microscopy (AFM)

257 Activation and coating of QCM-D sensor were realized as described in Section 2.9. QCM-D  
258 sensors were prepared overnight before AFM analysis and kept in water to avoid SMIL retraction.  
259 Atomic force microscope measurements were performed using a Multimode 8 Instrument  
260 controlled by Nanoscope 5 electronic software. Peak Force Tapping mode cantilevers  
261 (Nanosensors) with scanasyst fluid spring (constant near 0.7 N/m) were used for the imaging at  
262 approximately 60 kHz in air and water. Images were analyzed using Gwydion software.

## 265 3. Results and discussion

266 In this work, a series of cationic polyelectrolytes, namely PAH, PEI,  $\epsilon$ PLL or  $\alpha$ PLL, were grafted  
267 with PEG (1kDa or 5 kDa) at different molar proportions. These polycations were selected  
268 because they contain primary amine groups that can be derivatized with lateral PEG chains. PAH,  
269 PEI,  $\epsilon$ PLL were derivatized using the NHS / primary amine reactivity, while  $\alpha$ PLL were

270 derivatized using a click-chemistry approach. The PEG grafted polycations are named  
271 “Polycation-g-(mPEG<sub>y</sub>)<sub>x</sub>” where  $y$  corresponds to the mPEG length (1kDa or 5 kDa) and  $x$  is the  
272 calculated PEG/polycation molar ratio. Various PEGylation molar ratios were achieved up to  
273 20%. All details about the synthesis, chemical structure and characterization, are given in the  
274 experimental sections 2.2 to 2.6 and in the SI (see Table SI 1 and subsequent sections in  
275 Supporting Information).

276 Non-PEGylated and PEGylated PAH, PEI,  $\epsilon$ PLL or  $\alpha$ PLL were used as a last layer deposited on  
277 a 4-layer PDADMAC/PSS SMIL sub-structure for capillary coatings, as depicted in Figure 1. The  
278 PDADMAC / PSS system was retained as a suitable sub-structure since it was described as a  
279 prime SMIL coating for CE applications [35]. To study the impact of the last polycationic layer  
280 on separation performances in CE, a mixture of 4 model intact proteins (Myo, RNase A, Lyz, TI  
281 at 0.25 gL<sup>-1</sup> each in 2 M acetic acid, pH 2.2) was selected.

282

### 283 3.1. Influence of the polycation nature on the last layer on the $H=f(u)$ curve and resolution

284 Before studying the impact of the PEGylation of the polycationic last layer on the CE separation  
285 performances, the 5-layer SMIL coating terminating with the non-PEGylated PDADMAC, PAH,  
286 PEI,  $\epsilon$ PLL or  $\alpha$ PLL were first compared. The electropherograms (five repetitions) obtained for  
287 the separation of the protein mixture at different separation voltages (i.e., -20 kV, -17.5kV, -15kV,  
288 -12.5kV and -10 kV, with typical electric currents of 18.1  $\mu$ A, 15.7  $\mu$ A, 13.4  $\mu$ A, 11.0  $\mu$ A and 8.8  
289  $\mu$ A, respectively) are shown in Supporting Information (see Figure SI 21 to Figure SI 25). An  
290 example of electropherograms, repetitions obtained at -10 kV is displayed in Figure 2A. Excellent  
291 repeatabilities were generally obtained as demonstrated by the very low RSD <sub>$t_m$</sub>  (typically lower  
292 than 0.6%) on migration times at -10 kV and -20 kV (see Table 1 for numerical data). The  
293 repeatability followed this ranking: PEI > PDADMAC ~ PAH >  $\alpha$ PLL ~  $\epsilon$ PLL. Lower stability  
294 was observed for PLL, and especially for  $\epsilon$ PLL at -10 kV. To get more information about the  
295 performances of the capillary coatings, the  $H=f(u)$  plots were systematically investigated by  
296 measuring the separation efficiency (plate height) at different separations voltages. Figure 2B  
297 shows typical  $H=f(u)$  plots in the case of Myo for all the polycations tested as a last layer (see  
298 Figure SI 26 for the experimental plots on TI, RNase A and Lyz). Using equation (1), all the  
299  $H=f(u)$  plots were fitted taking  $k$  and  $A$  as variable parameters and by neglecting the third term in  
300 the right-hand side of the equation which is related to the resistance in mass transfer in stationary  
301 phase. Table 1 gathers the numerical adjusted data for  $k$  and  $A$  for the 4 proteins. From Figure 2A,  
302 it clearly appears that PAH, and to a lower extend PDAMAC are the best coatings leading to the

303 lower  $H$  values (see Figure SI 26). This conclusion is also confirmed by the ranking based on the  
 304  $k$  values (see Table 1): PDADMAC ( $6.5 \times 10^{-2}$ )  $\sim$  PAH ( $6.9 \times 10^{-2}$ )  $<$  PEI ( $7.3 \times 10^{-2}$ )  $<$   $\alpha$ PLL ( $7.5$   
 305  $\times 10^{-2}$ ). Regarding  $\epsilon$ PLL, poor curve fitting was obtained together with much lower repeatabilities  
 306 on separation efficiency (see larger error bars in Figure 2B) which can be considered as a signature  
 307 of a lower stability of the coating. This finding can be explained by the low polymer molar mass  
 308 (ca.  $4000 \text{ g mol}^{-1}$ ) of  $\epsilon$ PLL compared to that of the other polymers (10 to 100 times longer chains)  
 309 that generated less robust SMIL coatings. The low  $k$  value obtained for  $\epsilon$ PLL ( $3.8 \times 10^{-2}$ ) should  
 310 be balanced by the much higher  $A$  value ( $6.9 \text{ }\mu\text{m}$ ). The  $A$  value is supposed to incorporate all  
 311 constant extra-column effects but the contribution to the  $A$  value in regard to the coating properties  
 312 remains unclear.

313

314 SMIL coatings induced differences in the electroosmotic mobility and thus migration time.  
 315 Therefore, the impact of the last polycationic layer was investigated on peak resolution ( $R_s$ ) based  
 316 on the Myo / RNase A pair (see Figure 2C).  $R_s$  depends both on the migration times and the  
 317 separation efficiency, as shown in equation 3:

$$318 \quad R_s = \frac{1}{4} \times \frac{\Delta\mu_{ep}}{\langle \mu_{app} \rangle} \times \sqrt{N} \quad (3)$$

319 where  $\langle \mu_{app} \rangle$  is the average apparent electrophoretic mobility of the two analytes (in TU),  $\Delta\mu_{ep}$   
 320 is the difference in effective electrophoretic mobility between the two analytes and  $N$  is the  
 321 average plate number. The highest  $R_s$  values were obtained with the PAH polycation followed by  
 322 PDADMAC or  $\epsilon$ PLL (see Figure 2C). Despite the lowest separation efficiencies obtained with  
 323  $\epsilon$ PLL, the resolution loss due to higher peak broadening was balanced by the decrease of the  
 324 electroosmotic flow ( $\mu_{eo}(\epsilon\text{PLL}) = -40.7 \text{ TU}$  vs  $\mu_{eo}(\text{PDADMAC}) = -45.5 \text{ TU}$ ) which increased the  
 325 apparent selectivity and migration times. A diminution of the resolution was observed for all the  
 326 coatings when the applied voltage increased (increasing  $u$ ) since the experimental data are located  
 327 in the ascending part of the  $H=f(u)$  curve. As a conclusion of this first part, it seems that PAH is  
 328 the best polycation for the last layer, and therefore the best candidate for subsequent PEGylation.

