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Optimization of capillary coating by hydroxyethyl methacrylate for capillary zone electrophoresis of proteins

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Abstract

This work describes further improvements of coating fused silica capillaries with 2-hydroxyethyl methacrylate (HEMA) by atom transfer radical polymerization (ATRP). First, endcapping with a sterically less bulky silanyl reagent reduces the electrosmotic flow (EOF) by 25% in addition to the 40% EOF reduction caused by HEMA coating compared to a bare fused silica capillary. An additional hydrolysis step was introduced into the preparation of HEMA coated capillaries and leads to better reproducible migration times. The influence of the solvent during ATRP and the resulting polymer coating was investigated by replacement of DMF with water or water–methanol mixtures. The quality of the optimized coating was characterized by protein separations at pH 3. HEMA coated capillaries reveal up to 746 000 plates. The polyvinyl alcohol (PVA) coated capillary provides only half of this efficiency. A long-term test at pH 9 shows good stability of the HEMA coated capillaries in basic medium. Also the numbers of plates in this medium was about 30% higher than for separations with the PVA capillary. In addition, the phosphate buffer was replaced by a volatile ammonium acetate buffer for later use with mass spectrometry (MS).

Keywords: Optimization; Capillary coating; Hydroxyethyl methacrylate; Proteins

1. Introduction

Nowadays, gel electrophoresis is routinely used for separation of proteins [1,2]. Difficulties are the reproducible preparation of the gel and the suppression of Joule heating. The replacement of gel electrophoresis by capillary electrophoresis (CE) can circumvent these disadvantages. A major problem with use of fused silica capillaries is adsorption of positively charged analytes onto the deprotonated silanol groups of the inner capillary wall. Adsorption could be successfully suppressed by permanent coating of capillaries [3–5], typically by polymers. In common fused silica capillaries an electric double layer is formed, based on negatively charged surface silanol groups and positively charged ions of the buffer solution. Under the action of an electrical field an electroosmotic flow originates. The strength of the electrosmotic flow (EOF) depends on the pH value and that determines the number of deprotonated silanol groups. The polymer coating suppresses the contact between the silanol groups and the buffer ions and a pH independent separation is feasible.

The atom transfer radical polymerization (ATRP) was invented by Matyjaszewski [6–9]. It is a further improvement of the atom transfer addition (ATRA). Polymeric coatings of low polydispersity are generated by a catalyst-controlled reaction mechanism. Wirth and co-workers [10] introduced HEMA polymerization using ATRP for capillary coating. Optimal conditions for these ATRP in capillaries were determined in our group by GC [11]. The advantage of the ATRP mechanism is the direct polymerization of HEMA on the covalently bound silanyl reagent on the capillary wall. This guarantees, that the danger of capillary clogging is lower. In this paper an improved method for the preparation of HEMA coated capillaries is described (Fig. 1).

This includes optimization of the surface functionalization by a bifunctional silane (step A) [12] which acts simultaneously as ATRP starter molecule. An increase of initiator molecules will be obtained by an additional reaction with a sterically less bulky silanyl reagent (step B). In addition, hydrolysis is introduced to form oxygen bridges between the individual silyl groups (step C). The solvent has a great influence on the development of the ATRP [7], because the structure of the catalyst may change in different solvents or side reactions are more or less suppressed. Therefore, polymerization (step D) in three different solvents (DMF, water, water–methanol) was investigated.

