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Original article

Improvement of injection conditions for zone electrophoresis of proteins in capillaries coated with N, N-didodecyl-N, N-dimethylammonium bromide

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Abstract: Fused silica capillaries treated with N, N-didodecyl-N, N-dimethylammonium bromide was used in capillary electrophoresis to separate basic proteins using ammonium acetate as BGE at pH 4.0, 70 mM. This surfactant forms a semi-stable coating that provides a strong anodic electroosmosis; however, such coating must regularly be regenerated to maintain efficiency and reproducibility. The temporal stability of the coating was greatly improved by a new procedure as described in previously study by our group. In this study, it is found such experimental conditions that the starting zone could be enriched without loss of sample. The injection of a short plug of the BGE diluted 100 times for 3 sec in 50 µm i.d. capillaries behind the sample zone at 15 kV gave the best performance, whereas, a plug of lower dilution degree (10 times) in 25 µm i.d injected for 3 sec at the same field strength gave the optimum results for this dimension. In conclusion, those conditions were found to effectively decrease the loss of sample and to give a higher separation performance in regard to peak efficiency and resolution compared to injecting the sample without a plug.

Introduction

Capillary electrophoresis (CE) is one of the driving forces in modern analytical chemistry for analysis of a wide variety of low to high molecular weight analytes, such as nucleic acids, peptides and proteins. Efficient separations of these substances are of a great interest in clinical and pharmaceutical research, as well as in other related fields [1 - 3]. CE has gained in a popularity due to its high separation performance, versatility, automation and short analysis time based on improved heat dissipation and thus the possibility of applying high electric fields [4, 5]. However, the tendency of positively charged analytes such as peptides and proteins to adsorb onto the deprotonated silanol groups of the inner wall of fused silica capillaries is of a major drawbacks of CE [6]. Adsorption of proteins to the capillary surface through hydrophobic, electrostatic interactions and/or hydrogen bonding lead to loss

of efficiencies, low protein recovery, poor reproducibility of migration time and lower sensitivity. Different approaches have been explored to minimize such adsorption effect. Capillary coating is one of the most commonly used approach which can either be covalent coupling to the ionized silanol groups on the capillary surface [7, 8] or non covalent coupling by using an adsorbed polymer or surfactant [9, 10]. Dynamic surfactant based coatings; using double chain "cationic" surfactants such as N, N-didodecyl-N, N-dimethylammonium bromide (DDAB) are becoming particularly attractive due to their versatility and simplicity. In addition, the surfactant need not be present in the BGE which might make the system possible to apply on-line to mass spectrometry [11 - 13]. The present investigation has been undertaken as a step to improve and complement a recently published study [14, 15]. The performance of protein separations in capillaries non-covalently coated with DDAB has been studied with the aim directed toward issues of prime importance for successful separations of proteins by CE: the conditions at the sample injection, providing the maximum sensitivity with acceptable efficiency.

Materials and methods

Chemicals and reagents: All solutions were prepared in the MQ water system (Millipore,

Bedford, MA, USA) and all reagents were of pro-analysis grade. The cationic surfactant N, N-didodecyl-N, N-dimethyl ammonium bromide (DDAB) (**Figure 1**) was received from (Aldrich, Milwaukee, WI, USA) and was used to coat the inner wall of fused silica capillaries at a concentration of 1.0 mM. Sodium chloride (NaCl) (Scharlau Chemie, Barcelona, Spain) and sodium hydroxide (NaOH) (Merck KGaA, Darmstadt, Germany) were used to flush the fused silica capillaries. Untreated fused silica capillaries with 25 µm and 50 µm inner diameters (i.d) and 365 µm outer diameters (o.d) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Acetic acid (HAc), ammonium acetate (NH_4Ac) and 25.0% ammonia solution (NH_3) (Riedel-de Haen, Germany) were used to prepare the CE buffer (ammonium acetate, NH_4Ac, pH 4, 70 mM). The Henderson-Hasselbalch equation was used to calculate the concentration of the ionic component of the buffer. The buffer solution was passed through a Minisart N syringe filtration unit with filters of a pore size of 0.45 µm (Sartorius AG, Göttingen, Germany). Mesityloxide (Aldrich) dissolved in the running buffer was used as the neutral EOF marker. Protein samples: cytochrome c (horse heart), α-chymotrypsinogen A (bovine pancreas) and lysozyme (chicken egg white) were purchased from Sigma (St. Louis, Mo, USA). The stock protein solutions were made at the concentration of 1 mg /mL (46 - 82 µM) and diluted in the BGE to the final concentration of 0.1 mg/ml.