329

### 330 3.2. Investigating SMIL growth by QCM-D

331 The SMIL film growth was investigated by QCM-D. Figure 3 shows the dissipation and frequency  
 332 shifts of the 3<sup>rd</sup> harmonic during the SMIL construction. Since films terminated by PEI and  $\alpha$ PLL

333 presented similar behavior as PAH, but with lower analytical efficiency, only films terminated by  
334 PAH were investigated by QCM-D. Frequency shifts were measured at each injection step of  
335 oppositely charged polyelectrolyte (see Section 3 in Supporting Information), signaling the  
336 adsorption of the next polyelectrolyte layer by charge overcompensation promoting [30]. The first  
337 increment corresponds to PDADMAC (at 3 gL<sup>-1</sup> in 20 mM HEPES) adsorption onto the bare  
338 sensor, followed by rinsing with 20 mM HEPES. Frequency and dissipation shift values of the  
339 first step were larger than those of following steps since the adsorption onto the bare QCM sensor  
340 is different than onto polyelectrolytes. Average PDADMAC and PSS shift in frequency were 9  
341 Hz and 2 Hz per step, respectively, showing growth of very thin film. As observed in Figure SI  
342 32, low final frequency (-27 Hz) and dissipation shift ( $3.4 \times 10^{-7}$ ) for the (PDADMAC/PSS)<sub>2</sub>  
343 SMIL indicated thin films, while overlapping overtones indicated rigid properties. Due to their  
344 high linear charge density, PDADMAC and PSS multilayered films are known to form thin and  
345 rigid films in low ionic strength conditions (typically 15 mM NaCl [41]). As the Sauerbrey  
346 equation is applicable for thin rigid films (see Section 2.9, eq. 2), an approximated deposited areal  
347 mass was calculated for the (PDADMAC/PSS)<sub>2</sub> SMIL coating: a frequency shift of  $\approx -26.98$  Hz  
348 was found, leading to 478 ngcm<sup>-2</sup> deposited areal mass (corresponding to a film thickness of 4.5  
349 nm), as presented in Table 2.

350 Next, the influence of the last deposited cationic layer on the film growth was investigated. εPLL  
351 deposition showed the smallest final frequency shift and final dissipation, corresponding to 98  
352 ngcm<sup>-2</sup> deposited areal mass. In the case of PDADMAC, the deposited areal mass was higher (121  
353 ngcm<sup>-2</sup>). In contrast, the deposition of PAH as a last layer led to harmonic differentiation and a  
354 concomitant increase in dissipation values (Figure SI 33), indicating a change in the viscoelastic  
355 properties of the film, attributed to a more hydrated structure. Yet, the ratio of final  $\Delta f_3/3$  divided  
356 by final  $\Delta D_3/3$  was around  $1.5 \times 10^{-7}$ , indicating that the Sauerbrey equation was still valid for  
357 this film, with a calculated deposited areal mass of 301 ngcm<sup>-2</sup>. For all systems, and with the film  
358 volumic mass ( $\rho$ ) approximated to 1 gcm<sup>-3</sup>, the thickness was determined using QTM software as  
359 about 7 nm (see exact values in Table 2).

360 AFM analyses were performed on bare and (PDADMAC/PSS)<sub>2.5</sub> SMIL coated QCM-D sensor in  
361 air and water medium to characterize SMIL covering. As shown in Figure SI 37, no significant  
362 impact was observed in the case of bare sensor when analysis was performed either in air  
363 (roughness of 0.9 nm) or in water (roughness of 1.1 nm). Comparing with the SMIL coated sensor  
364 in water (Figure SI 38), an increase of 2.3 nm in rugosity was observed, related to the presence of  
365 the SMIL at the surface. Finally, AFM experiment was performed on the same QCM-D sensor,

366 after complete evaporation of water (Figure SI 39). Bare silica (dark area) was observed on the  
367 left of the picture, while the dried SMIL coating remained on the right. The in-between light zone  
368 corresponds to SMIL retraction. To measure SMIL height, topographic measurement was  
369 performed on the bare silica zone and on the SMIL coating zone, leading to average  $8.7 \pm 3.8$  nm  
370 and  $7.2 \pm 5.0$  nm differences (see Table SI2), in agreement with the value obtained by QCM-D  
371 experiment (6.8 nm).

372 To conclude, with average film thickness of around 7 nm and final dissipation below  $7 \times 10^{-6}$ , all  
373 films showed rigid properties. PDADMAC and  $\epsilon$ PLL yielded thinner film coating, with lower  
374 deposited areal mass and dissipation shift than PAH. The glassy nature of these films contributes  
375 to refraining any diffusion of proteins within the multilayer, while the SMIL's polycationic last  
376 layer grants antifouling properties, as experimented in CE (Figure 2). However, the low molar  
377 mass of  $\epsilon$ PLL led to less robust films and thus to lower repeatability in CE measurements [36,  
378 42]. When PAH was used as the last layer, higher dissipation shift and higher deposited masses  
379 were observed, but similar or slightly higher CE performances were obtained compared to  
380 PDADMAC.

381 Due to thin film growth, rigid behavior, excellent analytical properties and ease of  
382 functionalization, PAH was selected as the capping layer reference. In the following, PEGylation  
383 of PAH, which is known to improve antifouling properties [43-52], was investigated, and the  
384 impact of PEGylation on electroosmotic mobility and CE separations was discussed.

### 385 386 *3.3. Influence of the PEGylation density and length on electroosmotic flow mobility ( $\mu_{eo}$ )*

387 PAH was grafted with PEG chains of two different lengths (1 kDa and 5 kDa) at various ratios  
388 (see Section 2.6 for polymer synthesis). PEGylated PAH were prepared from 1 kDa PEG chains  
389 (targeting 10% and 20% grafting ratios) and from 5 kDa PEG chains (targeting 1%, 2%, 3.5%,  
390 5%, 7.5%, 10%, 20% and 30% grafting ratios). Effective % of PEGylation was measured by  $^1\text{H}$   
391 NMR or SEC/MALLS, as described in Sections 2.2 and 2.4, and results were gathered in Table  
392 SI 1.

393 As shown in Figure 4, no influence of the PEGylation of the PAH last layer on the EOF was  
394 observed for 1 kDa PEG lateral chains:  $\mu_{eo}$  was found stable at  $\approx -47$  TU, even at 23.5% grafting  
395 proportion. In contrast, in the case of PAH grafted with 5 kDa mPEG, PEGylation strongly  
396 impacted the EOF, even at the lowest PEG ratio. This is in good agreement with the hydrodynamic  
397 diameter of a 5kDa PEG ( $\approx 6$  nm [53]) which is higher than the Debye length (4 nm at  $I = 6.4$  mM

398 ionic strength, 2 M acetic acid BGE), while the typical size of 1kDa PEG chain (around 1 nm  
399 [53]) is significantly smaller than the Debye length. The impact of PEGylation on the EOF is  
400 based on the hydrodynamic screening of the charges contained in the double layer by the PEG  
401 chains. This hydrodynamic screening is only effective when the PEG dimension is larger than the  
402 Debye length in the experimental conditions.

