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Novel adsorptive polyamine coating for enhanced capillary electrophoresis of basic proteins and peptides

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Abstract

In capillary electrophoresis (CE), the anionic and hydrophobic nature of the fused-silica capillary surface has long been known to present a problem in protein and peptide analysis. The use of capillary surface coating is one of the approaches to avoid the analyte–wall interactions. In this study, a new polymer, poly-LA 313, has been synthesized, physico-chemical characterized, and applied as polyamine coating for CE separations. The coating process is highly reproducible and provides fast separations of peptides and proteins in a few minutes and with high efficiency. The physically adsorbed polymer gives rise to a durable coating in the range of pH 2–10, in the presence of organic modifiers (acetonitrile and methanol) and with complex biological samples. The efficiency of the new cationic polymer was also tested performing protein and peptide separations with capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS).
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1. Introduction

Capillary electrophoresis (CE) has been developed into a robust separation technique, mainly employed for the analysis of peptides, proteins, and nucleic acids, due to its high efficiency, short analysis time, ease of automation, and capacity to perform separations in aqueous solutions. Further, the small sample volumes employed make CE the separation technique chosen in applications which are sample-limited. However, in spite of the advantages of the CE, the anionic and hydrophobic nature of the silica of the capillaries employed in CE, gives rise to analyte–wall interactions, where basic analytes such as basic peptides and proteins, present the greatest problem. Since interaction forces increase with molecular size, basic proteins are generally more adversely affected, compared to basic peptides. However, although cationic proteins are especially problematic to analyze, any protein with a region of net positive charge or external hydrophobic domain can be adsorbed on the silica surface. Over the years, several strategies have been employed to avoid the adsorption of peptides and proteins on the capillary silica wall. These strategies involve either the use of extreme pH values, high salt concentrations, and additives such as amines and zwitterions added to the background electrolyte (BGE), or the permanent or semi-permanent coating of the silica surface. Amines and zwitterions added to the BGE behave as "dynamic" coatings. When mass spectrometry detection (MS) with electrospray ionization (ESI) is used to analyze peptides or proteins, the preferred strategy is the permanent or semi-permanent capillary coating, because the other strategies are usually not compatible with ESI-MS or can denature the analytes. Several reviews have been published where coating of capillaries for CE has been considered [1–5].

The analysis of proteins and peptides in complex samples requires the use of mass spectrometry (MS) detection, which is usually not compatible with the modifications of the BGE previously mentioned. Thus, the use of coatings of the silica wall is the best choice when MS is employed. These coatings should be stable without any bleeding that could produce high background signal interfering with the analyte detection. Besides, when sheathless ESI is employed, the electroosmotic flow at

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coated capillaries should be high enough to promote a stable electrospray, and the coating should be stable against the organic modifiers added to the BGE.

Nowadays, there are different kinds of coatings employed to prevent the adsorption of peptides and proteins to the capillary silica wall. Coatings covalently bonded to the silica wall are often prepared by bonding either monomers such as 3-aminopropyltriethoxysilane (APS) [6], [3-(methacryloylamino)propyl]trimethylammonium chloride (MAPTAC) [7] and [(acryloylamino)propyl]trimethylammonium chloride [7], or polymers such as methylcellulose [8], hydroxypropylmethylcellulose (HPMC) [9], polyvinylalcohol (PVA) [10], polyacrylamide (PAA) [8] and trimethoxy silane-modified polydimethylacrylamide [11]. The use of these neutral polymers is restricted to sheathflow ESI interfaces when MS is employed due to the suppression of the EOF. The main disadvantages of the covalently bonded coatings are the limited pH range of use and the time required to prepare them.

In comparison with covalent coatings, physically adsorbed coatings are easily prepared by fast and simple procedures. Up to now, there are lot of physically adsorbed polymers employed to avoid the adsorption of peptides and proteins on fused silica capillaries in CE-UV applications such as derivatized cellulose polymers [12], poly(ethylene oxide) polymer (PEO) [13], PVA [14], polyethyleneimine (PEI) [15,16], polybrene [17,18], successive multiple ionic layer (SMIL) coatings formed by polybrene/dextran sulfate [19] or polybrene/polyvinylsulfonate [20], poly(diallyldimethylammonium chloride) (PDADMAC) [21], polyarginine [22], chitosan [23], quaternary ammonium-substituted agarose [24], liposomes [25], phospholipid bilayers [26] and poly-E-323 [27], polymer of 1,2-bis(3-aminopropylamino)-ethane and epichlorohydrin.

However, the number of physically adsorbed cationic polymers successfully employed with MS detection is much lower than with UV detection. Polybrene [28], polybrene/dextran sulfate in successive multiple ionic layer (SMIL) [29,30], poly-E-323 [31], and copolymer of derivatized methacrylate and dimethylacrylamide [32], are the main cationic polymeric coatings successfully employed in CE-ESI-MS applications. However, some of these coatings present different shortcomings. Polybrene coating has several drawbacks such as lack of stability against methanol [18], one of the main organic modifiers employed at BGE when sheathless ESI interface is used, or the need of the regeneration of the coating between every run [7,33,34]. Copolymer of derivatized methacrylate and dimethylacrylamide coating also needs to be regenerated between every run, though the process is very simple, and the EOF shifts from negative to positive in the range pH 2–10, indicating that the silanol groups are not completely blocked, though the coating is efficient avoiding the adsorption of proteins on the silica wall. Polybrene/dextran sulfate in successive multiple ionic layer (SMIL) coating shows strong adsorption of compounds present in plasma samples which make it compulsory to rinse the coating with 0.1 M NaOH between every run when these kind of samples are analyzed [35]. Thus, the development of more cationic polymer coatings is necessary to improve the application of CE-ESI-MS to the analysis of complex samples.