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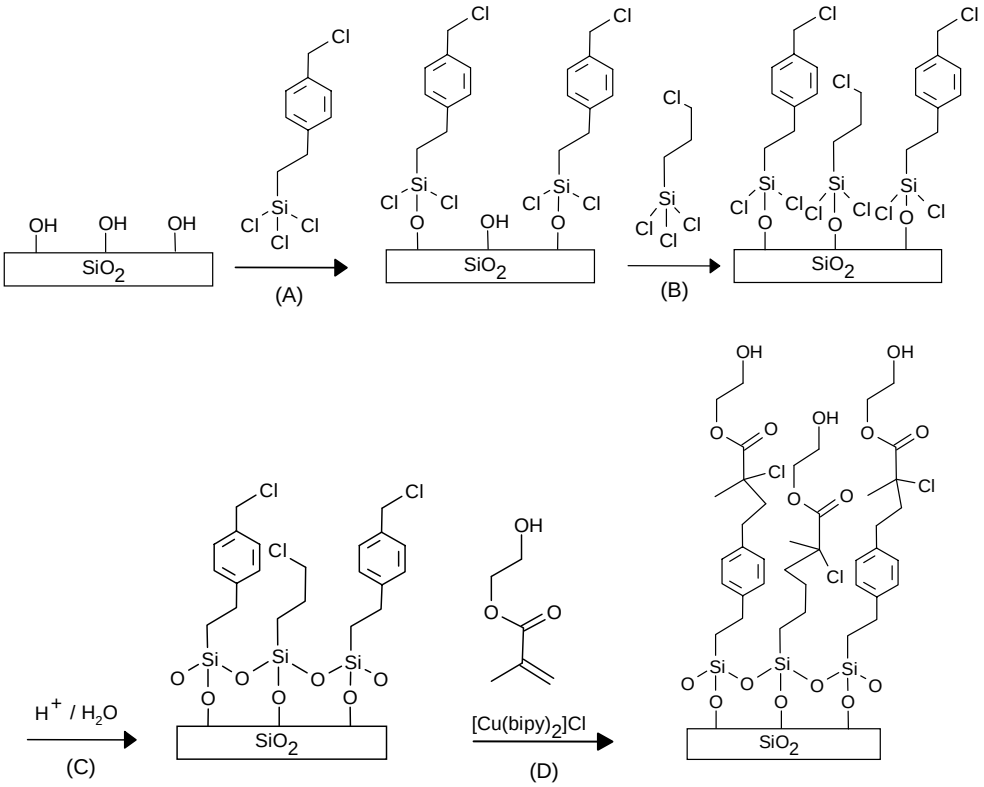

Fig. 1. The developed preparation steps: (A) silanization of capillary inner wall with trichlorosilyl(*m/p*-chloromethyl-phenyl)ethane, (B) endcapping of the remaining silanol groups with (3-chloropropyl)-trichlorosilane, (C) hydrolysis with diluted acetic acid, (D) atom-transfer radical polymerisation (ATRP) with 2-hydroxyethyl methacrylate (HEMA). The picture at the bottom right describes the reaction of one monomer, the chain reaction proceeds at the chlorinated tertiary carbon atom.

The coated capillaries were characterized at first by measurements of the EOF in a pH range from 3 to 10 [13], secondly by protein separations at pH 3, and finally their stability at high pH values was shown using a long-term test at pH 9.

An important requirement for coupling of CE with mass spectrometry (MS) is the use of a volatile electrophoresis buffer [14]. The buffer has to ensure likewise an optimal electrophoretic separation and an appropriate ionization and desolvation. To enable MS coupling of the investigated capillaries the replacement of phosphate buffer by ammonium acetate was also studied.

2. Experimental

2.1. Chemicals and materials

N,*N*-Dimethylformamide was purchased from Riedel-de Haen (Seelze, Germany), ultra resi analysed toluene from J.T. Baker (Phillipsburg, NJ, USA), methanol gradient grade from Merck (Frankfurt, Germany), hydrochloric acid and sodium hydroxide from Fluka (Buchs, Switzerland), 2-hydroxyethyl methacrylate (HEMA, ophthalmic grade, >99.8%) from Polyscience Europe GmbH (Eppelheim Germany), trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane from ABCR GmbH (Karlsruhe, Germany), (3-chloropropyl)-trichlorosilane from Fluka, bipyridine from Polish Chemicals (Gleiwitz, Poland). Copper(I) bromide 99,999% and copper(I) chloride >98% were from Aldrich (Milwaukee, WI, USA). All proteins were purchased from Sigma (Deisenhofen, Germany). Pure water was obtained from a Milli-Q water system (Millipore, Milford, MA, USA). (11-(2-Bromo-2-methyl)propionyloxy)undecyltrichlorosilane was prepared according to a publication from Matyjaszewski et al. [15]. The fused silica capillary (50 μm i.d. × 360 μm o.d.) was purchased from Microquartz GmbH (Muenchen, Germany) and polyvinyl alcohol (PVA) CE capillary (50 μm i.d., 56 cm) from Agilent Technologies GmbH (Karlsruhe, Germany).