403 Since the electrophoretic mobility ( $\mu_{ep}$ ) of the four proteins tested were 23.7 TU for TI, 27.2 TU  
404 for Myo, 28.2 TU for RNase and 31.6 TU for Lyz, the electrical polarity must be reversed from  
405 5kDa mPEG PAH with grafting ratio equal or higher than 5%. This leads to a complete inversion  
406 of the protein migration order. A plateau was reached when PEGylation density was higher than  
407 11%, where essentially a neutral coating behavior was observed ( $\mu_{eo} \approx -2$  TU). PEGylation was  
408 also performed on PEI and similar impact on EOF was observed (see Figure 4), but with a faster  
409 EOF decrease with the grafting density of the 5 kDa mPEG.

410

#### 411 3.4. Impact of the PEGylation of PAH on $k$ and $R_s$

412 Figure 5A shows the impact of PEGylation for the PAH grafted with 1kDa PEG chains in the last  
413 layer. A slight reduction of  $\mu_{eo}$  was observed ( $\approx -41.5$  TU). However, intra-capillary repeatability  
414 was lower (see  $RSD_{tm}$  values in Table 3). In contrast, little impurity peaks around the Lyz peak  
415 were detected in the case of PAH-g-(mPEG<sub>1kDa</sub>)<sub>0.235</sub> in the last layer unlike PAH, due to better  
416 selectivity due to the slight EOF reduction.

417 Figure 5B shows the impact of PEGylation for the PAH grafted with 5kDa PEG chains in the last  
418 layer. Interestingly, SMIL with PAH-g-(mPEG<sub>5kDa</sub>)<sub>0.021</sub> gave similar  $\mu_{eo}$  than PAH-g-  
419 (mPEG<sub>1kDa</sub>)<sub>0.138</sub> and <sub>0.235</sub>, but with better repeatability. SMIL with PAH-g-(mPEG<sub>5kDa</sub>)<sub>0.11</sub> showed a  
420 quasi-neutral behavior coated capillary with  $\mu_{eo}$  at  $\approx -5.5$  TU. The order of detection of the proteins  
421 was thus reversed using positive polarity, and the analysis time was drastically reduced (from 12  
422 min for the non-PEGylated PAH to only 7 min for the PAH-g-(mPEG<sub>5kDa</sub>)<sub>0.11</sub>) in the last layer.

423 Regarding the impact of PEGylation on  $H$ , 1kDa PEGylated PAH led to lower average  $k$  values  
424 but slightly higher  $A$  values compared to the unmodified PAH, as shown for instance in Figure  
425 6A for Myo, Figure 6B for Lyz and Table 3. Average  $k$  value (tested on the four proteins) was  $7$   
426  $\times 10^{-2}$ ,  $4.4 \times 10^{-2}$  and  $6.05 \times 10^{-2}$  for PAH, (PAH-g-(mPEG<sub>1kDa</sub>)<sub>0.138</sub>)<sub>1</sub> and (PAH-g-  
427 (mPEG<sub>1kDa</sub>)<sub>0.235</sub>)<sub>1</sub>, respectively. Average  $A$  values changed from 1.9  $\mu\text{m}$  for PAH to 4.4  $\mu\text{m}$  for  
428 13.8% mPEG grafted PAH and 3.3  $\mu\text{m}$  for 23.5% mPEG grafted PAH. On the whole, the  
429 separation efficiencies were very similar to those obtained with unmodified PAH, but the

430 resolution was significantly higher with 1kDa PEGylation (see Figure 6C), especially at high  
431 voltage due to flatter  $H=f(u)$  curve and slightly lower EOF.

432 Increasing mPEG length from 1kDa to 5kDa was first detrimental on the average  $k$  value at  
433 intermediate grafting ratio ( $k = 9.4 \times 10^{-2}$  for 2.1% PEG) but reached similar value ( $k = 4.8 \times 10^{-2}$ )  
434 for higher ratios (11% PEG). Higher average  $A$  values were obtained, i.e. 4.65  $\mu\text{m}$  at 2.1% and  
435 5.3  $\mu\text{m}$  at 11%. This non-linear effect of PEGylation on both the slope ( $k$ ) and the constant value  
436  $A$  of the  $H=f(u)$  curve may be not only related to residual protein adsorption. The coating  
437 homogeneity may also affect the characteristics of  $H=f(u)$  curve.

438 On the whole, the PEGylation of the PAH last layer was found to lead to much flatter  $H=f(u)$   
439 curve, except at intermediate 5 kDa grafting ratio (2.1%). Therefore, the resolution was much less  
440 affected by increasing voltages on PEGylated SMIL compared to the non-PEGylated PAH last  
441 layer (see the variation of the resolution between Myo and RNase A with the voltage on Figure  
442 6 C).

443 Figure 7 compares the  $(\text{PDADMAC/PSS})_2\text{-(PAH)}_1$  SMIL building to two other films obtained  
444 with PEGylated PAH as the last layer. The PAH backbone interacts electrostatically with the last  
445 PSS layer, while neutral mPEG pendant chains in PEGylated PAH could extend at the SMIL/BGE  
446 interface. As the last layer, PAH (unmodified or PEGylated) led to films with slightly less rigid  
447 behavior and frequency overtone spreading (Figures 3 and SI 33) compared to PDADMAC last  
448 layered SMIL. When adsorbing PEGylated PAH chains on SMIL, two adverse effects can be  
449 inferred: on the one hand the molar mass of each polycationic chain is significantly increased by  
450 grafted neutral pendant chains, leading to thicker films and larger mass deposition. On the other  
451 hand, the charge density of grafted PAH is decreased while steric hindrance increases, which can  
452 limit polymer chain adsorption and lead to thinner films. When comparing SMILs capped by  
453 either unmodified PAH or 5kDa PEGylated PAH (11% grafting ratio) as the last layer, the latter  
454 exhibited a larger frequency shift concomitant with a larger overtone spread (Figure SI35);  
455 however similar dissipation values were observed in both cases. In contrast, the frequency shift,  
456 overtone spreading (Figure SI 36) and dissipation values, were similar or inferior to that of PAH  
457 when 1 kDa PEGylated PAH (20% grafting rate) were adsorbed as a capping layer. These results  
458 indicate that thicker films are promoted by PAH bearing longer pendant chains with a moderate  
459 grafting degree while thinner films are formed with shorter pendant chains and higher grafting  
460 degree. Since the final dissipation and frequency shifts obtained for all films remained in the rigid  
461 domain, the Sauerbrey equation was applied to estimate deposited areal masses and the  
462 corresponding thicknesses were calculated (Table 4). While films capped with PAH and 1 kDa

463 PEGylated PAH chains (23% grafting rate) reached similar thicknesses ( $\approx 7$  nm), demonstrating  
464 that less chain adsorption had occurred with the latter, the SMIL capped with 5 kDa PEGylated  
465 PAH (11% grafting rate) exhibited a deposited areal mass 50 % higher, leading to a  $\approx 11$  nm thick  
466 film. In that case, the thickness of the last deposited layer accounts thus for around 7 nm, a result  
467 that confirms that the mPEG impact on EOF was observed for PEG chains longer than the Debye  
468 length (Figure 4). Such a significant increase in the film thickness when 5 kDa PEGylated PAH  
469 (11% grafting rate) was deposited, suggests that mPEG chains adopt a non-collapsed  
470 conformation on top of the coating. Combination of the rigid film structure of (PDADMAC/PSS)<sub>2</sub>,  
471 known for its high stability, robustness and analytical separation efficiency [3, 4, 36], with a  
472 slightly hydrated polycation in the last layer where the water thin layer reduced adhesion or  
473 adsorption phenomena [54-56], is thus key to coatings with antifouling properties.