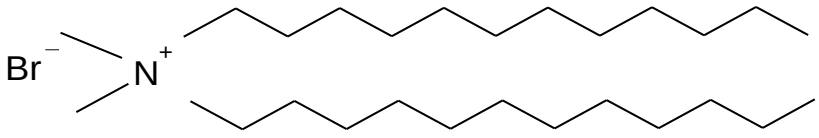


Figure 1: Structure of N, N-didodecyl-N, N-dimethylammonium bromide coating

Instrumentation and separation conditions: An automated Agilent capillary electrophoresis system, Agilent^{3D} CE (Palo Alto, CA, USA), interfaced with a HP Pentium II personal computer was used in all CE experiments. The system was equipped with a photodiode-array recording the UV absorbance at a wavelength of 200 nm for protein separations and the EOF marker at 254 nm. The total and effective lengths of the capillaries were 48.5 cm and 40 cm, respectively. 0.25 cm section was burned off by electric heating for on-capillary UV detection. The separations were carried out at 25 ^oC and at field strengths of 250 - 450 V/cm with 10 sec ramping. The protein samples were dissolved in the running buffer to a final concentration of 0.1 mg /ml and

injected hydrodynamically at 50 mbar for 6 - 30 sec. The capillary was flushed with fresh and degassed BGE for 2 min prior to each run. The stability of the baseline was found to be strongly dependent on the buffer freshness; stable baseline was obtained only by using fresh BGE in each run with RSD < 02.0% for the repeatability of the migration times (t_m) of the analyte. The plate numbers were calculated by statistical moments method provided by standard Agilent software.

The non-covalent coating with DDAB and the EOF measurements: The coating procedure was performed as previously described [14, 15]. The capillaries were pre-treated prior to the coating by consecutive washings at 935 mbar for 20 min with 1 M NaOH, 0.1 M NaOH and MQ water, respectively (time = 60 min). This was followed by flushing the capillary at 935 mbar with at a water solution of 1.0 mM DDAB for 5 min and an equilibration step for 5 min with a total repetition of 3 cycles. Finally, the capillary was rinsed at 935 mbar with the BGE for 3 min, followed by the application of voltage (15 kV for 10 min) for equilibration. The quality of the coating was tested by injection of the EOF marker mesity-loxide (5 µL in 2 ml BGE) at 50 mbar for 6 sec and the electroosmotic mobility was calculated.

Results

The effect of injecting a plug of diluted buffer behind the sample: A buffer plug behind the sample zone was introduced to prevent or at least minimize losses of sample that could occur due to many factors like sedimentation, adsorption and volume expansion caused by Joule heating. These investigations give information on the optimum dilution factor and the injected plug volume for capillaries with different in inner diameters.

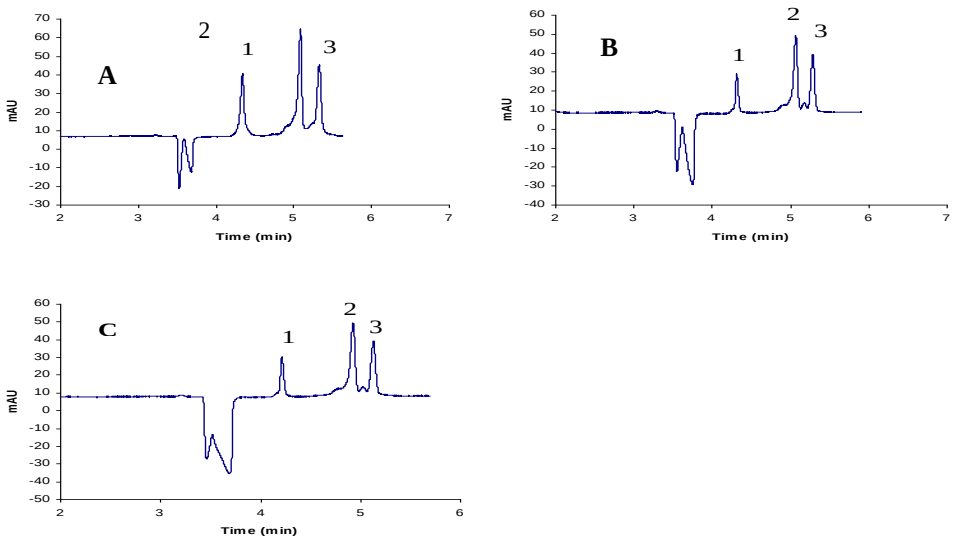
Capillaries with 50 µm i.d: The effects of injecting a short plug of BGE diluted 10 and 100 times behind the protein zone dissolved in 10 times diluted BGE (0.1 mg/mL) were investigated (**Table 1**). In all the experiments, the injection of a plug results in larger amounts of proteins in the sample zones compared to injection of the sample without plug as shown in **Table 2**. Accordingly, plugs behind the sample increased the peak areas indicating loss of injected proteins when no plug is used. Also, at the higher field strength (412 V/cm), the largest dilution gave the highest recovery that in some cases was almost 10 times higher compared to injection of sample without plug; although the differences obtained with the different plugs were quite small. For comparison, the effect of an undiluted buffer plug behind the sample was also calculated; however, as expected it gave instead a negative effect: the peak heights decreased by a third compared to the injection of the sample without plug (**Figures 2A and 2B**). The presence of 100 times diluted plug in front of the sample also had a negative effect on recoveries (**Figure 2C**). Obviously, other factors that decrease the recoveries dominate, like for instance adsorption of proteins to the surface at the position of the interface between the sample zone and the BGE zone. Mismatch of EOF between the different zones has probably an effect on the efficiencies.