The objective of this work is to introduce a new polycationic polymer, poly-LA 313. The use of cationic modifications of the silica capillary wall means the shift of the normal adsorption problem to adsorption of acid compounds. However, the development of this work was focused in avoiding the adsorption of basic peptides and proteins. The synthesis and the physical adsorption process to coat the capillaries are described. Poly-LA 313 was characterized by matrix-assisted laser/ionization mass spectrometry (MALDI-MS) and static light scattering. The performance of the poly-LA 313-coated capillaries was evaluated analyzing a mixture of basic proteins. Reproducibility of the coating process and stability of the coated capillaries against different BGE in a wide pH range including the use of organic modifiers was tested. Compatibility of the poly-LA 313 coating with MS with electrospray ionization (ESI) analyzing peptides and proteins was also evaluated. Finally, the ability of the coated capillaries to handle biological samples of complex matrix such as plasma or cerebrospinal fluid (CSF) was demonstrated. Poly-LA 313 belongs to the same family of polycationic polymers as poly-E 323, earlier developed in house, but the structure of the new polymer is tailored to better interact with hydrophobic surfaces such as poly(dimethylsiloxane) (PDMS), where negative charges are induced by NaOH etching to bind the polycationic polymer to this surface by electrostatic interactions [36,37]. The increase in the distance between the positive charges in poly-LA 313 (1,4-butanediol diglycidyl ether as cross-linker) in comparison to poly-E 323 (epichlorohydrin as cross-linker), makes the new polymer better to interact with surfaces with lower density of negative charges such as PDMS activated by NaOH etching. This will enable the new polymer to be used as non-covalent coating for PDMS channels employed in electrophoretic separations on chip with high performance. In this work, experimental conditions similar to those employed with poly-E 323 polymer were used to facilitate the comparison of the both polymers.

2. Materials and methods

2.1. Chemicals and materials

3,3′-Diamino-*n*-methyldipropylamine (CAS: 105-83-9) of 96% purity was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). 1,4-Butanediol diglycidyl ether (CAS: 2425-79-8) of 95% purity was obtained from Fluka Chemie (Butchs, Switzerland). All other chemicals were of analytical-reagent grade. Acetic acid, ammonium acetate, ammonia solution 25%, dimethyl sulfoxide, formic acid, isopropanol, methanol and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Ammonium formate was purchased from BDH (Poole, England). All the buffers and solutions were prepared in ultrapure water obtained from a Milli-Q unit (Millipore, Bedford, MA, USA). Calculations to prepare all buffers were performed with the PHoEBuS software program, version 1.3 (Analis, Orleans, France). Fused-silica capillary tubing of 50 μm i.d. × 185 μm or 365 μm o.d. was obtained from Polymicro Technologies (Phoenix, AZ, USA). Methionine-enkephalin (M-6638), leucine-enkephalin (L-9133), oxytocin (O-6379), angiotensin II (A-9525), LHRH (L-7134), bradykinin

Table 1
Molecular masses (M_r) and isoelectric points (pI) of the peptides and proteins used

	M_r	pI
Peptides		
Methionine-enkephalin	574	5.1
Leucine-enkephalin	556	5.2
Oxytocin	1,007	5.2
Angiotensin II	1,046	7.8
LHRH	1,182	9.7
Bradykinin	1,060	12.5
Proteins		
α-Lactalbumin	14,200	4.5
α-Chymotrypsinogen A	25,700	8.5
Carbonic anhydrase	28,982	6.4
Myoglobin	18,800	6.8
Ribonuclease A	13,700	8.6
Cytochrome *C*	12,327	9.5
Lysozyme	14,300	10.9
Human serum albumin	66,500	5.7

(B-3259), α-chymotrypsinogen A (C-4879), α-lactalbumin (61289), ribonuclease A (R-5500), cytochrome *C* (C-2037), lysozyme (L-6876), myoglobin (M-1882), carbonic anhydrase (C-7500), and human serum albumin (A-2817), were purchased from Sigma–Aldrich Chemie (Steinheim, Germany) as lyophilized powders, and used without further purification. Molecular weights and isoelectric points of peptides and proteins, see Table 1, were obtained from bibliography [31] and from the manufacturer. Stock solution of proteins and peptides were prepared by dissolving proteins and peptides in Milli-Q (MQ) water at a concentration range of 1–6 mg/mL. The protein and peptide solutions were stored at −20 °C and −80 °C, respectively, and adjusted to room temperature prior use. Collection of plasma and CSF samples was performed as described by Bergquist et al. [38].

2.2. Synthesis of poly-LA 313

In a 250 mL flask, 1.24 g (8.5 mmol) of 3,3′-diamino-*n*-methyldipropylamine was mixed with 6.67 g of MQ water and 1.69 g (8.3 mmol) of 1,4-butanediol diglycidyl ether during intensive magnetic stirring. The flask was sealed and the mixture was continuously stirred at room temperature during 5 h. After that, 51.23 g of MQ water were added and the reaction was left alone for 48 h until the reaction mixture was thickened. To be sure that the reaction was completely finished, 3.6 g of acetic acid were added and the mixture was heated to 50 °C for 1 h. Finally, the mixture was left with magnetic stirring for 66 h at room temperature. The polymer solution was stored at +4 °C and used without further purification. The whole reaction process lasted for 120 h. According to the polymer synthesis stoichiometry, the concentration of poly-LA 313 in the solution is 4.5% (w/w).

2.3. Capillary coating process

Optimization of the capillary coating process was performed with 50 μm i.d. × 33.5 cm length fused silica capillaries. Prior to the coating process, a 5 mm optical window was prepared on the fused-silica capillaries by using an optical fiber splicer (Model PFS-200 series; Power Technology, Little Rock, AR, USA). Initially, the internal surface of the silica capillary was activated by flushing NaOH, followed by MQ water. After that, the pre-treated capillary was flushed with either the 4.5% (w/w) poly-LA 313 solution obtained in the polymerization reaction or dilutions of that with pH adjusted at pH 7 by the addition of 1 M NH_4OH. Excess polymer solution was washed out sequentially with MQ water and with 50 mM ammonium acetate buffer, pH 5. Different parameters tested in the coating process such as concentration of chemicals, pressure applied to flush the capillary and time of every coating step, are shown in Table 2.

