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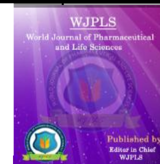

ENHANCEMENT OF COATING STABILITY FOR CAPILLARY ZONE ELECTROPHORESIS OF PROTEINS IN DDAB MODIFIED SURFACES

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ABSTRACT

Separation of basic proteins with capillary electrophoresis was performed using a cationic double chain N, N-didodecyl-N, N-dimethylammonium bromide (DDAB) as coating reagent for fused silica capillaries and ammonium acetate as background electrolyte (BGE) at pH 4.0, 70 mM. This double-chained surfactant forms a semi-stable coating that provides a strong electro-osmosis flow (EOF) toward the anode. To maintain the highest efficiency and reproducibility, such coating must regularly be regenerated. The temporal stability of the coating was improved by a new procedure which increases the hydrophobic interaction between the DDAB coating and the silica wall and thus makes the coating more stable and at the same time the electrostatically adsorbed proteins are released. The procedure includes rinsing the capillaries, which were kept overnight in 1.0 mM DDAB solution, with (1:1 v/v) 0.5 M NaCl in the BGE. Rinsing the capillaries with this solution gave a stable coating which permitted the separation of proteins for up to 3 consecutive days without recoating and with maintained good precision in migration times of the analytes and EOF measurements compared to the previously published method in which the capillary can be used for 9 hours only.

KEYWORDS: Coating Stability, Sodium Chloride Rinsing, Efficiency.

1 INTRODUCTION

In recent year, capillary electrophoresis (CE) has become one of the most powerful analytical tools for the analysis of a wide variety of peptides and protein mixtures. Besides high efficiency, CE offers additional advantages in the area of analytical biotechnology such as very low requirement of sample volume and rapid separation time.[1-3] Excellent separation efficiencies can be obtained using CE because the small diameter of capillaries allows for a very efficient heat dissipation and as a result band broadening due to Joule heating is minimized and high electric field can be applied.[3, 4] However, positively charged analytes, such as peptides and proteins have the tendency to adsorb onto the deprotonated silanol groups of the inner wall of fused silica capillaries which considered as the major drawbacks of CE.[5] Such adsorption effect of positively charged analytes to the capillary surface via hydrophobic, electrostatic interactions and/ or hydrogen bonding lead to loss of efficiencies, low recovery, poor reproducibility of migration time and lower sensitivity. Different studies have shown that capillary coating is one of the most commonly used approaches to minimize such adsorption effect.[6] Capillary coatings can either be covalent coupling to the ionized silanol groups on the capillary surface or non covalent coupling by using an adsorbed polymer or surfactant.[7,8] Dynamic surfactant based coatings using double chain surfactants are becoming particularly attractive due to their simplicity and the possibility of coupling the technique on line to the mass spectrometer since the surfactant need not be present in the BGE.[9-11]

However, cationic surfactants, such as N, N-didodecyl-N, N-dimethylammonium bromide (DDAB), form semi-stable coatings.[12,13] which at certain intervals must be regenerated to maintain efficiency, robustness and reproducibility. A number of approaches have been

employed to improve the stability of the DDAB coating including for instance an increase in ionic strength and pH of the BGE and/ or a decrease in the capillary diameter.[14] The use of surfactants with longer chains was also shown to give more stable coatings.[14,15] The present investigations have been undertaken as steps to improve and complement a recently published study.[12,13] 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 generation of a stable non-covalent coating that can stand numerous injections of samples providing the maximum acceptable efficiency.

2 Experimental Conditions

2.1 Chemicals and Reagents

All reagents were of pro analysis grade and MQ water system (Millipore, Bedford, MA, USA) was used to prepare all needed solutions. 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). 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.

Acetic acid (HAc), ammonium acetate (NH_4Ac) and 25 % ammonia solution (NH_3) (Riedel-de Haen, Germany) were used to prepare the CE buffer (ammonium acetate, NH_4Ac, pH 4, 70 mM). 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). The Henderson-Hasselbalch equation was used to calculate the concentration of the ionic component of the buffer. 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.