Table 1: Peak efficiencies using plug of BGE behind the sample diluted capillary

Proteins	Sample without plug	10 times diluted plug		100 times diluted plug	
		3 sec	6 sec	3 sec	6 sec
α-chymotrypsinogen A	105.000 (14.200)	215.000 (25.150)	164.000 (19.000)	**314.000 (32.000)**	266.000 (22.000)
Lysozyme	221.600 (78.000)	266.000 (86.500)	193.000 (60.000)	**297.000 (89.200)**	270.000 (68.000)
Cytochrome c	195.000 (89.000)	284.000 (123.000)	199.000 (77.000)	**372.500 (130.500)**	293.000 (96.000)

The true efficiencies (number of plates per meter based on the electrophoretic mobility) are given within brackets.

Figure 2: Separation of mixture of proteins on DDAB coated capillary



A) Sample without plug, B) Injection of undiluted plug of BGE behind the sample and C) Injection of a 100 times diluted plug of BGE in front of the sample. Condition: 50 µm i.d. capillary, BGE; NH_4Ac at pH 4.0, 70 mM, field strength: 310 V/cm, protein samples dissolved in 10 times diluted BGE (0.1 mg/mL) and injected at 50 mbar for 6 sec. The amount of sample injected was 0.95 ng. Sample: 1) α-chymotrypsinogen A, 2) lysozyme and 3) cytochrome c.

Table 2: Measured relative sample sing short plug behind the sample at different injection times in capillary

Proteins	Sample without plug	10 times diluted plug		100 times diluted plug	
		3 sec	6 sec	3 sec	6 sec
α-chymotrypsinogen A	3.6 × 10-4 (1)	4.6 × 10-4 (1)	3.7 × 10-4 (1)	**5.1 × 10-4 (1)**	4.9 × 10-4 (1)
Lysozyme	4.9 × 10-4 (1.6)	5.6 × 10-4 (1.4)	5.2 × 10-4 (1.7)	**7.9 × 10-4 (1.8)**	7.4 × 10-4 (1.7)
Cytochrome c	2.8 × 10-4 (0.95)	3.2 × 10-4 (0.86)	3.2 × 10-4 (1.1)	**4.6 × 10-4 (1.09)**	4.4 × 10-4 (1.06)

Equation 1 was used to calculate the amount of protein in a zone. The highest values are marked in bold and the relative peak areas are given within brackets.

Capillaries with 25 µm i.d: The injection of 10 and 100 times diluted plug of the BGE behind the sample was also studied using a narrower capillary. The results indicated that 10 times diluted plug was beneficial regarding peak heights (**Figure 3**), the best effect was obtained for the short plug (3 sec) with an increase in peak heights of about 30.0 - 45.0%. However, 100 times diluted plug had no positive effect, irrespective of the plug length (**Figure 4**). The true and apparent efficiencies were generally higher using a plug irrespective of plug length (**Table 3**), the only exception being the 9 sec plugs at 100 times dilution. The relative amounts observed were somewhat different for the 25 i.d compared to the 50 µm i.d capillary. The 10 times diluted plug applied for 3 sec at a field strength of 310 V/cm was the best choice regarding recovery (6 - 9 times higher than without plug) (**Table 4**). However, increasing the field strength to 412 V/cm, the larger diluted plug gave about the same recovery as the 10 times diluted one (2.5 - 3.6 times higher).