Table 2
Conditions tested in the coating procedure of fused silica capillaries (50 μm i.d. × 33.5 cm length)

Step	Reagent	Concentration	Time (min)	Pressure (bar)
1	NaOH	0.1–1 M	10–100	3.5
2	MQ water	–	5	3.5
3	Poly-LA 313	0.2–4.5% (w/w)	5–20	4.5
4	MQ water	–	4, 15	4.5
5	Ammonia acetate pH 5	50 mM	5, 15	4.5

When MS detection was employed, the effective length of the fused silica capillaries was either 45 cm or 57 cm and the optimized capillary coating process was adjusted according to the increase in the dimensions of the silica capillaries.

2.4. Instrumentation

Poly-LA 313 was characterized by MALDI-MS and static light scattering. Mass spectra were recorded on a Brucker Ultraflex MALDI-TOF/TOF instrument (Bruker, Daltonics, Bremen, Germany). The wavelength of the laser was 337 nm and an acceleration voltage of 25,000 V was used. The software packages Ultraflex version 2.0 and Flex Analysis version 2.0 were used to record and analyze mass spectra, respectively.

Intensity light scattering measurements were made using a photon-counting apparatus supplied by Hamamatsu to register the scattered signal. The light source was a 3 mW He–Ne laser. The optical constant for vertically polarized light is $K = 4\pi^2 n_0^2 (dn/dc)^2/(N_A \lambda^4)$, where n_0 is the solvent refractive index, dn/dc the measured refractive index increment and λ is the wavelength (632.8 nm). The reduced scattered intensity, Kc/R_θ was derived where c is the concentration and R_θ is the Rayleigh ratio obtained through calibration using toluene. $R_{90^\circ} = 13.59 \times 10^{-6}$ cm^{-1} at 25.0 °C. The solvent scattering was subtracted from the scattered light from the solution. The refractive index increment was measured using a differential refractometer with Rayleigh interference optics.

For CE with UV detection, an HP^{3D} capillary electrophoresis instrument equipped with UV diode-array detection and HP ChemStation software for data collection was used. Capillaries of 33.5 cm total length, 25 cm effective length, 50 μm i.d. and 365 μm o.d. were employed for the CE-UV separations.

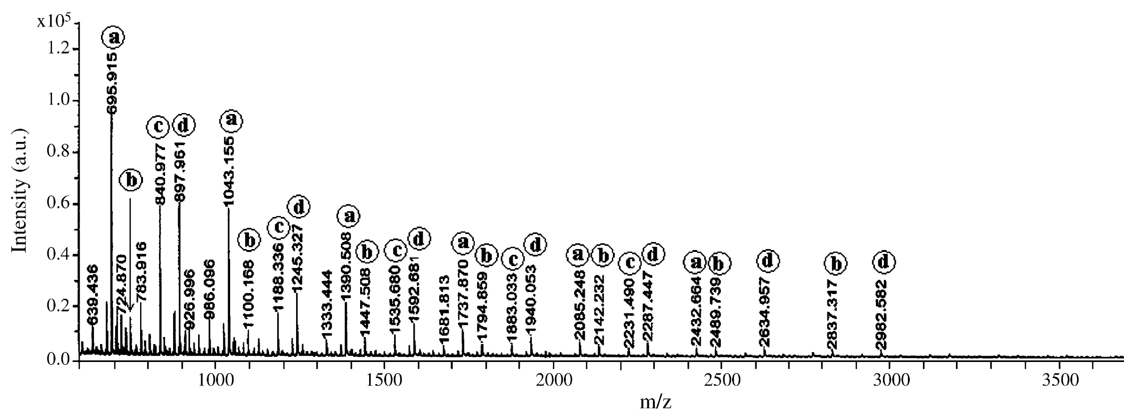

Fig. 1. MALDI-MS spectrum of poly-LA 313 solution. The spectrum was acquired using α-cyano-4-hydroxycinnamic acid as matrix. Four series of molecules were identified and are labeled in the MALDI-MS spectrum with a–d, respectively.

For CE with MS detection, the CE system previously described was coupled to an orthogonal acceleration time-of-flight mass spectrometer (oa-TOF MS), Agilent LC/MSD TOF (Agilent Technologies, Santa Clara, CA, USA). Fused silica capillaries with 50 μm i.d., 365 μm o.d. and either 45 cm or 57 cm length were used with the CE-MS system. Detection was performed in positive ion mode. The voltage applied at the sampling capillary at the entrance of the mass spectrometer was in the range 4.2–5.0 kV. Nitrogen at 130 °C and 5 L/min was used as drying gas. Voltage fixed at fragmentor, skimmer and octopole guides were 175 V, 60 V and 250 V, respectively. The ion pulser at the TOF analyzer was set up to a measurement frequency of 1–2 cycles/s. Agilent TOF software and Agilent™ QS software were used to record and analyze mass spectra, respectively. CE system was coupled to oa-TOF MS by a sheathflow interface. Make-up flow liquid was delivered by a Jasco PU-980 HPLC pump (Jasco Inc, Easton, MD, USA). Sheath-liquid flow rate was 1 μL/min. The outlet end of the silica capillary was sharpened to obtain more stable electrospray and to try to avoid any possible band broadening at the interface.

3. Results and discussion

3.1. Characterization of poly-LA 313

In order to characterize the structure and the average size of poly-LA 313, MALDI-MS and light scattering measurements were performed. One of the attractive features of MALDI-MS is the spectral simplicity, due to generation of predominantly singly charged molecules, which makes it easier to interpret the MS spectra of mixtures of molecules like in case of a reaction mixture [39]. Fig. 1 shows the MALDI-MS spectrum of poly-LA 313 solution obtained after the synthesis reaction without any further purification. Analyzing the MALDI-MS spectrum, it is possible to observe four series of peaks, labeled with a–d (see Fig. 1). The mass peaks in every series are related to each other by the addition or subtraction of one monomer molecule, and they correspond to singly charged molecules. The variation of the number of monomers of the polymer molecule gives rise to the different mass peaks in every series. The general formula of the polymeric molecules is shown in Fig. 2.1. The head molecules

