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# Successive Multiple Ionic-Polymer Layer (SMIL) Coatings for intact protein analysis by Capillary Zone Electrophoresis - Mass Spectrometry – Application to Hemoglobin Analysis

L. Salzer, A. Stolz, L. Dhellemmes, A. Höchsmann, L. Leclercq, H. Cottet, C. Neusüß

## Abstract

Successive multiple ionic-polymer layer (SMIL) coatings are frequently used for protein analysis in capillary electrophoresis resulting in high repeatability and separation efficiency. Here, the coating procedure of a 5-layer SMIL coating is described using quaternized diethylaminoethyl dextran (DEAEDq) as polycation and poly(methacrylic acid) (PMA) as polyanion. Depending on the analyte, different polyions may be used to increase separation efficiency. However, the coating procedure remains the same. To demonstrate the possibilities of SMIL coatings, human hemoglobin was measured by CE-MS in 2 M acetic acid BGE. DEAEDq-PMA coating was found to be the most suitable for hemoglobin analysis due to relatively low reversed electroosmotic mobility leading to increased electrophoretic resolutions. Thereby, not only alpha and beta subunit of the hemoglobin could be separated, but also glycosylated and carbamylated species were separated within 24 min.

**Key words** SMIL coatings, polyelectrolytes, static adsorbed capillary coatings, CZE, CE-MS, hemoglobin

## 1 Introduction

Capillary coatings are frequently used in capillary electrophoresis (CE) for protein analysis to lower analyte adsorption on the capillary wall [1]. The results are increased reproducibility and separation efficiency, which is especially important in CE coupled to mass spectrometry (CE-MS) [2–6]. There are covalently bonded coatings, dynamic coatings or static-adsorbed coatings such as successive multiple ionic-polymer layers (SMIL) [2]. SMIL coatings are frequently used in CE analyses due to their high stability, simple preparation and possible regeneration [3, 7–9]. Katayama et al. introduced an anionic 2-layer SMIL coating of polybrene (PB) and dextran sulfate (DS), which was stable over 100 runs of acidic protein separations in phosphate buffer [10]. However, several studies have been investigating the influence of SMIL conditions on the protein analysis in CE. A cationic polymer as the last layer leads to a reversed electroosmotic flow (EOF), which lowers protein adsorption due to repulsive charges in acidic conditions [11].

The nature of polyanions and polycations also plays an important role in separation efficiency and stability. Schlenoff et al. found out that strong polyelectrolytes, such as poly(diallyldimethylammonium) chloride (PDADMA) and poly(styrene sulfonate) (PSS), result in efficient protein separations [12]. Excellent results can be obtained with 5-layer coatings using 1.5 M ionic strength coating solutions and a polyelectrolyte concentration of 0.5% w/v, using phosphate BGE for peptide and protein separations [13, 14]. The influence of the background electrolyte (BGE) on electroosmotic mobility was found out by Swords et al. to be significant in the case of weak but not of strong polyelectrolytes [15].

Leclercq et al. investigated the separation performance based on the separation efficiency and repeatability of a test mixture of 5 standard proteins in 5-layer SMIL coatings using different polyanions and polycations in acetic acid BGE [16]. The nature of the polycation allows the

modulation of the electroosmotic mobility over a wide range <sup>\*\*\*ref16\*\*\*</sup>. Best separation efficiencies were obtained using coatings with poly(L-lysine citramide) (PLC) and polycations with low to moderate linear charge densities as PB or  $\epsilon$ -poly(lysine) ( $\epsilon$ -PLL). It was found that the nature of the polyanion was also crucial for the separation efficiency, and not only the nature of the polycationic last layer.<sup>\*\*\*\*ref 7 and 16\*\*\*\*</sup>

For hemoglobin CE-MS analysis, it was found that quaternized diethylaminoethyl dextran (DEAEDq) as the polycation and poly(methacrylic acid) (PMA) as the polyanion in a 2 M acetic acid BGE led to the highest resolutions due to both low electroosmotic mobility leading to high selectivity, and high separation efficiencies. Hemoglobin is the main component of erythrocytes and can achieve concentrations up to 120 g/L in our blood [17, 18]. It is a globular protein consisting of four subunits, two alpha and two beta subunits, which differ in their amino acid sequence but have a similar tertiary structure [9, 19]. Moreover, there are several variants that may also be clinically relevant, such as aminoacid exchanges or posttranslational modifications (PTMs) such as glycation or carbamylation [20, 21]. Due to their great similarity, the CZE analysis of hemoglobin requires a capillary coating with very high separation efficiency to separate its subunits or other hemoglobin variants, such as glycation or carbamylation [22].

Here, the coating procedure of 5-layer SMIL coatings is described using DEAEDq and PMA. However, the procedure can be adapted to any other combination of polyanion and polycation. The polyions should be selected individually, depending on the protein to be analyzed. The separation efficiency, and the selectivity (due to modulation of the electroosmotic mobility), may be increased by changing the polyions.

In addition, this chapter shows the CE-MS analysis of a glycated human hemoglobin sample with DEAEDq-PMA in 2 M acidic acid as the volatile BGE. It was not only possible to separate alpha and beta subunit of the hemoglobin; moreover, glycated species could be separated.