474

#### 475 **4. Conclusion**

476 The first step of this work was to investigate the impact of the last polycationic layer on separation  
477 performances in CE using SMIL coated capillaries. For that purpose, five different  
478 polyelectrolytes were tested on a (PDADMAC/PSS)<sub>2</sub> SMIL coating, namely PDADMAC, PAH,  
479 PEI,  $\epsilon$ PLL and  $\alpha$ PLL. With similar properties as PDADMAC, PAH was found the best candidate  
480 to investigate the impact of PEGylation in the last layer. 5 kDa PEGylation generated reduced  
481 EOF, even at low PEG ratios (typically between 1 and 5 % grafting), while the impact of 1 kDa  
482 PEGylation on EOF was much lower even at high grafting rates (i.e. 10-25%), with a decrease of  
483 the electroosmotic mobility by only about 5%. This finding can be explained by the hydrodynamic  
484 screening of the PEG chains in the electrical double layer by sufficiently long PEG chains. An  
485 electroosmotic mobility plateau was reached for 5 kDa PEG chains, when the PEGylation rate  
486 was above 11%. The SMIL coating then adopts a neutral behavior with very low electroosmotic  
487 mobility ( $\sim 2$  TU) and a reverse migration order of detection of the proteins.

488 Regarding the analytical behavior of PEGylated last layered SMIL, flatter  $H=f(u)$  curves were  
489 generally obtained leading to lower  $k$  values in agreement with antifouling properties. However,  
490 a slight increase of the  $A$  values was observed, for unknown reason. On the whole, a lower impact  
491 of increasing applied separation voltage (electric field strength) on the separation performances  
492 could be obtained using the PEGylated SMIL. The use of (PDADMAC/PSS)<sub>2</sub>-(PAH-g-  
493 (mPEG<sub>5kDa</sub>)<sub>0.11</sub>)<sub>1</sub> SMIL coating is a very convenient way to build a performant and almost neutral  
494 coating, which is not possible to obtain with classical SMIL coatings terminating with positive or  
495 negative surface charges. In conclusion, SMILs with PEGylated polycations as the last layer can



496 be used to improve the CE resolution via EOF modulation without compromising the separation  
497 efficiency; or as an alternative to neutral coatings for 5kDa PEGylated last layer. As a perspective,  
498 it would be interesting to study and to better understand the contribution coming from capillary  
499 coatings in the constant term of the  $H=f(u)$  curve.

500

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505

506

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## 672 **Captions to Figures and Tables:**

673

674 **Figure 1. Schematic representation of SMIL coatings used in this work based on (PDADMAC/PSS)<sub>2</sub>**  
675 **substructure.** The last polycationic layer can be composed of unmodified PDADMAC, PAH, PEI, εPLL  
676 and αPLL, or their respective PEGylated chains with different chain lengths.

677 **Figure 2. Impact of the nature of the last layer on protein separation at -10kV (A), on the  $H=f(u)$**   
678 **curve in the case of Myo (B) and on the resolution of the Myo / RNase A pair (C).** Experimental  
679 conditions: (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL coated capillary, 40 cm total length (30 cm to  
680 detector) × 50 μm i.d. BGE: 2 M acetic acid, pH 2.2. Hydrodynamic injection of proteins at 0.25 gL<sup>-1</sup> each  
681 in BGE: 2.7 kPa, 4s (0.61% V<sub>tot</sub>). Co-injection of 0.001% DMF in BGE (w/w): 2.7 kPa, 0.4s. Protein  
682 mixture: (1) TI, (2) Myo, (3) RNase A and (4) Lyz. Applied voltages: -10kV, -12.5kV, -15kV, -17.5kV  
683 and -20kV. Temperature: 25°C. See Section 2.7 for the coating procedure.

684 **Figure 3. Evolution of frequency (continuous lines) and dissipation (dashed lines) shifts for the 3<sup>rd</sup>**  
685 **harmonic during SMIL construction procedure in QCMD.** Experimental conditions:  
686 (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL deposited on a silica-coated QCM sensor. Hydrodynamic flow:  
687 0.05 mLmin<sup>-1</sup>. Temperature: 23°C. Average (PDADMAC/PSS)<sub>2</sub> layers construction in grey. See Section  
688 2.9 for the coating procedure.

689 **Figure 4. Impact of the PAH and PEI PEGylated polycation last layer on the electroosmotic flow.**  
690 Experimental conditions: (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL capillary coated, 40 cm total length  
691 capillary (30 cm to detector) × 50 μm i.d. BGE: 2 M acetic acid (pH 2.2). Hydrodynamic injection: 2.7  
692 kPa, 4s. Co-injection of 0.002% DMF water (w/w). Protein mixture: (1) TI, (2) Myo, (3) RNase A and (4)  
693 Lyz, at 0.25 gL<sup>-1</sup> each in BGE. Temperature: 25°C. See Section 2.7 for the coating procedure.

694 **Figure 5. Impact of PEGylation of the last PAH layer with 1kDa PEG (A) and 5kDa PEG (B).** Same  
695 experimental conditions as in Figure 2. Reversed polarity was used for (PDADMAC/PSS)<sub>2</sub>-(PAH-g-  
696 (mPEG<sub>5kDa</sub>)<sub>0.11</sub>)<sub>1</sub>.

697 **Figure 6. Impact of the PEGylation for the last PAH layer on Myo separation efficiency (A), Lyz**  
698 **separation efficiency (B) and peak resolution for the Myo / RNase A pair (C).** Same experimental  
699 conditions as in Figure 2. Reversed polarity was used for (PDADMAC/PSS)<sub>2</sub>-(PAH-g-(mPEG<sub>5kDa</sub>)<sub>0.11</sub>)<sub>1</sub>.  
700 See Figure SI 31 for impact on TI and RNase A.

701 **Figure 7. Evolution of shift frequency (continuous lines) and shift dissipation (dashed lines) for the**  
702 **3<sup>rd</sup> harmonic during SMIL construction procedure in QCMD.** Experimental conditions: see Figure 3.  
703 See detailed construction in supporting information Figure SI 32 to SI 34.

704

705 **Table 1. Impact of the chemical nature of the last polycationic layer on protein retention factor (*k*)**  
706 **and on RSD on migration times at -10kV and -20kV.** Experimental conditions: see Figure 2.