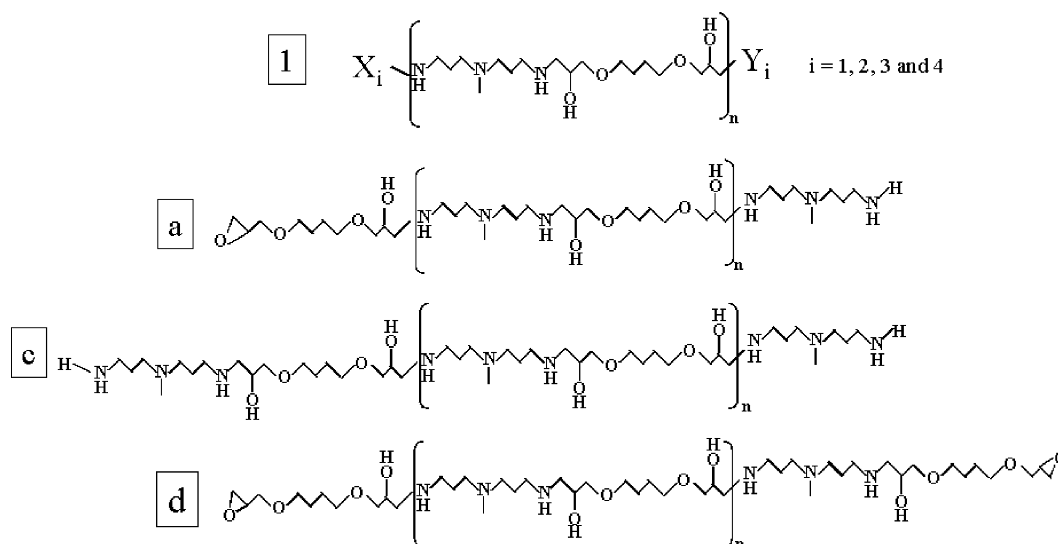

Fig. 2. Structures of the polymer molecules corresponding to the mass peaks observed in the MALDI-MS spectrum in Fig. 1. The general polymer structure is shown in part 2.1 and head molecules of the series a, c and d are shown in parts a, c and d, respectively.

of series a, c and d are shown in the Fig. 2a, c and d, respectively. With these results, the poly-LA 313 structure is characterized as four series of linear polymeric molecules.

Static light scattering measurements were performed to estimate the average molecular weight of poly-LA 313 using the Zimm Plot method [40]. Initially, the refraction index (n) for different concentrations (c) of poly-LA 313 in 0.2 M NaCl and pH 5 is measured to calculate the refraction index increment (dn/dc) parameter. These experimental results are fitted to a straight line ($y = 0.00096751 + 0.28882x$ (mL/g); $r^2 = 0.99994$) whose slope provides the dn/dc parameter. The variation of the reduced scattered intensity, Kc/R_θ, as a function of the scattered angle θ for different concentrations of poly-LA 313 in 0.2 M NaCl and pH 5 were plotted and fitted to straight lines by the minimum squared method. The equations corresponding to the straight lines were $y = 9.123e^{-7} + 5.7764e^{-6}x$ ($r^2 = 0.99878$); $y = 1.1913e^{-6} + 6.1148e^{-6}x$ ($r^2 = 0.99541$); $y = 1.2656e^{-6} + 5.6466e^{-6}x$ ($r^2 = 0.99495$); and $y = 1.9548e^{-6} + 5.1057e^{-6}x$ ($r^2 = 0.99712$), for 0.193 g/L, 0.385 g/L, 0.625 g/L and 1.25 g/L poly-LA 313 concentration, respectively. $Kc/R_{\theta=0}$ is obtained by extrapolation at $\theta = 0$ for every line at every concentration measured. The variation of $Kc/R_{\theta=0}$ values as a function of the concentration is fitted by the minimum square method, to a straight line which equation is $y = 7.4877\,e^{-7}$ (mol/g) + 0.00094861 (mol/g)x; $r^2 = 0.98017$. The average molecular weight of the polymer, 1.3×10^6 g/mol, was obtained from the inverse of the ordinate at the origin. Gyration radius of the polymer, $R_g = 1800$ Å, was calculated from the slope of the adjustment.

Comparison of the results obtained by MALDI-MS and light scattering shows that only small polymer molecules were ionized and detected at the MALDI-MS measurements. However, these measurements confirm the structure of poly-LA 313.

3.2. Capillary coating preparation

The polymeric coating is formed by multisite electrostatic interaction between silanol groups and positively charged nitrogen atoms, and hydrogen bonding due to the hydroxyl groups present in the polymer. The steps of the capillary coating process were previously described in Section 2.3. The influence of every step on the efficiency of coated capillaries to resolve a mixture of basic proteins (α-chymotrypsinogen A, ribonuclease A, cytochrome C and lysozyme) was tested. Experiments were performed with CE-UV, using acetate or formate buffers as background electrolytes (BGE).

The effect of the concentration of the polymer solution in the coating process was studied (Fig. 3). The polymer was diluted and adjusted to pH 7 by adding 1 M NH_4OH in the 0.2–4.5% (w/w) concentration range. The best results were obtained with 3.75% and 4.5% (w/w) polymer solutions, though the efficiency values were similar in the 1–4.5% (w/w) polymer concentration. A clear decrease in the efficiency was observed with polymer concentration lower than 1%. Nevertheless, efficiency at 0.2% concentration was slightly higher as compared with 0.5% concentration, also seen in earlier studies [31]. Solution of 3.75% (w/w) polymer concentration was chosen for the following optimization studies because the pH was better controlled.