Figure 3: Separation of a mixture of proteins on coated capillary using a short plug behind the sample diluted 10 times for A) 3 sec, B) 6 sec and C) 9 sec.

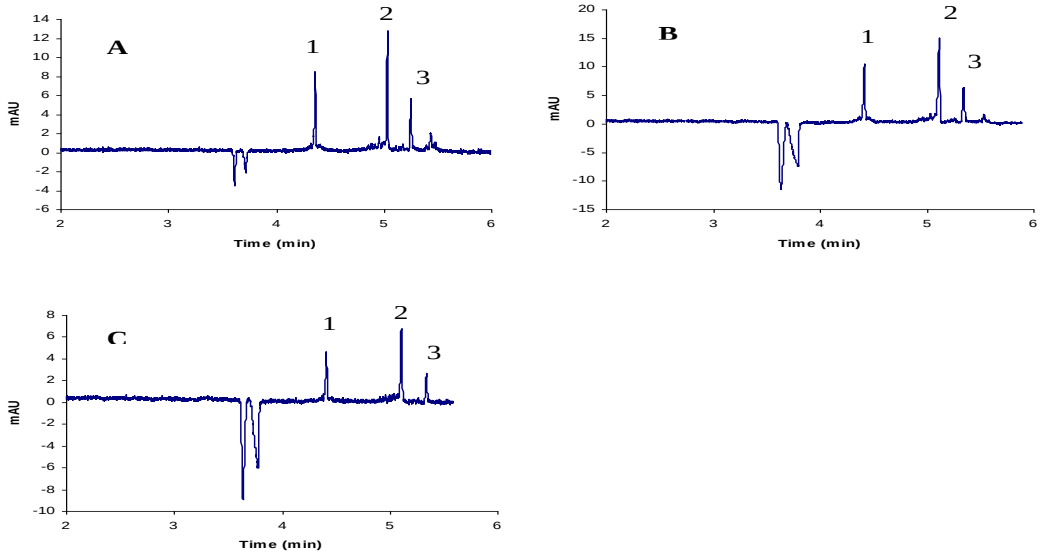


Table 3: Efficiencies using plug of BGE behind the sample diluted capillary

Proteins	Sample without plug	10 times diluted plug			100 times diluted plug		
		3 sec	6 sec	9 sec	3 sec	6 sec	9 sec
α -chymotry-psinogen A	769.000 (43.250)	**1.486.500 (83.250)**	1.120.300 (63.000)	763.500 (43.000)	963.500 (54.000)	939.900 (53.000)	235.000 (13.000)
Lysozyme	762.400 (125.000)	**3.250.000 (533.000)**	1.520.200 (249.500)	1.255.000 (249.500)	1.198.000 (196.500)	1.023.900 (168.000)	618.000 (101.50)
Cytochrome c	938.000 (208.250)	**2.321.400 (515.500)**	1.168.000 (259.500)	1.397.000 (310.000)	1.080.000 (240.000)	1.048.400 (233.000)	289.000 (64.000)

The highest values are marked in bold. The true efficiencies (number of plates per meter based on the electrophoretic mobility) are given within brackets.

Figure 4: Separation of mixture of proteins on coated capillary using short plug diluted 100 times behind the sample for A) 3 sec, B) 6 sec and C) 9 sec.

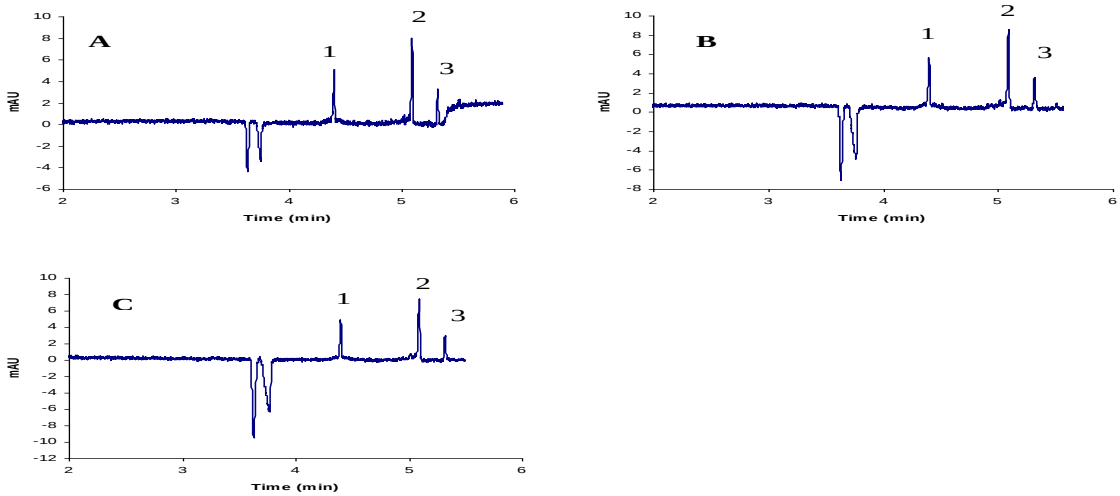


Table 4: Measured relative sample amounts using short plug behind the sample at different injection times in capillary