2.2. Preparation of the capillary coating

2.2.1. Pretreatment

A fused silica capillary was filled with 1.0 M sodium hydroxide solution, sealed with silicon rubber, and placed in an oven at 100 °C for 2 h. The capillary was then washed with deionized water for 30 min, with 0.2 M hydrochloric acid for 15 min and once more with deionized water for 15 min. This sequence is important, otherwise undesirable

salts might be formed [16]. The capillary was dried under nitrogen purge in an oven at 120 °C for 1 h. Finally an optical window was formed by burning away the polyimide coating.

2.2.2. Silanization

A solution of 200 μl trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane or (11-(2-bromo-2-methyl)propionyloxy) undecyltrichlorosilane and 600 μl toluene (dried over 5 Å molecular sieve) was prepared and the capillary was rinsed for 10 min at 4 bar with this solution. Then, the filled capillary was sealed and incubated at room temperature for 18 h. After rinsing with dried toluene (at 4 bar, for 20 min) the same procedure follows with (3-chloropropyl)-trichlorosilane for endcapping.

2.2.3. Hydrolysis

The silanized capillary was filled with 0.1 M acetic acid, sealed and placed in an oven at 80 °C for 1 h. After this, the capillary was flushed with acetone for 10 min at 4 bar and heated under nitrogen purge in an oven at 120 °C for 1 h.

2.2.4. Surface-initiated atom transfer radical polymerization

Polymerization with DMF as solvent [11]: A solution of 2 mg/ml copper(I) chloride, 6.32 mg/ml bipyridine and 50:50 (v/v) of HEMA in DMF was prepared and degassed in an ultrasonic bath for 10 min. The silanized capillary was filled, sealed and placed in an oven at 80 °C for 4 h. Thereafter, the capillary was flushed with DMF, methanol and water at 4 bar for 20 min each.

Polymerization with water–methanol as solvent: In a degassed mixture of 250 μl water and 250 μl methanol, 14.7 mg/ml copper(I) chloride, 40 mg/ml bipyridine and 500 μl HEMA are dissolved. The silanized capillary was filled, sealed and left at room temperature for 2 h. Finally, the capillary was flushed with water at 4 bar for 20 min.

2.3. Preparation of protein standard and buffer solutions

Commercial protein standards (cytochrome *c*, lysozyme, β-lactoglobulin A and B, lactalbumin and α-chymotrypsinogen) were dissolved in pure water to 1 mg/ml. The phosphate buffer stock solution was obtained by adjusting the pH of 0.1 or 1.0 M phosphoric acid solution to the desired value with 0.1 or 1.0 M sodium hydroxide solution. The desired concentration was achieved by dilution with the appropriate amount of deionized water. Furthermore, a solution of 200 mM acetic acid and a solution of 200 mM ammonium acetate were prepared. By mixing both solutions the desired pH 3 for the ammonium acetate stock solution was obtained. All buffers were filtered through 0.45 μm membrane filters and sonicated.

2.4. Capillary electrophoresis system and separation conditions

All analyses were performed by using an HP^{3D} capillary electrophoresis system from Hewlett-Packard (Waldbronn, Germany).

2.4.1. EOF measurements and protein separation

The EOF measurements were performed using 41 cm/33 cm long capillaries (total length L_{tot}/effective length L_{eff}), the protein separations were carried out using 64.5 cm/56 cm capillaries. All experiments were performed at 25 °C separation temperature, 30 kV voltage and detection at 200 nm (bandwidth 10 nm). The protein standard solution was injected by applying a pressure over time (40 mbar × 3 s), that corresponds to an injection volume of nearly 3.5 nl [3] and amounts of 0.1–0.3 pmol protein. The capillaries were conditioned by rinsing with buffer solution for 10 min and after changing the buffer, the capillaries were additionally rinsed with deionized water for 10 min.

2.4.2. Long-term test

Separations were carried out at pH 9 with 100 mM sodium phosphate buffer at −12 kV voltage (anode as outlet an cathode as inlet). After 15 min a pressure of 20–30 mbar was overlaid. The capillary (L_{tot} = 64.5 cm/L_{eff} = 56 cm) was conditioned by rinsing with buffer for 5 min. The buffer was replaced after three separations. A solution of 1 mg/ml lactoglobulin A + B and lactalbumin was injected at 50 mbar × 4 s corresponding to nearly 5.5 nl, respectively, 0.3 pmol lactoglobulin A + B and about 0.4 pmol lactalbumin. All other conditions were used as for EOF measurements.