2.2 Instrumentation and Separation Conditions

In all CE experiments, an automated Agilent capillary electrophoresis system, Agilent ^{3D} CE (Palo Alto, CA, USA), interfaced with a HP Pentium II personal computer was used. 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. A 0.25 cm section was burned off by electric heating for on-capillary UV detection. The total and effective lengths of the capillaries were 48.5 cm and 40 cm, respectively. The separations were carried out at 25 ^{0}C 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 BGE for 2 min prior to each run. A stable baseline was obtained by using fresh BGE in each run with RSD < 2.0 % for the repeatability of the migration times (t_m) of the analyte. The plate numbers were calculated by the statistical moments method provided by the standard Agilent software.

2.3 The DDAB coating and the EOF measurements

The non-covalent coating procedure with DDAB reagent was performed as previously described.[12,13] 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 (total time 60 min). This was followed by flushing the capillary at 935 mbar with 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 mesityloxide (5 µL in 2 mL BGE) at 50 mbar for 6 sec and the electroosmotic mobility was calculated.

3 RESULTS AND DISCUSSIONS

3.1 Improvement of the DDAB coating stability

Adsorption of the cationic DDAB coating to the ionized silica wall makes its surface charge strongly positively charged which creates high electroosmotic mobility with a flow towards the anode. Monitoring the stability of the electroosmotic mobility over time gives thus indications on the coating stability.[14, 16] According to an earlier protocol.[12, 13], separations with high repeatability of the migration time of the analyte could generally be performed up to 7 hours following the coating. In those studies, the capillaries were stored overnight in MQ water; however, the separation performance was severely decreased during the following days. Thus, we investigated some procedures that could maintain the original good performance of the capillaries with regard to the EOF stability and the repeatability of t_m of the analytes. The first attempts involved keeping the capillaries overnight in a DDAB solution and then recoating the capillaries the following day. However, this procedure did not work well since the EOF was lower than the original value, the peaks were broader, the migration times were longer and the resolution decreased compared to those obtained in a freshly coated capillary (Figure 2 and Table 1). Furthermore, the EOF continuously decreased at subsequent separations (the $\mu_{EOF} = 6.3\times10^{-4}$ (cm ^{2} /Vs) in the first day and $\mu_{EOF} = 4.8\times10^{-4}$ (cm ^{2} /Vs) on the third day) and the

separation performance decreased the following days although the capillary was kept overnight in DDAB and then subjected to a complete recoating procedure on the next day. This indicates a less complete coverage of the capillary surface with DDAB. Therefore, an alternative procedure was investigated in order to improve the coating stability and permit the use of the same coated capillary for a longer period (few days) without recoating in between the runs or days. The new procedure involved washing the capillary, which was kept overnight in DDAB, with a 1:1 v/v solution of 0.5 M NaCl in the BGE for 10 min and then for an additional 2 min with the BGE without recoating with DDAB was performed. Significant improvement in the EOF and also the coating stability was obtained using this procedure, providing an EOF that was very close to that obtained by the freshly coated capillary (Figure 3). The EOF mobility was $\mu_{EOF} = 6.5\times10^{-4}$ (cm^2/Vs) in the first day and $\mu_{EOF} = 6.2\times10^{-4}$ (cm^2/Vs) on the third day of use. This procedure permitted separation of proteins for up to 3 consecutive days without recoating with maintained good repeatability of the migration times of the analyte and EOF mobility (Figure 4 and Table 2). The results indicated high peak efficiency can also be obtained using the new rinsing procedure as it can be seen in Table 3 compared to previously published procedure. Moreover, the third day of using the same capillary gives good peak shape similar to freshly coated capillary (Figure 5). The experiments also indicated that the inner diameter of the capillary did not influence the outcome of this optimized coating procedure.

It was assumed earlier.[12, 13] that the DDAB coating is interacting with the silica surface mainly by electrostatic interactions. It was shown that the presence of ammonium ions in the BGE used in experiments to coat the capillaries prevented the build-up of a stable coating.[12] However, by exchanging the ammonium ions for sodium ions a more stable coating was obtained. The hypothesis is that there is a competition between the ammonium ions and the DDAB ions for the binding sites on the surface compared to other cations.[12] The main purpose of flushing the capillary with NaCl was to increase the hydrophobic interaction between the hydrophobic groups in the DDAB and the silica wall and thus to make the coating more stable. This type of interaction increases with an increase of the ionic strength. This procedure appeared to work satisfactory; the net result was the formation of a more stable coating: the same capillary can be used for at least three consecutive days without recoating in between the runs or between the days. This procedure was therefore used in all subsequent experiments in this study.