707 **Table 2. Impact of last polycationic layer on SMIL construction and characteristics.** Experimental  
708 conditions: see Figure 3.

709 **Table 3. Impact of PEGylated polycation in the last layer on protein retention factor (*k*) and RSD<sub>*t<sub>m</sub>*</sub>**  
710 **at -10kV and -20kV.** Corresponding electropherograms and  $H=f(u)$  graphs are available in Supporting  
711 Information, from Figures SI 27 to Figure SI 30.

712 **Table 4. Impact of the last polycationic layer on SMIL construction.** Experimental conditions: see  
713 Figure 3.

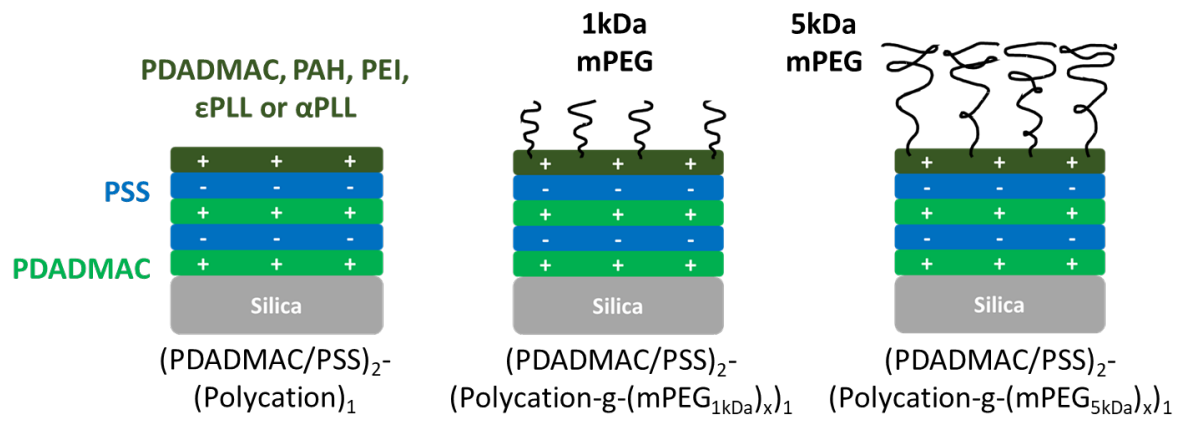
714

# Figures and Tables



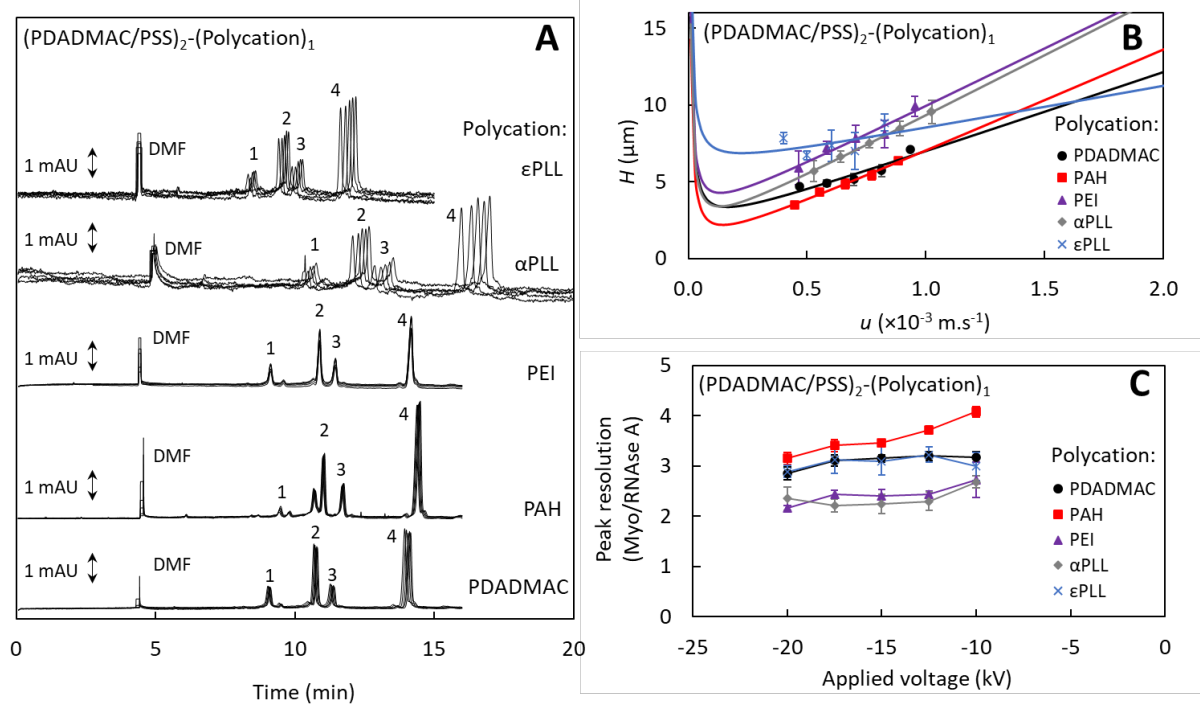
# Figure 1

**Figure 2. Schematic representation of SMIL coatings used in this work based on (PDADMAC/PSS)<sub>2</sub> substructure.** The last polycationic layer can be composed of unmodified PDADMAC, PAH, PEI, εPLL and αPLL, or their respective PEGylated chains with different chain lengths.



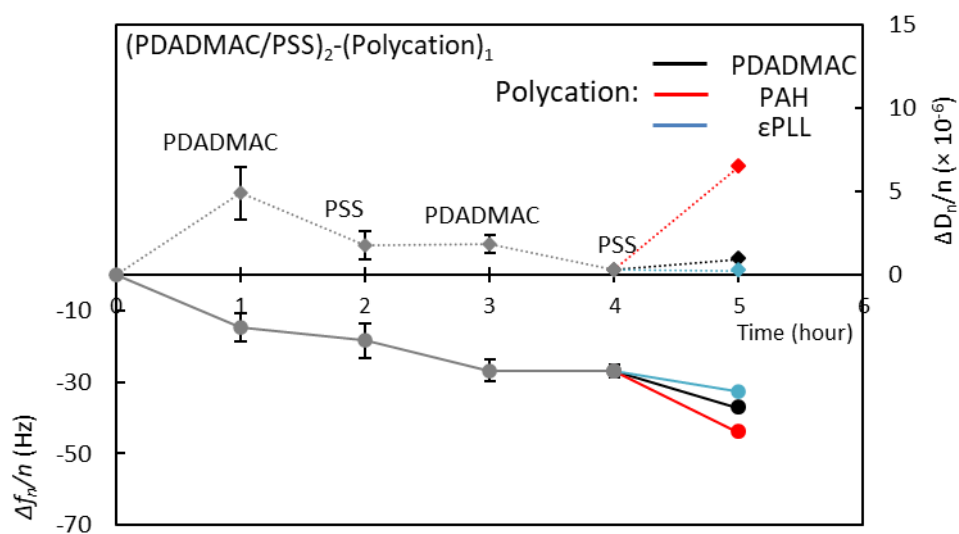
## Figure 2

**Figure 2. Impact of the nature of the last layer on protein separation at -10kV (A), on the  $H=f(u)$  curve in the case of Myo (B) and on the resolution of the Myo / RNase A pair (C).** Experimental conditions: (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL coated capillary, 40 cm total length (30 cm to detector) × 50 μm i.d. BGE: 2 M acetic acid, pH 2.2. Hydrodynamic injection of proteins at 0.25 gL<sup>-1</sup> each in BGE: 2.7 kPa, 4s (0.61% V<sub>tot</sub>). Co-injection of 0.001% DMF in BGE (w/w): 2.7 kPa, 0.4s. Protein mixture: (1) TI, (2) Myo, (3) RNase A and (4) Lyz. Applied voltages: -10kV, -12.5kV, -15kV, -17.5kV and -20kV. Temperature: 25°C. See Section 2.7 for the coating procedure.