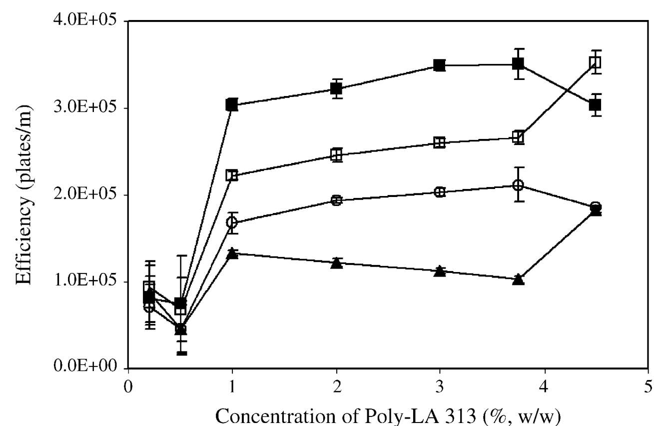

Fig. 3. Efficiency of the electrophoretic separation as a function of poly-LA 313 concentration (%, w/w) in the coating solution. Error bars represents mean ± S.E.M. ($n = 12$). Coating process: The capillary was coated by the sequential treatment with 1 M NaOH at 3.5 bar for 10 min, MQ water at 3.5 bar for 5 min, polymer solution at 4.5 bar for 10 min, MQ water at 4.5 bar for 4 min and BGE (50 mM ammonium acetate pH 5) at 4.5 bar for 5 min. Mixture of 0.17 mg/mL proteins: ribonuclease A (■), α-chymotrypsinogen A (□), lysozyme (◯) and cytochrome C (▲). Conditions: Capillaries, 50 μm i.d., 360 μm o.d., 33.5 cm total length and 25 cm useful length; injection at 50 mbar during 5 s; separation at −450 V/cm; detection at 191 nm.

Activation with NaOH, poly-LA 313 rinsing time, MQ water and 50 mM ammonium acetate pH 5 rinsing steps were studied according to the values in Table 2. Maximum efficiency and minimum time were the criteria to select the experimental conditions for these steps. The optimized process thus consists of a sequential treatment of the fused silica capillary with 1 M NaOH at 3.5 bar for 10 min, MQ water at 3.5 bar for 5 min, 3.75% (w/w) polymer solution with pH 7 at 4.5 bar for 10 min, MQ water at 4.5 bar for 4 min and 50 mM ammonium acetate pH 5 at 4.5 bar for 5 min. Separation of the mixture of proteins using a capillary coated with the optimized process is shown in Fig. 4. This process is fast, taking only 34 min, which is shorter than common covalent coating process (with 23 h coating time [6]), and than other polyamine dynamic coatings (50 min) such as the one previously described by our group [27,31]. The efficiency of the separation is in the range 1.7×10^5 (cytochrome C)–3.9×10^5

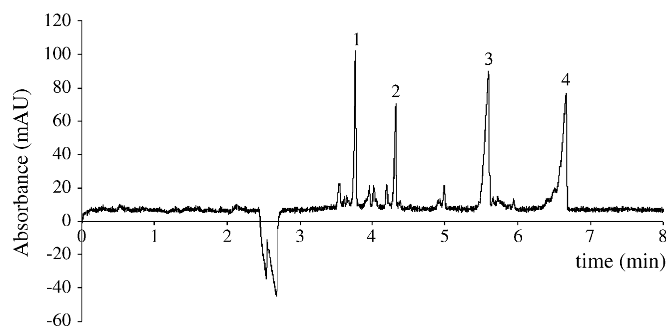

Fig. 4. Separation of α-chymotrypsinogen A (1), ribonuclease A (2), cytochrome C (3) and lysozyme (4), by CE-UV using a capillary coated with a 3.75% (w/w) poly-LA 313 solution. Rest of the experimental conditions are the same as in Fig. 3.

(ribonuclease A) plates/m, better than with poly-E 323 coating, and the variation coefficient (CV) for the electroosmotic flow (EOF) mobility is in the order of 0.5%. The intraday reproducibility of the coating process, when CV of EOF mobility is the evaluation parameter, is 0.4% ($n = 2$ capillaries). The interday reproducibility of the coating process, when CV of EOF mobility is the evaluation parameter, is 1.2% ($n = 17$ capillaries, coated at 14 different days during 1 year). The CV for EOF mobility and reproducibility of the poly-LA 313 coating is in the same range as other polycationic polymer coatings (e.g. poly-E 323), when CE separations in coated silica capillaries are carried out. However, the structure of poly-LA 313 is more suitable than poly-E 323 to interact with PDMS channels, today widely used in the field of on-chip electrophoretic separations.

This new polymer is not commercially available yet and it must thus be synthesized in house. Although the synthesis process is long, it is easy and it is performed with minimum labor. Once the polymer is synthesized, it can be stored and used for long periods (longer than one year) and only a few microliters of polymer solution are consumed for every coating process. It means that the use of the polymer is economically favorable.

3.3. Stability of the coating

3.3.1. Intra- and interday stability and regeneration of the coating

In order to evaluate the stability of the coating, the mixture of proteins was analyzed in coated capillaries at different storage times. The intraday stability was good, 0.2% (CV of EOF mobility as evaluation parameter) or 1.2–4.6% (CV of efficiency of the separation of proteins as evaluation parameter). The interday stability of coated capillaries was less convincing. Three capillaries were coated, firstly tested with the mixture of proteins and then stored without any preventive measurement (with MQ water and at room temperature) during 13–16 days. Unfortunately, the same efficiency values as before storage, were obtained only for one of the stored capillaries (data not shown). Thus, methods to regenerate the coating of the capillaries were developed and evaluated. Regeneration of the surface of the coated capillaries may also be required when complex samples like biological body fluids are analyzed due to compounds present in these samples could adsorb on the capillary wall decreasing its performance for the electrophoretic separation. Several regeneration procedures with the use of 1 M NaOH and 0.1 M HCl, commonly employed to break electrostatic interactions between proteins and the capillary surface, were tested. Coated capillaries were treated with several sequences consisting of four analyses of proteins and a regeneration procedure. Best result was obtained with the following process: 1 M NaOH at 3 bar for 5 min, MQ water at 3 bar for 2 min, 0.1 M HCl at 3 bar for 2 min, MQ water at 3 bar for 2 min, 3.75% (w/w) polymer solution at 4.5 bar for 10 min, and BGE (50 mM ammonium acetate pH 5) at 4.5 bar for 5 min. Following this procedure, it was possible to fully regenerate the coating. CV of EOF mobility after one regeneration step was 0.6%. Efficiency values for the separation of four proteins before and after the regeneration process were compared by a test of parametric hypothesis for independent results, obtaining that at 95% significance level, there were no significant differences between both groups of results. Thus, it is possible to use the same coated capillary during a working day and if the performance of the capillary decreases due to either long storage or analysis of complex samples, it is possible to regenerate the coating.