3. Results and discussion

3.1. EOF measurement on different coated capillaries

The EOF is a function of pH and the amount of dissociated silanol groups on the surface. The more silanol groups react with the silanyl reagent (e.g. trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane), the less the EOF depends on the pH of the separation buffer. Fig. 2 shows the mobility (μ_{EOF}) of the neutral marker DMSO, calculated by Eq. (1), where t_m is the migration time of DMSO and U represents the voltage.

$$\mu_{EOF} = \frac{L_{eff} L_{tot}}{t_m U} \quad (1)$$

The EOF is reduced by up to 40% by HEMA coating compared to the bare fused silica capillary. An additional endcapping with (3-chloropropyl)-trichlorosilane after the reaction with trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane reduces the EOF once more by 25%. All results are reported for at least three measurements of the migration time for each pH value. To characterize the reproducibility of mi-

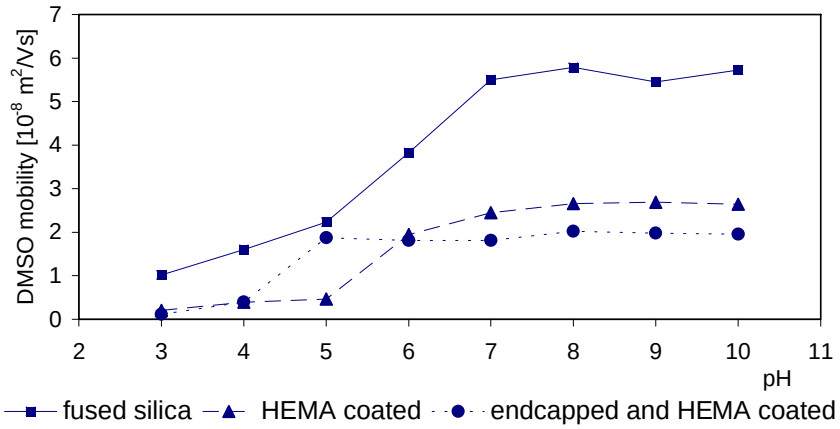

Fig. 2. Electroosmotic mobility in different capillaries (20 mM phosphate buffer at 25 °C, the EOF marker was DMSO (1 μL/mL), injection 40 mbar × 3 s), mean of a least 3 measurements.

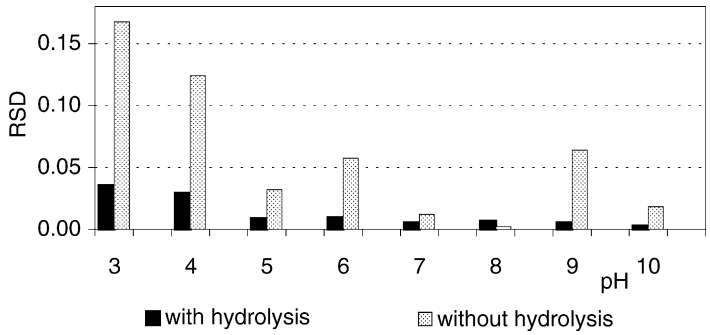

Fig. 3. Relative standard deviation (3 measurements) of DMSO migration times at different pH values on HEMA coated capillaries.

gration times the relative standard deviation (R.S.D.) was calculated ranging between R.S.D. = 0.036 at pH 3 and 0.003 at pH 10. Without hydrolysis the buffer reacts with the remaining chlorine atoms at the silicon atoms and forms oxygen bridges between the covalently bound silyl groups. As a result the migration times vary. Hydrolysis (cf. Fig. 1, reaction C) in diluted acetic acid leads to better reproducible migration times of DMSO. This is shown in Fig. 3, characterized by relative standard deviations of the DMSO mobility.