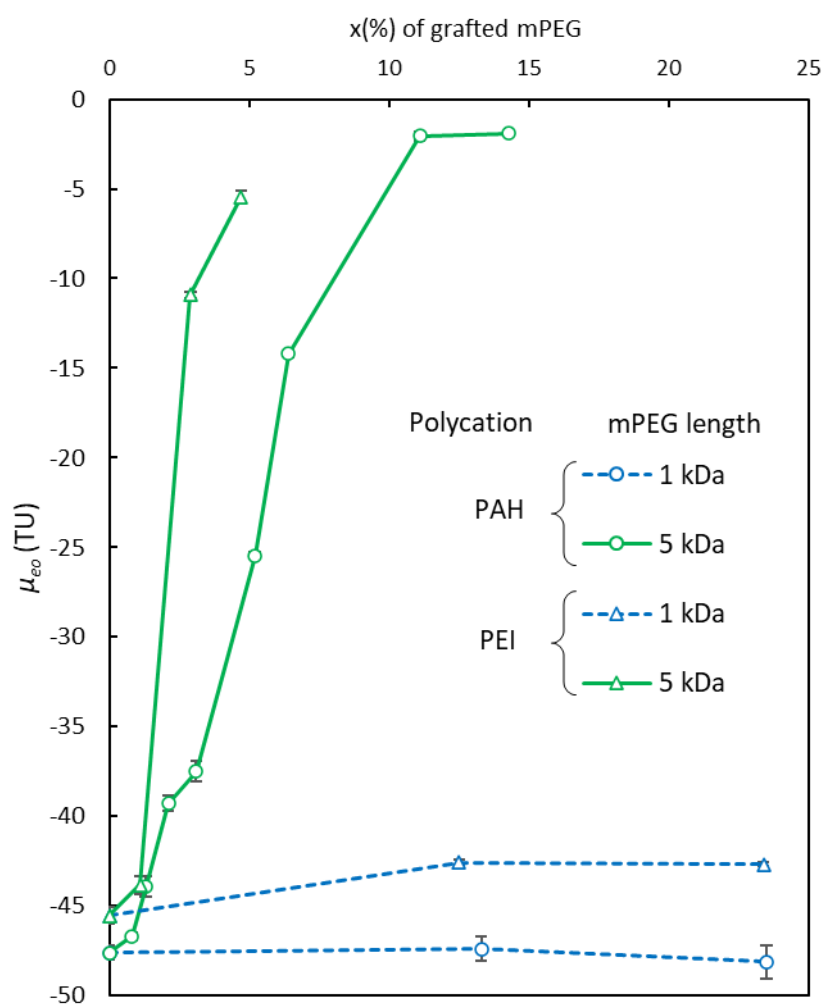
## Figure 3

**Figure 3. Evolution of frequency (continuous lines) and dissipation (dashed lines) shifts for the 3<sup>rd</sup> harmonic during SMIL construction procedure in QCMD.** Experimental conditions: (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL deposited on a silica-coated QCM sensor. Hydrodynamic flow: 0.05 mLmin<sup>-1</sup>. Temperature: 23°C. Average (PDADMAC/PSS)<sub>2</sub> layers construction in grey. See Section 2.9 for the coating procedure.