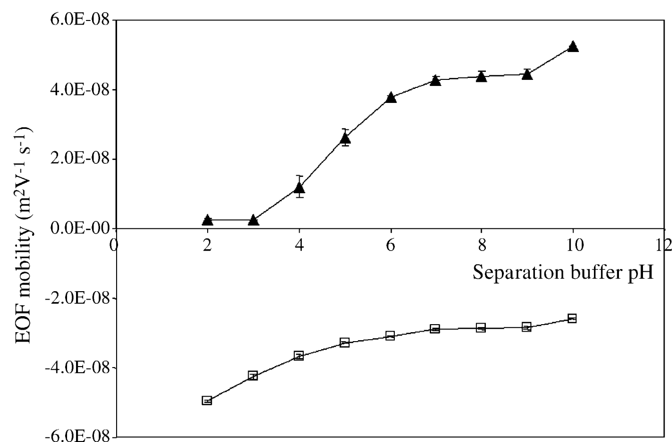

Fig. 5. EOF mobility as a function of the BGE pH using a bare fused silica capillary (▲) and a poly-LA 313-coated capillary (□). Conditions: capillaries as in Fig. 4; injection, MQ water at 50 mbar for 5 s; applied positive or negative voltage of 450 V/cm; detection at 191 nm. All the buffers were 20 mM ionic strength either ammonium acetate (pH 4–10) or ammonium formate (pH 2–3). MQ water was used as EOF marker. The number of injections was either 5 (bare silica capillary) or 10 (coated silica capillary). Error bars represent mean ± S.E.M.

3.3.2. Effect of BGE on capillary coating

3.3.2.1. Effect of pH. The stability of the EOF generated by the coating is very important, especially when ESI-MS is used for the detection step. The EOF mobility was measured and compared at different pHs using bare fused silica capillaries and poly-LA 313 coated capillaries. Results are shown in Fig. 5. The fused silica capillary shows that the higher the pH of BGE, the higher the EOF mobility due to the well-known increased ionization of silanol groups at higher pH. The behavior of the poly-LA 313 coated capillary is just the opposite of the bare fused silica capillary, due to the positive charges of the polymer which promotes anodal EOF. Thus, at high pH values most of the positive charges of the polymer are neutralized and the absolute value of EOF mobility tends to decrease. The EOF of poly-LA 313 coated capillaries is in the range 150–260 nL/min over the range of pH 2–10. Thus, the coated capillaries can be used for fast protein separations in a wide pH range. Besides, the EOF mobility is quite stable, although not constant, in the range of pH 4–10.

The stability of the coating was tested at extreme pH values, pH 2 and pH 10. The CV of the EOF was the parameter selected to perform this study, using MQ water as the EOF marker. Each pH value was tested in a CE-UV system during 4 h, corresponding to 50 injections of MQ water, using 20 mM ionic strength either ammonium formate or ammonium acetate. The CV of EOF at pH 2 and 10 was 0.5% and 0.8%, respectively, showing a high stability of the coating when extreme pHs are used. Thus, the pH working range of poly-LA 313 coated capillaries

is wider than covalent, cationic monomer coatings prepared by silanization. This feature makes the poly-LA 313-coated capillaries useful in applications where, e.g., physiological pH BGE should be employed in the analysis of biomolecules to avoid their denaturation or in the analysis of non-covalent complexes, such as antigen–antibody, which are stable at this pH value.

3.3.2.2. Effect of organic modifiers. When ESI-MS is used as detection system, sheathless interface is one of the interfaces usually employed to couple CE and MS. The use of coated capillaries with sheathless ESI-MS implies that the coating should tolerate the use of organic modifiers such as acetonitrile (MeCN) or methanol (MeOH), and still providing stable EOF. Thus, poly-LA 313-coated capillaries were evaluated against BGE containing MeCN and MeOH. BGEs consisting on 50 mM ammonium acetate pH 5 with either 20% of MeOH or 50% MeOH or 20% MeCN or 50% MeCN were tested by the injection of MQ water in a CE-UV system for 3 h, corresponding to 30 injections. CVs of EOF were 0.4% and 1.5% when BGE contained 20% and 50% of MeOH, respectively, and 0.4% and 1.2% when BGE contained 20% and 50% MeCN, respectively. These results show the stability of the EOF and the coating against both tested organic modifiers in a concentration up to 50%. Thus, poly-LA 313 coating is suitable to be used with sheathless ESI-MS and does not show the reported restriction of the polybrene coatings that are highly unstable when MeOH is used as organic modifier of the BGE [18].

3.4. CE-MS using poly-LA 313-coated capillaries

Nowadays, the analysis of complex samples demands the use of universal detectors such as MS detectors, which could also provide structural information. Thus, compatibility between coated capillaries and MS detectors should be a desirable feature for any new coating. The coating should be very stable to promote stable signal and to avoid any bleeding from the coated capillary to the MS, since it will contaminate the ion source and the MS sensitivity will decrease. Thus, poly-LA 313 was tested in CE-ESI-MS separations.

Initially, the MS spectrum of poly-LA 313 was measured by direct infusion. Thereafter the stability of the coating was tested by monitoring the capillary eluent direct infused or electromigrated through coated and uncoated silica capillaries, respectively. During monitoring, the main masses corresponding to the MS spectrum of poly-LA 313 were measured. Comparison of the signals from both kind of capillaries showed the absence of significant differences between them, indicating good stability of the coating without any observed bleeding. This requirement is especially important when sheathless ESI-MS is employed. Thus, the poly-LA 313 coating is stable enough and the EOF promoted by this coating is also stable enough to be employed in CE-ESI-MS systems. This is in contrast to other polymer coatings, where polymer interferences with analyte detection has been reported [41].