Furthermore, different methods were investigated to produce HEMA coated capillaries under mild conditions. The first aim was to change the solvent because for the successful polymerization of HEMA in the solvent DMF a time of 4 h and a temperature of 80 °C is necessary. Robinson et al. [17] published a controlled polymerization of HEMA in either methanol–water (1:1, v/v) mixture or in pure water at room temperature. Under similar conditions Perruchot et al. [18] synthesized polymer-grafted silica particles. This knowledge was used for coating of capillaries. The capillaries were modified as follows: silanization with trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane, endcapping with (3-chloropropyl)-trichlorosilane and hydrolysis with diluted acetic acid. Then, the polymerization solution was filled in the capillaries and reacted as described above in Section 2.2. Next, the silanyl reagent, which acted as the ATRP initiator, was varied. An alkylhalogenide containing a Br atom instead of a Cl atom should guarantee a faster reaction time. Because the carbon-bromine bonding is weaker the abstraction of the halide group by the transition metal complex (CuCl/bipy) is simplified [19]. Also the capillary inner wall was modified by using the self-prepared (11-(2-bromo-2-methyl)propionyloxy)undecyltrichlorosilane. Table 1 reveals differently coated capillaries.

Table 1
Differently coated capillaries A, B, C, D and E

Capillary number	Silanization reagent	Solvent for polymerization
A	Fused silica capillary	Fused silica capillary
B	Trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane	DMF
C	Trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane	Water/methanol (1:1; v/v)
D	Trichlorosilyl(*m*/*p*-chloromethyl-phenyl)ethane	100% water
E	(11-(2-Bromo-2-methyl)propionyloxy)undecyltri-chlorosilane	DMF

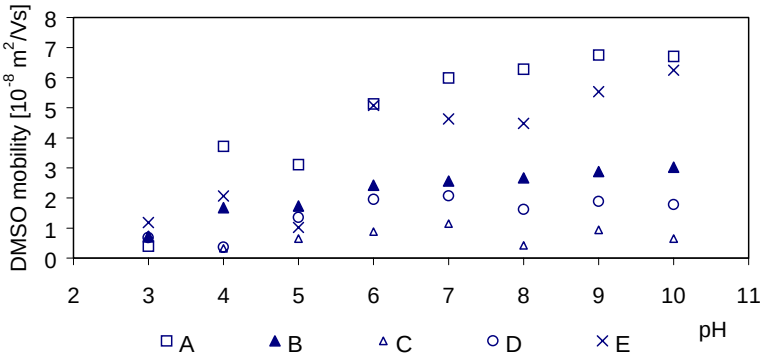

Fig. 4. Electroosmotic mobility (mean of 3 measurements) in differently coated capillaries. Letters A to E mark the coating procedure, see Table 1. Conditions: 20 mM phosphate buffer, 25 °C, EOF marker: DMSO (1 μL/mL), injection 40 mbar × 3 s.

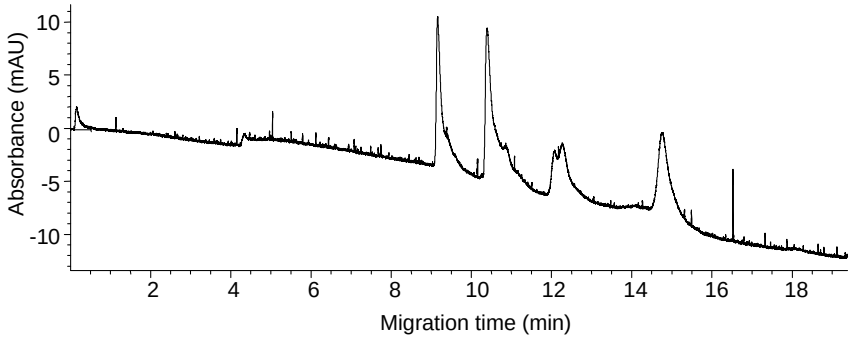

Fig. 5. Protein separation with bare fused silica capillary (20 mM phosphate buffer pH 3, 25 °C, injection 50 mbar × 4 s, protein standard solution with 1 mg/mL). Sequence of migration in the electropherogram: cytochrome *c*, lysozyme, β-lactoglobulin A and B, α-chymotrypsinogen.

The success of the coating was determined by measurements of the DMSO mobility. The results are shown in Fig. 4.