Proteins	Sample without plug	10 times diluted plug			100 times diluted plug		
		3 sec	6 sec	9 sec	3 sec	6 sec	9 sec
Chymotrypsinogen A	5.3 × 10-6 (1)	**4.8 × 10-5 (1)**	3.4 × 10-5 (1)	1.3 × 10-5 (1)	1.5 × 10-5 (1)	1.3 × 10-5 (1)	1.4 × 10-5 (1)
Lysozyme	7.3 × 10-6 (1.6)	**5.9 × 10-5 (1.7)**	4.9 × 10-5 (1.4)	1.9 × 10-5 (1.6)	2.4×10-5 (1.5)	1.2×10-5 (1.9)	2.2 × 10-5 (1.85)
Cytochrome c	3.1 × 10-6 (0.7)	**2.0 × 10-5 (0.73)**	1.9 × 10-5 (0.5)	8.0 × 10-6 (0.66)	9.3 × 10-6 (0.75)	7.2 × 10-6 (0.8)	8.3 × 10-6 (0.65)

Equation 1 was used to calculate the amount of protein in a zone.
The highest values are marked in bold and the relative peak areas are given within brackets.

Discussion

The use of 10 fold diluted plug resulted on average in an increase of peak heights and areas of about 15.0% compared to the injection without a plug. In comparison, injection of a short plug diluted 100 times and injected for 3 sec at the field strength 310 V/cm larger effects were obtained; increases of about 60.0% of the peak heights and areas in addition to a significant increase in the peak efficiency (**Table 1**). The highest efficiencies were obtained with those conditions while increasing the time to six sec decreased the efficiency. Furthermore, the efficiencies were generally 4 - 6 times lower in runs at field strength of 412 V/cm compared to runs at 310 V/cm. Almost equivalent decrease of the number of plates was obtained by decreasing the field strength to 247 V/cm. The effect of a 100 times diluted plug injected behind the sample in those experiments was as beneficial as the above run at 310 V/cm; the efficiency increased with 50 - 90% (not shown). The relative amount of protein in a zone was calculated using Equation 1:

$$Q = \pi \cdot R^2 \cdot v \cdot A \qquad (1)$$

Where Q = relative amount of protein (g) in the zone

v = the velocity of the zone (peak) ($cm/\sec$)

R = the capillary radius (cm)

A = the peak area ($g \cdot cm^{-3} \cdot s$)

The presence of a plug of 100 times diluted BGE behind the sample zone increased the amount of protein in the zone with 40.0 - 65.0% when the injection time was 3 sec (6 sec gave a similar result) using a field strength of 310 V/cm and 50 µm i.d capillary (**Table 2**). The same experiments were conducted using 25 µm i.d. capillaries and the obtained results were fairly different. The results indicated that losses of the proteins occur at the separations and the amount of the sample lost with the 25 µm i.d capillary was higher compared to that of 50 µm i.d capillary. Further evaluations and studies of the phenomenon are ongoing.

Conclusion: Novel and simple procedure to improve the injection condition of non-covalently bound DDAB was studied. Injection of a plug of the BGE behind the protein samples diluted 10 and 100 times in 25 and 50 µm i.d. capillary was explored in order to prevent sample losses. The findings indicated that injection of short plug of BGE (70 mM NH_4Ac at pH 4) diluted 10 and 100 times for short time (3 sec) behind the sample in 25 and 50 µm i.d. capillaries, respectively, is the best way to avoid sample losses and to give high efficiencies. The precision of the migration times seems to be independent on the degree of the dilution in the plugs.

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Author contributions: All authors substantially contributed to the conception, compilation of data, checking and approving the final version of the manuscript, and agreed to be accountable for its contents.
Ethical issues: Including plagiarism, informed consent, data fabrication or falsification and double publication or submission have completely been observed by authors.
Author declarations: The authors confirm all relevant ethical guidelines have been followed and any necessary IRB and/or ethics committee approvals have been obtained.

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