## 2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M $\Omega$  cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

### 2.1 SMIL coating

1. CE electrophoresis instrument (*see Note 1*).
2. Capillary: 70 cm total length x 50  $\mu$ m I.D. Cut a 70 cm  $\times$  50  $\mu$ m I.D. fused silica capillary (*see Note 2*).
3. HEPES buffer: 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethane sulfonic acid (HEPES) buffer, pH 7.4. Weigh 47.66 mg HEPES and add 10 mL water. Mix and adjust pH with NaOH (*see Note 3*). Store at 4 °C.
4. Polyanion solution: 3 g/L poly(methacrylic acid) (PMA,  $M_n$  50,000 g/mol, PDI = 2.5,  $pK_a$  = 4.2-4.8,  $\xi$  = 2.88 was synthesized as described by Leclercq et al. [23]) in HEPES buffer (*see Note 4*). Weigh 3 mg PMA and dissolve it in 1 mL 20 mM HEPES buffer pH 7.4. Store at -18 °C (*see Note 5*).
5. Polycation solution: 3 g/L quaternized diethylaminoethyl dextran (DEAEDq,  $M_w$  500,000 g/mol,  $\xi$  = 0.35 was obtained from Pharmacia (Uppsala, Sweden)) in HEPES buffer (*see Note 4*). Weigh 3 mg DEAEDq and dissolve it in 1 mL 20 mM HEPES buffer pH 7.4. Store at -18 °C (*see Note 5*).
6. Sodium Hydroxide: 1 M NaOH
7. Background Electrolyte (BGE): 2 M acetic acid (AA) in water

## 2.2 Hemoglobin CE-MS analysis

1. Glycated human hemoglobin: 2 g/L of lyophilized powder of glycated human hemoglobin (HbA<sub>1c</sub>) standard is diluted in deionized water.
2. Mass spectrometer (*see Note 6*)
3. CE-ESI-MS interface, triple-tube design (*see Note 7*)
4. Sheath-liquid: water and 2-propanol (1:1 v/v) and 0.5% formic acid (FA)
5. Syringe pump equipped with a 5mL syringe

## 3 Methods

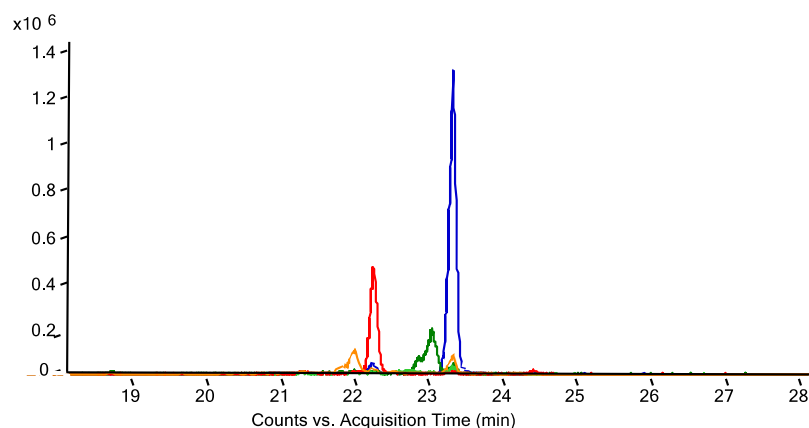
Carry out all procedures at room temperature unless otherwise specified.

### 3.1 5-layer SMIL coating

1. Prepare 1 mL of 1 M NaOH, water, HEPES buffer, BGE, 0.8 mL of polyanion solution and polycation solution (*see Note 8*), and 0.5 mL of water as waste, in CE vials and close them with caps.
2. The capillary is installed in the CE using a cassette. The cassette is tempered at 25°C (*see Note 9*)
3. Flush the capillary with external pressure of 2 bar 20 min with 1 M NaOH (*see Note 10*).
4. Flush the capillary with external pressure of 2 bar 5 min with deionized water.
5. Flush the capillary with external pressure of 2 bar 10 min with HEPES-buffer.
6. Flush the capillary with external pressure of 2 bar 7 min with the Polycation.
7. Flush the capillary with external pressure of 2 bar 3 min with HEPES-buffer.
8. Flush the capillary with external pressure of 2 bar 7 min with the Polyanion.
9. Flush the capillary with external pressure of 2 bar 3 min with HEPES-buffer.
10. Flush the capillary with external pressure of 2 bar 7 min with the Polycation.
11. Flush the capillary with external pressure of 2 bar 3 min with HEPES-buffer.
12. Flush the capillary with external pressure of 2 bar 7 min with the Polyanion.
13. Flush the capillary with external pressure of 2 bar 3 min with HEPES-buffer.
14. Flush the capillary with external pressure of 2 bar 7 min with the Polycation.
15. Flush the capillary with external pressure of 2 bar 3 min with HEPES-buffer.
16. Flush the capillary with external pressure of 2 bar 10 min with BGE.