Fig. 4 shows, that the same good results will be obtained with a polymerization in water (capillary D) or water–methanol mixture (capillary C) like in the solvent DMF. It can be seen that by a polymerization in water–methanol mixture (capillary C) the DMSO mobility at pH 10 is reduced by one order of magnitude in comparison to a fused silica capillary (capillary A). In an experiment, where temperature and reaction time were varied, it was discovered, that higher temperatures and a longer reaction time do not guarantee a better polymerization. Consequently, the polymerization step with HEMA under ambient temperatures can be carried out in 1 h. The silanization with the bromosilane and subsequent polymerization is presently unsatisfactory and needs further investigations.

3.2. Protein separation

To judge the quality of the different HEMA coatings separations of proteins were studied at pH 3 and compared to bare fused silica and PVA coated capillaries. A separation by use of a fused silica capillary reveals the necessity of a coating (Fig. 5).

To receive peaks of a sufficient height the five proteins must be injected at 50% higher concentrations than used for other capillaries. In addition, migration times are large and the pronounced peak tailing leads to low peak intensities. The bad separation is caused by adsorptions of positively charged analytes on the negatively charged silanol groups on the capillary inner wall. Coating the capillaries with HEMA or PVA results in complete separation (Fig. 6).

For HEMA polymerized in the solvent water/methanol (capillary C) 746 000 theoretical plates could be obtained.

Table 2
Separation efficiencies N/m for proteins in different capillaries, separation conditions see Fig. 6

Protein	Fused silica	HEMA DMF	HEMA water/methanol	PVA
Cytochrome *c*	50 000	140 000	746 000	347 000
Lysozyme	32 200	412 000	367 000	355 000
β-Lactoglobulin A	77 100	372 000	512 000	364 000
β-Lactoglobulin B	33 600	301 000	574 000	152 000
α-Chymotrypsinogen	24 000	496 000	384 000	262 000

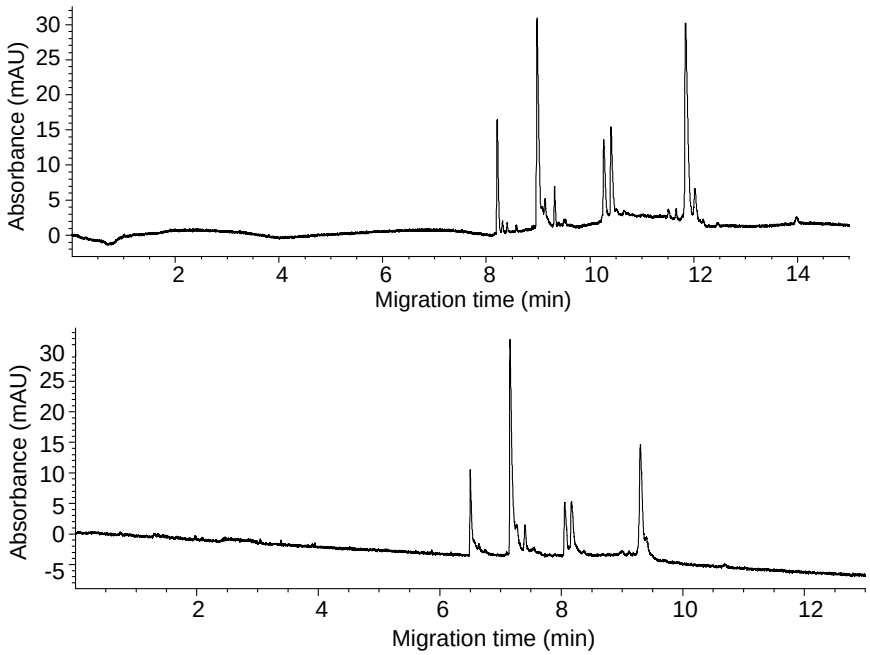

Fig. 6. Protein separation with HEMA (polymerized in water/methanol) coated (top) and PVA capillary (bottom). Conditions: 20 mM phosphate buffer pH 3, 25 °C, injection 40 mbar × 3 s, protein standard solution with 1 mg/mL. Sequence of migration in the electropherogram: cytochrome *c*, lysozyme, β-lactoglobulin A and B, α-chymotrypsinogen.

The peaks are high, narrow and symmetric. The separation in the PVA capillary is faster, but, less efficient. A comparison of the theoretical plates for the different capillaries is summerized in Table 2.