In the optimization of the CE-ESI-MS method for the analysis of mixtures of either peptides or proteins, several parameters were varied such as BGE (formic acid, acetic acid, buffer formate and buffer acetate, at different concentration and pH), organic acid in sheath-liquid (10, 20 mM acetic acid and 5, 10, 20, 25 mM formic acid), organic modifier and its percentage in the sheath-liquid (methanol, isopropanol), and voltage for the CE separation. Analysis of a standard mixture of six neuropeptides with molecular weight in the range of 556–1182 Da and with p*I* in the range of 5.1–12.5 was performed (Fig. 6). Using poly-LA 313-coated capillaries, it is possible to resolve the mixture of six neuropeptides in 13 min, with a minimum Rs of 1.4, and with efficiency values in the range of $1.0–3.7 \times 10^5$ plates/m. The limit of detection for the mixture of peptides was in the range of 0.3 μM (methionine-enkephalin)–2.5 μM (angiotensin II), LOD = 2 S/N calculated by Analyst™ QS software. Intra-capillary CV for the retention time of neuropeptides was in the range of 0.9–3.4% ($n = 6$).

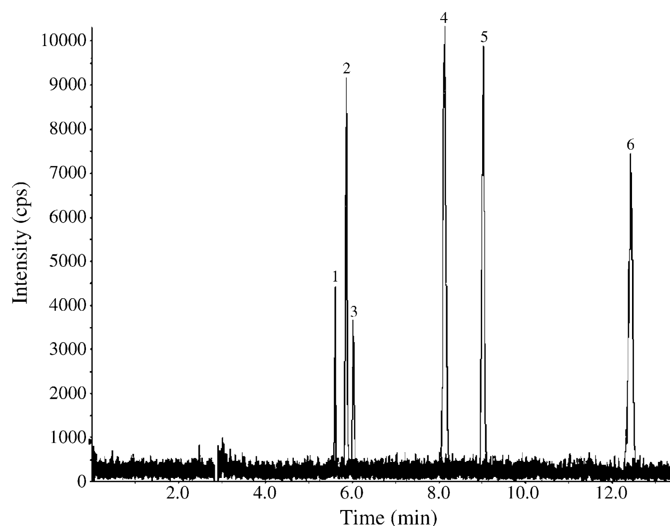

Fig. 6. CE separation of neuropeptides on a poly-LA 313-coated capillary using sheathflow ESI-MS. Overlay of the extracted ion electropherograms of every neuropeptide. Coating process: The capillary (50 μm i.d., 365 μm o.d., 57 cm total length) was coated by the sequential treatment with 1 M NaOH at 5 bar for 12 min, MQ water at 5 bar for 6 min, 3% (w/w) polymer solution at 8 bar for 10 min, MQ water at 8 bar for 4 min and 50 mM ammonium acetate pH 5 at 8 bar for 5 min. Analysis conditions: Injection by pressure at 50 mbar during 5 s of mixture of peptides in the range 3–14 μM; applied negative CE voltage of −526 V/cm; BGE, 10 mM formic acid. Detection conditions: Sheath liquid, mixture of 20% of 10 mM formic acid and 80% of isopropanol at 2 μL/min; nebulization, nitrogen at 2 psig; voltage at the sampling capillary at the entrance of the MS, +4.2 kV; frequency at the ion pulser at the TOF analyzer, 3.54 cycle/s. Peak identification: (1) methionine-enkephalin, (2) leucine-enkephalin, (3) oxytocin, (4) angiotensin II, (5) LHRH and (6) bradykinin.

Poly-LA 313-coated capillaries were also employed to resolve a mixture of proteins using CE-ESI-MS. Due to the general instability of proteins in presence of organic modifiers promoting their denaturation and precipitation, sheathflow interfaces are more suitable when proteins are analyzed by CE-ESI-MS. Proteins with molecular weight in the range of 12–26 kDa and p*I* in the range of 4.5–8.6 were analyzed. Fig. 7 shows the electrophoretic separation of the protein mixture using sheathflow ESI-MS detection with the optimized conditions for the analysis. The mixture of proteins was resolved in 11 min, with a minimum Rs of 1.51 ($n = 3$) between carbonic anhydrase and ribonuclease, and with efficiency values up to 1.1×10^5 plates/m

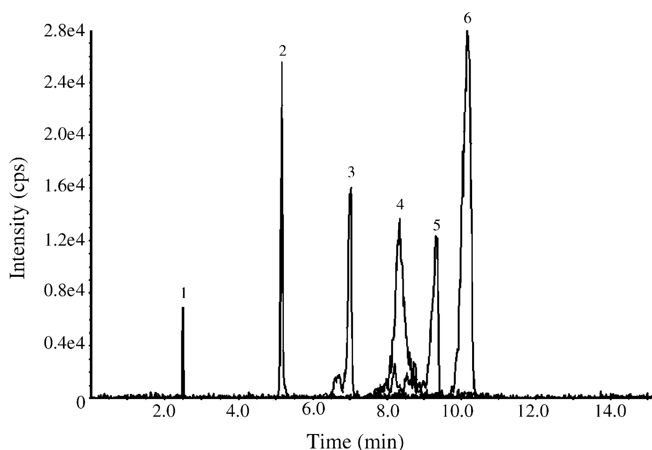

Fig. 7. CE separation of proteins on a poly-LA 313-coated capillary using sheathflow ESI-MS. Overlay of the extracted ion electropherograms of every protein. Coating process: The capillary (50 μm i.d., 365 μm o.d., 45 cm total length) was coated by the sequential treatment with 1 M NaOH at 5 bar for 9.3 min, MQ water at 5 bar for 4.7 min, 3% (w/w) polymer solution at 8 bar for 7.8 min, MQ water at 8 bar for 3.1 min and 50 mM ammonium acetate pH 5 at 8 bar for 3.9 min. Analysis conditions: Injection by pressure at 10 mbar during 1.5 s of mixture of proteins in the range 6–13 μM; applied negative CE voltage of −550 V/cm; BGE, 16 mM formic acid. Detection conditions: Sheath liquid, mixture of 20% of 25 mM formic acid and 80% of isopropanol at 1 μL/min; without nebulization; voltage at the sampling capillary at the entrance of the MS, +4.5 kV; frequency at the ion pulser at the TOF analyzer, 1 cycle/s. Peak identification: (1) dimethyl sulfoxide, (2) α-lactalbumin, (3) α-chymotrypsinogen A, (4) carbonic anhydrase, (5) ribonuclease A and (6) myoglobin.