Figure legends

Figure 1. Structure of N, N-didodecyl-N, N-dimethylammonium bromide (DDAB) coating.

Figure 2. Separation of a mixture of proteins on A) freshly coated capillary with 1.0 mM water solution of DDAB, B) capillary recoated on the second day and C) capillary recoated on the third day; C-1) the first run and C-2) the second run. 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.

Figure 3. Coating stability of DDAB demonstrated by the anodic EOF as a function of days in 50 µm i.d fused silica capillary coated with 1.0 mM water solution of DDAB. BGE: 0.07M ammonium acetate pH 4, field strength 310 V/cm (10 s ramping). Sample: mesityloxide injected by pressure (50 mbar for 6 s). A) Capillary recoated every day and without washing with NaCl and B) Capillary stored in 1.0 mM DDAB and then rinsed only with 0.5M NaCl without recoating.

Figure 4. Separation of a mixture of proteins on DDAB coated capillary (50 µm i.d.) using the new rinsing procedure. A) Freshly coated capillary and B) capillary used for the third day without recoating. Other conditions as given in Fig 2.

Figure 5. A comparison of the separation of a mixture of proteins on 50 µm i.d capillary on the third day of use without recoating. A) First run and B) second run. Rest of the condition as given in Fig 2.

Table legends

Table 1. A comparison of migration time and peak height of proteins when the capillary was freshly coated and when kept overnight in DDAB and then recoated. The protein was injected hydrodynamically (50 mbar for 6 s) in a 50-µm fused silica capillary coated with 1.0 mM water solution of DDAB; BGE: 0.07 M ammonium acetate pH 4.0. Field strength: 310 V/cm. Sample: 1) α-chymotrypsinogen A, 2) lysozyme and 3) cytochrome c, dissolved in 10 times diluted BGE at a concentration of 0.1 mg/mL, the amount of sample injected: 0.95 ng.

Table 2. A comparison of migration time and peak height of proteins using the new rinsing procedure with NaCl. Rest of condition as given in table 1.

Table 3. A comparison of efficiencies using the new rinsing procedure in a 50 µm i.d capillary. The highest values are marked in bold. The true efficiencies (number of plates per meter based on the electrophoretic mobility) are given within brackets.

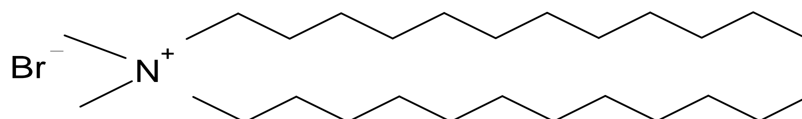

Figure 1.