3.3. Long-term test

Many commercially available coated capillaries cannot be used at pH values higher than 8. In order to test the performance of HEMA coated capillaries at pH values above 8 a long-term test consisting of 102 measurements was carried out at pH 9. For comparison two HEMA coated capillaries (one polymerized in the solvent DMF and the other one in the solvent water/methanol) an a commercially available PVA capillary were tested. Fig. 7 shows the last runs of this long-term test.

The capillary B (HEMA coating in DMF) separated completely the three proteins up to the last run. A better efficiency over all runs could be obtained with a polymerization of HEMA in the solvent water/methanol (capillary C), however the peaks of the 102nd run are of a lower intensity. Probably the quality of the coating is worse than for the other HEMA capillary. The polymer layer might be damaged, and adsorption of the proteins becomes possible, recognizable by higher peak widths. With a PVA capillary the lactoglobulins A and B were never completely separated by the baseline. Furthermore, the high number of theoretical plates could not be obtained as found for the other two capillaries (Table 3).

Table 3
Separation efficiencies (mean values) of proteins for the long-term test in different capillaries, separation conditions see Fig. 7

Capillary/solvent for polymerization	β-Lactoglobulin A	β-Lactoglobulin B	Lactalbumin
Run 1–10 (N/m)			
HEMA/DMF	71 600	65 800	92 000
HEMA/water/methanol	95 800	95 700	113 000
PVA	68 700	69 900	68 500
Run 93–102 (N/m)			
HEMA/DMF	55 000	50 500	63 800
HEMA/water/methanol	44 200	38 400	88 300
PVA	30 200	56 600	77 300
Overall runs (N/m)			
HEMA/DMF	58 400	54 100	84 400
HEMA/water/methanol	73 300	71 200	93 800
PVA	50 500	57 300	73 800