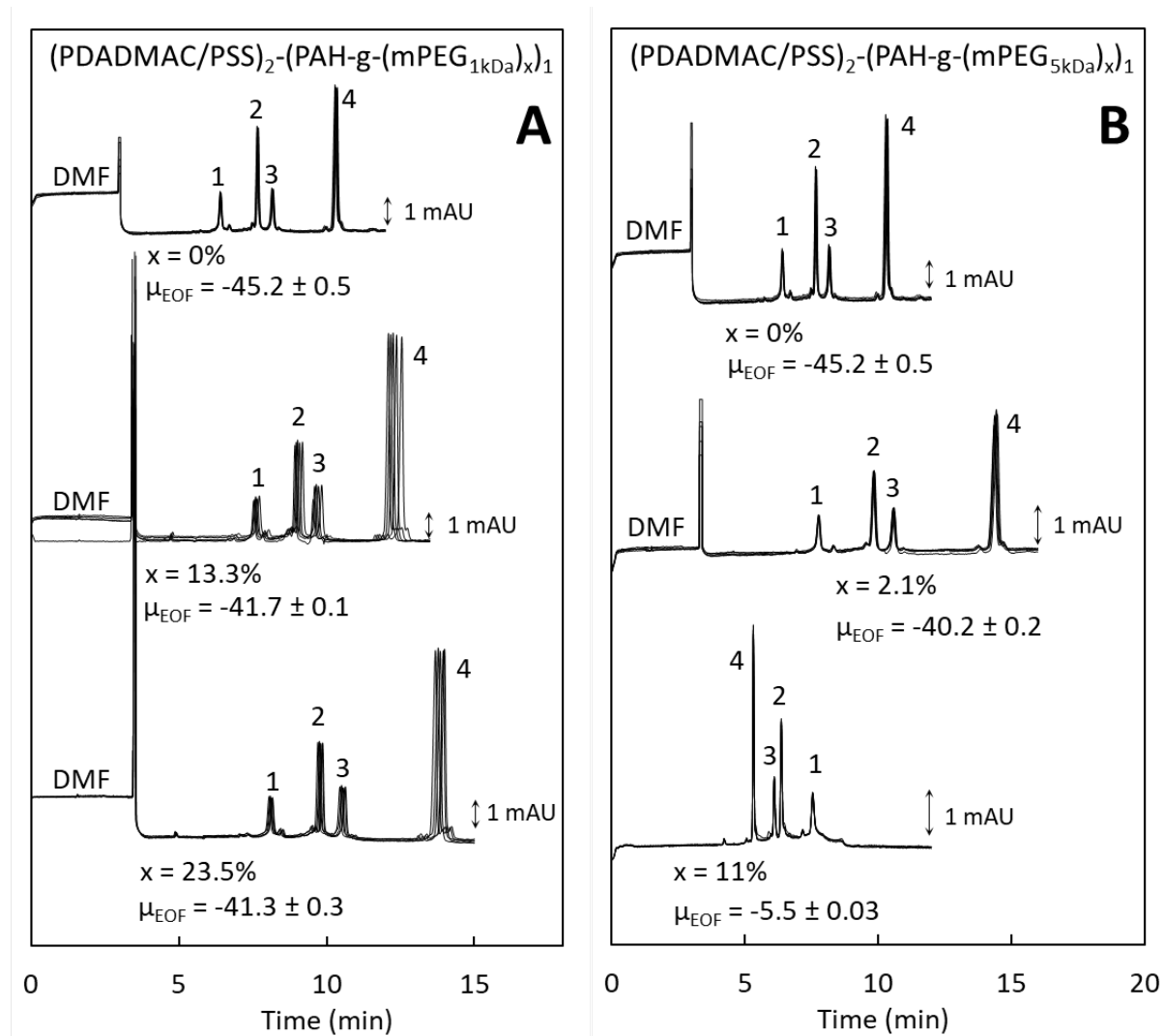
## Figure 4

**Figure 4. Impact of the PAH and PEI PEGylated polycation last layer on the electroosmotic flow.** Experimental conditions: (PDADMAC/PSS)<sub>2</sub>-(Polycation)<sub>1</sub> SMIL capillary coated, 40 cm total length capillary (30 cm to detector) × 50 μm i.d. BGE: 2 M acetic acid (pH 2.2). Hydrodynamic injection: 2.7 kPa, 4s. Co-injection of 0.002% DMF water (w/w). Protein mixture: (1) TI, (2) Myo, (3) RNase A and (4) Lyz, at 0.25 gL<sup>-1</sup> each in BGE. Temperature: 25°C. See Section 2.7 for the coating procedure.



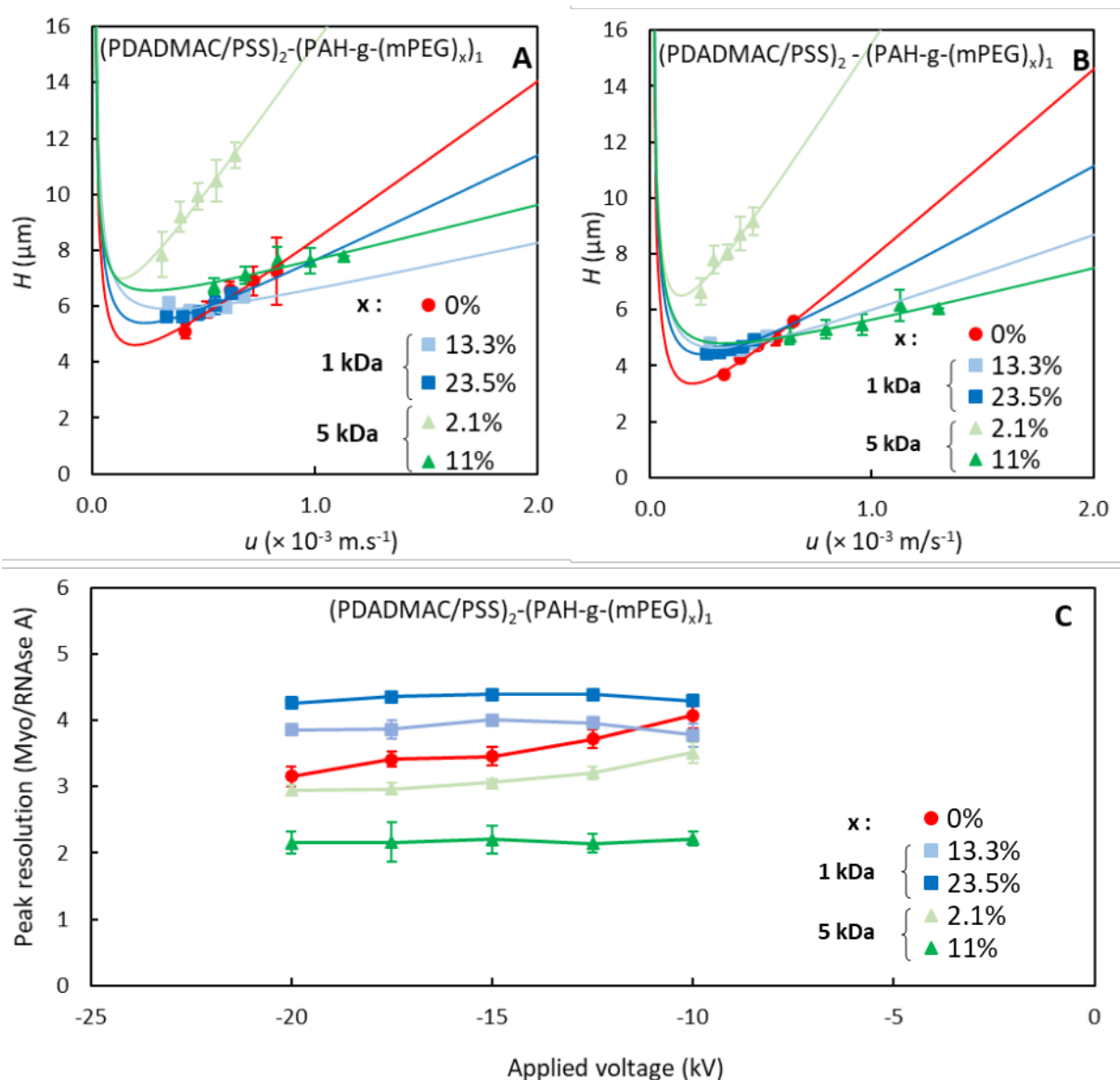
## Figure 5

**Figure 5. Impact of PEGylation of the last PAH layer with 1kDa PEG (A) and 5kDa PEG (B).** Same experimental conditions as in Figure 2. Reversed polarity was used for  $(\text{PDADMAC/PSS})_2\text{-(PAH-g-(mPEG}_{5\text{kDa}})_x)_1$ .



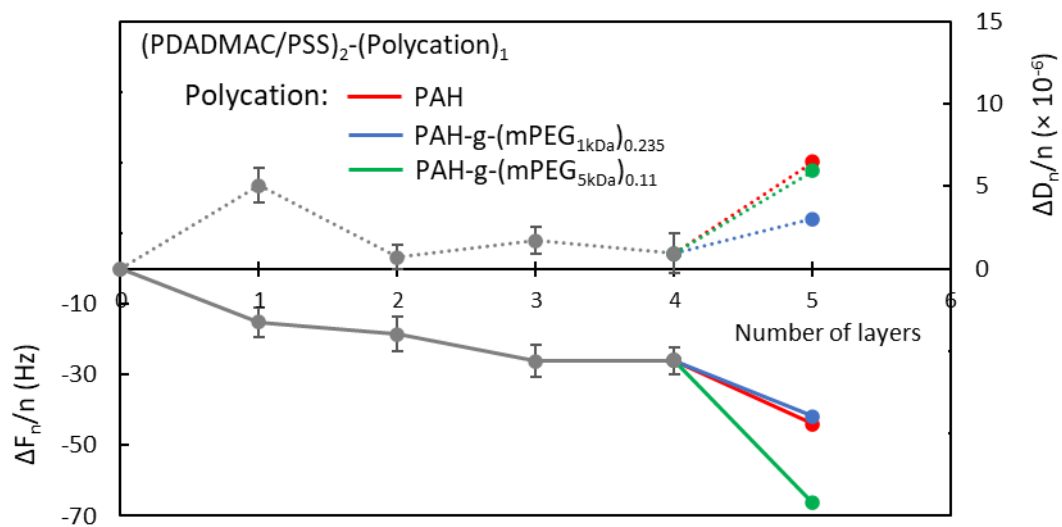
# Figure 6

**Figure 6. Impact of the PEGylation for the last PAH layer on Myo separation efficiency (A), Lyz separation efficiency (B) and peak resolution for the Myo / RNase A pair (C).** Same experimental conditions as in Figure 2. Reversed polarity was used for  $(PDADMAC/PSS)_2-(PAH-g-(mPEG)_{5kDa})_{0.11}1$ . See Figure SI 31 for impact on TI and RNase A.