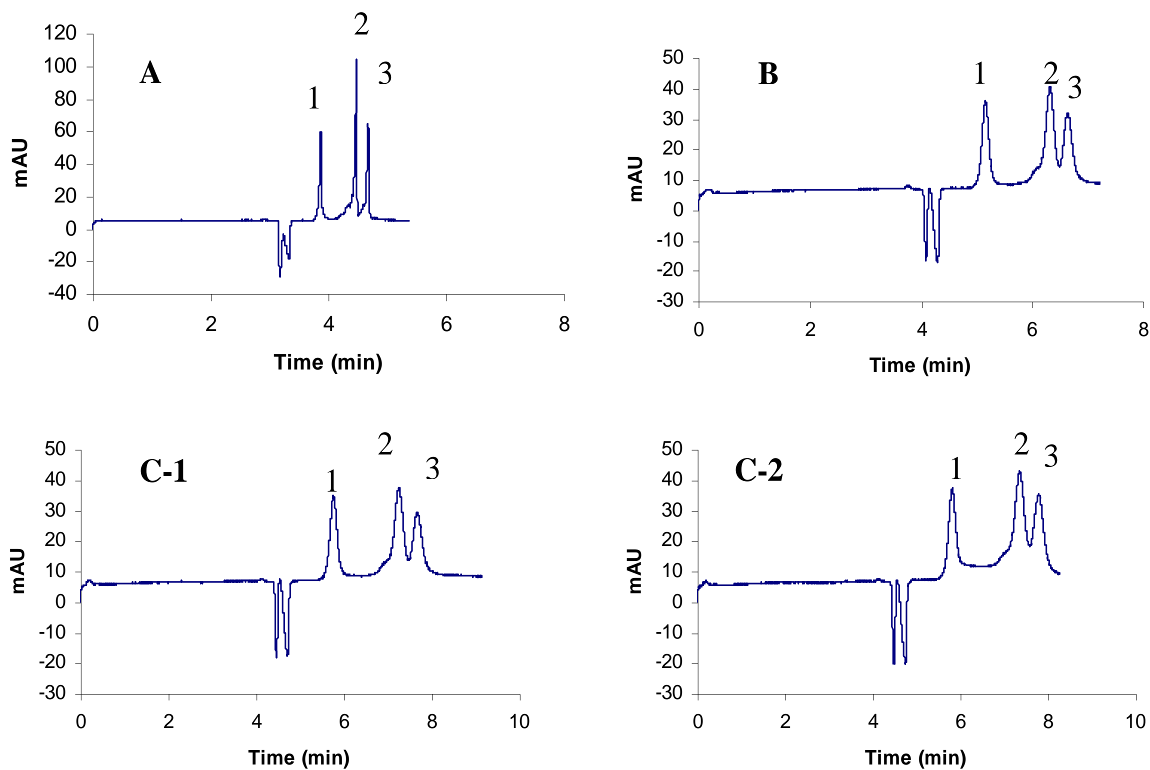

Figure 2.

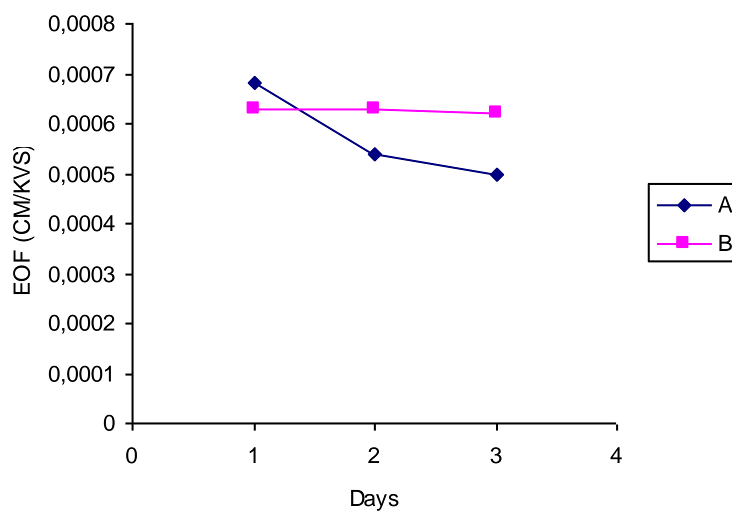

Figure 3.

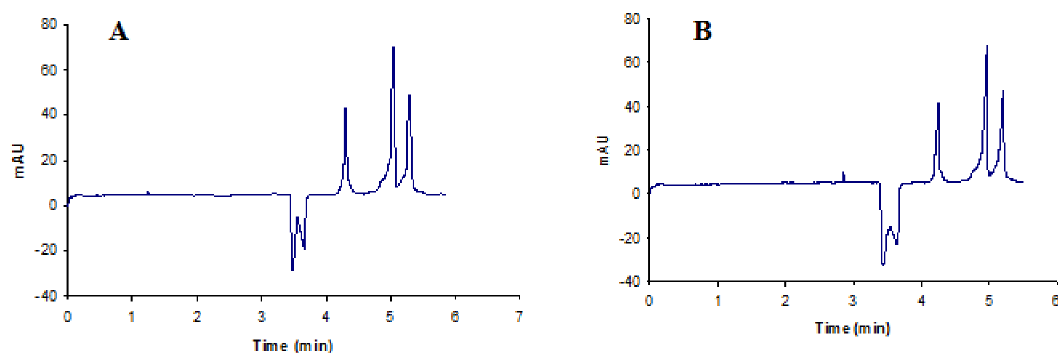

Figure 4.