($n = 3$). LOD for the mixture of proteins was in the range of 0.199 μM (α-chymotrypsinogen A)–0.678 μM (ribonuclease A), LOD = 2 S/N calculated by Analyst™ QS software. Intra-capillary CV for the retention time of proteins was in the range of 0.5–1.0% ($n = 3$). The efficiency of the separation of proteins by CE-ESI-MS was lower than by CE-UV. The inherent restrictions in MS detection, as the use of volatile buffers with low ionic strength and the balance between CE separation and ionization in the MS interface, are the main reasons for the decrease in the efficiency of the separation. Furthermore different proteins, capillary lengths and separation voltages were employed in the separations shown in Figs. 4 and 7. When MS detection is used, the comparison between the results obtained with poly-LA 313 and poly-E 323 is not straightforward. In reference [31], poly-E 323-coated capillaries, conditions for the separation of peptides are different to the ones employed in this work, and no data about efficiencies were provided. Besides, the proteins analyzed in this work are different to the ones in ref. [31], except for ribonuclease A. The resolving power, in combination with the high efficiencies achieved, makes the poly-LA 313 coating useful for the analysis of complex samples.

3.5. Separation of biological samples

Human body fluids, such as CSF and plasma, are great sources of potential biomarkers for disease. Thus, the analysis of these body fluids is of high interest from a clinical point of view. Due to the complexity of these samples and the need to get structural information from the compounds presents in the samples, MS is often the detection technique of choice. However, the range of concentration of the compounds in the samples is extremely wide and the presence of high concentrations of salts, amines, carbohydrates and lipids, may promote difficulties to employ MS detection. Besides, adsorption of compounds on the silica wall of the capillary can occur, inducing lack of reproducibility in the separation and memory effects. The stability of poly-LA 313-coated capillaries was evaluated analyzing plasma, diluted 1/10 in MQ water, and CSF samples by CE-UV. The capillary was coated with 3.75% (w/w) poly-LA 313 solution, 7 mM ionic strength formate buffer pH 2.2 was employed as BGE and the rest of experimental conditions are given in legend of Fig. 3. The coating showed high stability against both complex samples. The CV values of EOF were 2.3% ($n = 10$) and 0.9% ($n = 9$), for CSF and plasma, respectively. No memory effect was observed when these samples were analyzed, opposite to other cationic polymers what made compulsory the regeneration of the coating between every run [35].

Human CSF diluted 1/2 (Fig. 8), and plasma diluted 1/100 (data not shown), were analyzed using poly-LA 313-coated capillaries by CE-ESI-MS with sheathflow interface. The main compound present in the sample is human serum albumin (HSA). In these analyses, especially for the CSF one, the capillary was overloaded with the sample, as it should be expected when the analysis of minority compounds is required. Besides, no pre-treatment or cleanup step, such as desalting, was performed on the sample which gives rise to high ionic strength in the sample. Both aspects may contribute to have not very high efficiency values (Fig. 8, 0.3×10^4 plates/m) in the separation. However, in spite of that, Fig. 8 show the usefulness of the employment of poly-LA 313-coated capillaries to perform CE-ESI-MS of proteins in body fluids. The coating avoids the adsorption of

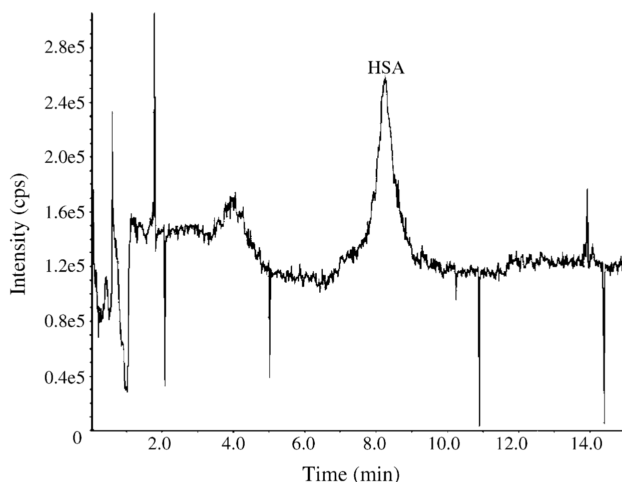

Fig. 8. Electropherogram of human CSF diluted 1/2 with MQ water, analyzed by CE-ESI-MS with sheath-flow interface and using a poly-LA 313-coated capillary. Selected ion profile: 900–2200 *m/z*. Capillary dimensions and coating process as in Fig. 7. Analysis conditions: Injection by pressure at 50 mbar during 5 s; CE voltage, −666 V/cm; BGE, 15 mM ionic strength formate buffer pH 2.8. Detection conditions: Sheath liquid, mixture of 50% of 10 mM formic acid and 50% of methanol at 1 μL/min; without nebulization; voltage at the sampling capillary at the entrance of the MS, +5.0 kV; frequency at the ion pulser at the TOF analyzer, 1 cycle/s.

compounds on the silica wall enabling the analysis of proteins as big as HSA with 66 kDa of molecular weight and the MS provides structural information of compounds in the sample and their identification. Consequently, the combination of CE and MS, using poly-LA 323-coated capillaries, provides an important analytical tool in protein analysis with potential use in screening of biomarkers.

4. Concluding remarks and future aspects

In this study, a novel cationic polymer, poly-LA 313, for non-covalent coating of fused silica capillaries in CE was introduced. Synthesis of poly-LA 313 is straightforward and the new polymer has been characterized by MALDI-MS and static light scattering. The capillary coating process is very fast and simple obtaining a polymeric coating which is stable in a wide pH range, and it is compatible with CE-ESI-MS due to robust anodal EOF and tolerance towards organic modifiers. Capillaries coated with poly-LA 313 enabled fast analyses by CE-UV and CE-ESI-MS, with high resolution and efficiency of proteins and peptides in a wide MW and p*I* range, mainly neutral and basic proteins and peptides. The efficiency of the separation and suppression of the non-specific adsorption is enhanced by using the poly-LA 313-coated capillaries in comparison to the use of bare fused silica capillaries. The coating showed high tolerance against complex biological samples (plasma and CSF), which makes poly-LA 313 a valuable coating for use in clinical analysis. Next step will be to apply the novel polymer poly-LA 313 to in-house fabricated microstructure devices in PDMS [36,37].

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