### 3.2 CE-MS of Hemoglobin

1. Couple CE to the MS using a CE-ESI-MS interface (*see Note 11*).
2. Set the sheath-liquid flow to 4 µL/min using the syringe pump. Set the nebulizer gas to 4 psi (*see Note 12*).
3. Turn on the MS using suitable MS parameters (*see Note 13*).
4. Place a BGE vial to the inlet position. Precondition the capillary with BGE for 5 min before each run (*see Note 14*).
5. Inject 2 g/L glycated human hemoglobin hydrodynamic 30 mbar for 8 sec (*see Note 15*).
6. Load BGE vial into inlet position.
7. Apply negative voltage of -30 kV (*see Note 16*) for 30 min to complete the separation (Fig. 1) (*see Note 17*).



**Fig. 1** Extracted ion electropherograms (EIEs) of 2 g/L glycated human hemoglobin standard hydrodynamically injected 30 mbar 8 sec in a 70 cm capillary x 50  $\mu\text{m}$  I.D. The applied voltage was -30 kV. The blue line is the EIE of human hemoglobin beta chain, green of glycated beta chain, red of alpha chain and the orange line is the EIE of glycated alpha chain (*see Note 18*)

## 4 Notes

1. We used a HP 3DCE electrophoresis instrument from Agilent Technology. Before implementing the CE method, it is recommended to clean, wash and dry the electrodes and prepunchers with water and isopropanol.
2. Before coating, it is recommended to cut off the end of the capillary, which will later be installed in the CE-MS Sprayer, very sharp-edged. Afterwards about 2 mm of the polyimide layer should be burned off with a lighter. The burnt polyimide is then wiped off with a lint-free tissue and some water.
3. 1 M NaOH can be used to adjust the pH. A concentration of 10 mM NaOH in 20 mM HEPES solution should adjust a pH of 7.46.
4. The polyion concentration is set to 3 g/L in HEPES buffer whatever the used polyion.
5. Polyion solutions are preferably stored at -18 °C to prevent hydrolysis. Polyion solutions can be thawed one hour before use by storing at room temperature.
6. We used an Agilent 6520 Accurate-Mass QTOF-MS
7. We used a commercial CE-ESI-MS interface from Agilent Technologies
8. Only 0.8 mL of the polyions are filled in 2 mL CE glass vials to prevent contact with the electrodes and thus carry-over / cross-contaminations between vials. If the vials should be reused for other coatings, always keep the same vial for the same solution. Take new vials and do not recycle by washing the NaOH or polyion vials.
9. The capillary is coated in CE mode, decoupled to the MS, to avoid contamination of the MS.
10. At each step, the waste vial is located on the outlet position.  
If no external pressure connection is available, it is also possible to flush at 1 bar and double the time. This also applies to the following steps. The disadvantage here is that the coating time increases significantly.
11. The capillary is installed in the interface so that only 1 mm of the cauterized capillary tip protrudes from the sprayer. This should increase the spray stability.
12. It has been found that the spray is the most stable under these conditions.  
It should also be noted that the sheath-liquid should be drawn up as bubble-free as possible with the syringe. It is also recommended to degas the sheath-liquid in an ultrasonic bath for 10 minutes before applying. Air bubbles in the sheath-liquid can significantly worsen the spray stability.

13. Following MS parameters have been used for the hemoglobin analysis on the Agilent QTOF-MS: Electrospray performed in positive mode, fragmentor voltage of 175V, a capillary voltage of 4000V, a skimmer voltage of 65V, octopole voltage of 750V, mass range of m/z 550–2000 and a data acquisition rate of four spectra/s.
14. The preconditioning step before each run is necessary to flush the remains of the sample from the capillary.
15. It has been found that this injection volume leads to best signal-to-noise ratios, without beginning peak broadening, at MS detection of the hemoglobin subunits.
16. A negative voltage is applied since the EOF is reversed at 5-layer SMIL coatings. -30 kV is the lowest applicable voltage at the HP 3DCE electrophoresis instrument from Agilent Technology.
17. The separation of Hb alpha and beta chain and its glycosylated species should be completed within 24 min at -30kV using 5-layer SMIL coated capillary with DEAEDEq and PMA at a 70 cm total length x 50µm I.D. capillary. The 5-layer SMIL can be stable over 100 runs [24]. However, if the last layer come off and thus migration times shifts to longer values, a simple recoating is possible. Therefore, the capillary is removed from the sprayer and flushed with polycation (DEAEDEq) for 10 minutes. Then it is flushed with BGE for 10 minutes and the capillary can be reinstalled in the sprayer. It can be continued with step 3.2.2.
18. EIEs of human hemoglobin subunits and glycosylated species. For alpha m/z values: 757.32, 797.12, and 841.35 ( $\pm 0.5$  m/z) were used, for alpha glycosylated m/z values 765.42, 805.65, and 850.35 ( $\pm 0.5$  m/z) were used, for beta m/z values: 756.58, 794.36, and 836.12 ( $\pm 0.5$  m/z) were used, and for beta glycosylated m/z values: 764.30, 802.46, and 844.64 ( $\pm 0.5$  m/z) were used.

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