# Figure 7

**Figure 7. Evolution of shift frequency (continuous lines) and shift dissipation (dashed lines) for the 3<sup>rd</sup> harmonic during SMIL construction procedure in QCMD.** Experimental conditions: see Figure 3. See detailed construction in supporting information Figure SI 32 to SI 34.



# Table 1

**Table 1. Impact of the chemical nature of the last polycationic layer on protein retention factor ( $k$ ) and on RSD on migration times at -10kV and -20kV.** Experimental conditions: see Figure 2.

Last layer polymer ( $\mu_{eo}$ )	Parameters	Proteins				
		TI	Myo	RNase A	Lyz	Average
PDADMAC (-45.5 TU)	$k \times 10^{-2}$	6.8	5.1	6.8	7.3	6.5
	A [ $\mu\text{m}$ ]	4.8	1.6	3.0	1.5	2.7
	RSD $t_m$ (-10kV)	0.41	0.49	0.48	0.61	0.50
	RSD $t_m$ (-20kV)	0.05	0.06	0.06	0.10	0.07
PAH (-45.2 TU)	$k \times 10^{-2}$	7.3	5.7	6.7	8.0	6.9
	A [ $\mu\text{m}$ ]	4.3	0.3	2.4	0.7	1.9
	RSD $t_m$ (-10kV)	0.34	0.41	0.44	0.54	0.43
	RSD $t_m$ (-20kV)	0.06	0.05	0.10	0.15	0.09
PEI (-44.4 TU)	$k \times 10^{-2}$	8.3	6.2	7.2	7.6	7.3
	A [ $\mu\text{m}$ ]	4.4	2.2	4.2	2.5	3.3
	RSD $t_m$ (-10kV)	0.03	0.04	0.02	0.04	0.03
	RSD $t_m$ (-20kV)	0.05	0.05	0.04	0.06	0.05
$\epsilon$ PLL (-40.7 TU)	$k \times 10^{-2}$	3.1	3.7	4.6	3.9	3.8
	A [ $\mu\text{m}$ ]	10.0	5.6	7.0	5.1	6.9
	RSD $t_m$ (-10kV)	1.73	1.98	2.16	2.75	2.15
	RSD $t_m$ (-20kV)	0.17	0.14	0.13	0.17	0.15
$\alpha$ PLL (-45.3 TU)	$k \times 10^{-2}$	8.7	6.3	7.3	7.9	7.5
	A [ $\mu\text{m}$ ]	3.1	1.3	4.0	1.6	2.5
	RSD $t_m$ (-10kV)	0.98	1.12	1.13	1.37	1.15
	RSD $t_m$ (-20kV)	0.28	0.29	0.36	0.40	0.33



## Table 2

**Table 2. Impact of last polycationic layer on SMIL construction and characteristics.** Experimental conditions: see Figure 3.

	Last layer			
	(PDADMAC/PSS) <sub>2</sub>	PDADMAC	PAH	εPLL
Final $\Delta F_3/3$ shift [Hz]	-27	-37.2	-44	-32.5
Deposited areal mass [ng cm <sup>-1</sup> ]	478	599	779	575
SMIL thickness [nm]	4.5	6.8	7.6	6.6

### Table 3

**Table 3. Impact of PEGylated polycation in the last layer on protein retention factor ( $k$ ) and  $RSD_{tm}$  at -10kV and -20kV.** Corresponding electropherograms and  $H=f(u)$  graphs are available in Supporting Information, from Figures SI 27 to Figure SI 30.

Last layer polymer ( $\mu_{eo}$ )	Parameters	Proteins				
		TI	Myo	RNAse A	Lyz	Average
PAH (-45.2 TU)	$k \times 10^{-2}$	7.3	5.7	6.7	8.0	6.9
	$A$ [ $\mu\text{m}$ ]	4.3	0.3	2.4	0.7	1.9
	$RSD_{tm}$ (-10kV)	0.34	0.41	0.44	0.54	0.43
	$RSD_{tm}$ (-20kV)	0.06	0.05	0.10	0.15	0.09
PAH 13.3% (-41.7 TU)	$k \times 10^{-2}$	5.8	3.0	3.7	5.1	4.4
	$A$ [ $\mu\text{m}$ ]	6.5	3.6	4.7	2.9	4.4
	$RSD_{tm}$ (-10kV)	1.34	0.19	0.20	0.22	0.49
	$RSD_{tm}$ (-20kV)	0.53	0.59	0.64	0.83	0.65
1kDa PAH 23.5% (-41.3 TU)	$k \times 10^{-2}$	8.3	4.1	5.5	6.3	6.1
	$A$ [ $\mu\text{m}$ ]	4.5	2.6	3.6	2.3	3.3
	$RSD_{tm}$ (-10kV)	1.36	1.54	1.62	2.03	1.64
	$RSD_{tm}$ (-20kV)	0.41	0.46	0.48	0.61	0.49
PAH 2.1% (-40.2 TU)	$k \times 10^{-2}$	9.4	7.8	9.5	10.8	9.4
	$A$ [ $\mu\text{m}$ ]	7.7	3.9	3.9	3.1	4.6
	$RSD_{tm}$ (-10kV)	0.08	0.08	0.09	0.11	0.09
	$RSD_{tm}$ (-20kV)	0.11	0.14	0.14	0.20	0.15
5kDa PAH 11% (-5.5 TU)	$k \times 10^{-2}$	7.6	4.5	3.1	3.9	4.8
	$A$ [ $\mu\text{m}$ ]	8.3	3.8	5.5	3.5	5.3
	$RSD_{tm}$ (-10kV)	0.02	0.02	0.01	0.02	0.02
	$RSD_{tm}$ (-20kV)	0.04	0.05	0.05	0.01	0.04

## Table 4

**Table 4. Impact of the last polycationic layer on SMIL construction.** Experimental conditions: see Figure 3.

	Last layer			
	(PDADMAC/PSS) <sub>2</sub>	PAH	PAH-g-(mPEG <sub>1kDa</sub> ) <sub>0.235</sub>	PAH-g-(mPEG <sub>5kDa</sub> ) <sub>0.11</sub>
Final $\Delta F_n/n$ shift [Hz]	-26	-44	-41.9	-66.3
Deposited areal mass [ng cm <sup>-1</sup> ]	460.6	779	741	1174
SMIL thickness [nm]	4.3	7.6	7.2	11.4