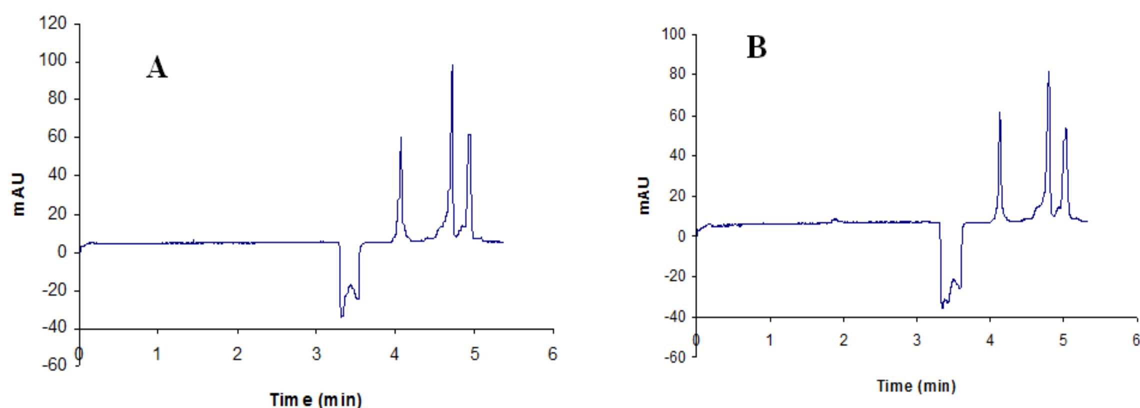

Figure 5.

Table 1.

Protein	Freshly coated capillary $\mu_{EOF} = 6.8\times10^{-4}$ (cm^2/Vs)			Recoated capillary $\mu_{EOF} = 5.4\times10^{-4}$ (cm^2/Vs)		
	t_m (min)	Peak height (mAu)	Peak area (mAu)	t_m (min)	Peak height (mAu)	Peak area (mAu)
α -chymotryp-sinogenA	4.1	61.7	143	5.2	28.2	232
Lysozyme	4.8	115	255	6.3	31.2	289
Cytochrome c	4.9	75.1	156	6.7	22.8	170

Table 2.

Protein	Freshly coated capillary $\mu_{EOF} = 6.5\times10^{-4}$ (cm^2/Vs)			NaCl treated capillary and no recoating (3rd day of use) $\mu_{EOF} = 6.2\times10^{-4}$ (cm^2/Vs)		
	t_m (min)	Peak height (mAu)	Peak area (mAu)	t_m (min)	Peak height (mAu)	Peak area (mAu)
α -chymotryp-sinogenA	4.2	63.0	141	4.7	60.2	147
Lysozyme	4.7	113	252	5.1	110	259
Cytochrome c	5.1	77.2	153	5.5	75.8	161

Table 3.

Proteins	Freshly coated capillary	2nd day of use without recoating and rinsed only with NaCl	3rd day of use without recoating and rinsed only with NaCl
α -chymotrypsino-gen A	779.000 (45.000)	765.000 (43.000)	763.000 (44.000)
Lysozyme	782.000 (115.000)	750.000 (113.000)	798.000 (119.000)

Cytochrome c	937.000 (205.000)	921.000 (195.000)	908.000 (190.000)

4 CONCLUSIONS

A new and simple rinsing procedure to improve the coating stability of the non-covalently bound DDAB was investigated. A DDAB coated capillary could be used providing the same performance for at least 3 days of continuous use without recoating. The improved procedure involves storing the capillary overnight filled with a DDAB solution and flushing with a mixture (1:1 v/v) of 0.5 M NaCl and 70 mM NH_4Ac at pH 4 the day before working with the capillary. This means a prolongation of the coating stability by at least a factor of three days compared to earlier published data.

5 ACKNOWLEDGMENTS

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