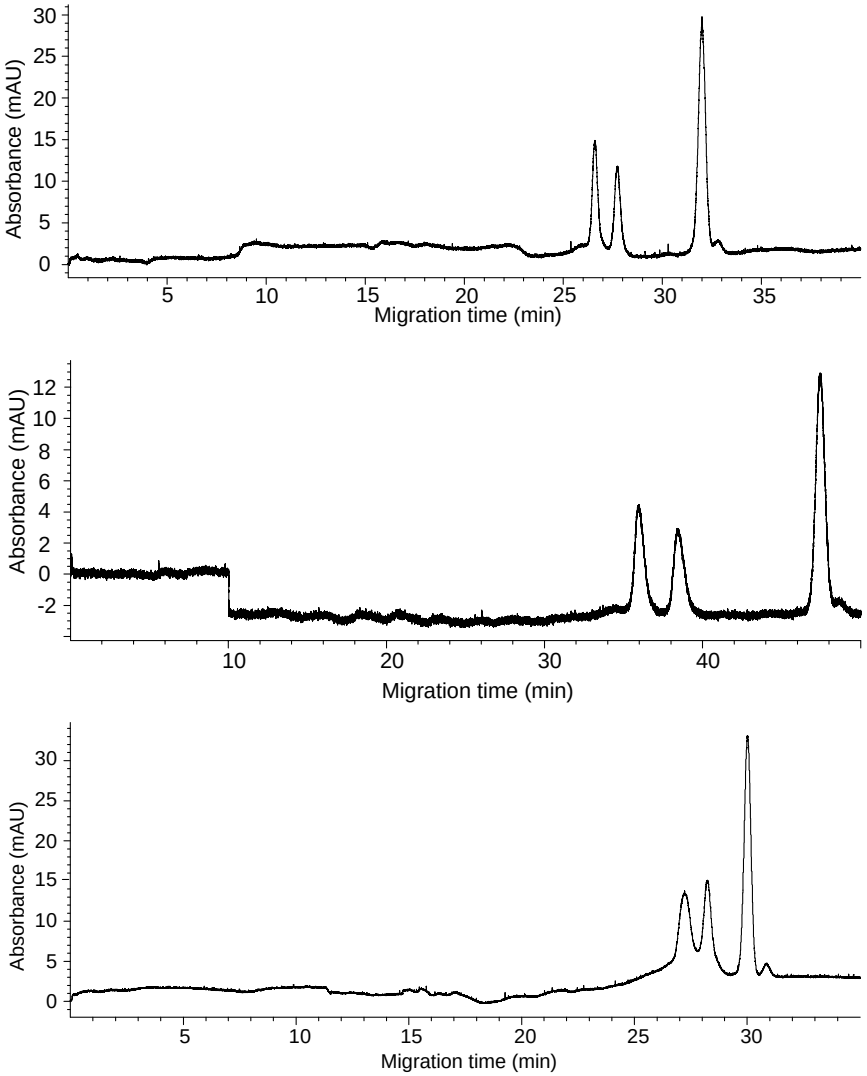

Fig. 7. 102nd runs of the long term test for different capillaries: HEMA polymerized in DMF (top), HEMA polymerized in water/methanol (middle) and PVA (bottom). Conditions: 100 mM phosphate buffer pH 9, 25 °C, injection 50 mbar × 4 s, protein standard solution with 1 mg/mL. Sequence of migration in the electropherogram: β-lactoglobulin A and B, lactalbumin.

The reproducibility of the migration time over the runs shows good results for all investigated capillaries. PVA was proved to be superior with respect to the relative standard deviations of the migration time (Table 4).

The ultimate goal in separation science is the resolution of samples. A calculation of the resolution according to Kaiser [20] for the PVA coated capillary resulted in 30% less resolution than obtained for HEMA coated capillaries. These investigations are very important, because the stability of the coating in a basic medium is important for many separation problems. For example capillary isoelectric focussing needs pH gradients, typically between 3 and 10. For good results the capillary coating must have the same property in every region of the pH gradient in order to suppress adsorptions.

Finally, it should be mentioned that comparison of the different capillaries was carried out without any cleaning steps, e.g. by 0.1 M sodium hydroxide solution, as done in previous investigations [11]. This leads to shorter total analysis times. However, if a cleaning step is required for some reason HEMA coated capillaries can cope with such treatment in contrast to PVA capillaries the live time of which would decrease substantially.

3.4. CZE separation of proteins with MS compatible buffer

For the coupling of CE with MS a volatile buffer is necessary. Therefore, a protein separation was performed at pH 3 with a 100 mM ammonium acetate buffer (Fig. 8).

The analytes were separated faster, but only 75% of plate numbers could be obtained in comparison to a separation with 20 mM phosphate buffer (Table 5).

Despite the minor efficiency the acetate buffer is well suitable for a coupling of CE with MS using the HEMA coated capillaries.

Table 4
Relative standard deviations of migration times of proteins in different capillaries

Capillary/solvent for polymerization	β-Lactoglobulin A (%)	β-Lactoglobulin B (%)	Lactalbumin (%)
Run 1–10 (R.S.D.)			
HEMA/DMF	3.3	3.6	6.4
HEMA/water/methanol	2.0	2.1	3.3
PVA	2.8	0.9	1.1
Run 93–102 (R.S.D.)			
HEMA/DMF	4.0	4.2	7.6
HEMA/water/methanol	2.0	2.3	3.6
PVA	1.7	1.8	3.1
Overall runs (R.S.D.)			
HEMA/DMF	4.5	4.9	7.3
HEMA/water/methanol	5.4	5.9	7.3
PVA	2.8	2.8	4.3

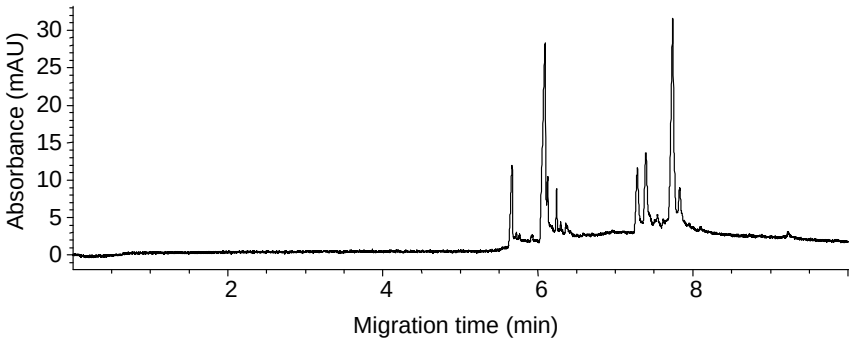

Fig. 8. CZE of proteins with 100 mM acetate buffer pH 3, 25 °C, injection 40 mbar × 3 s, protein standard solution with 1 mg/mL. Capillary coated with HEMA polymerized in water/methanol. Sequence of migration in the electropherogram: cytochrome *c*, lysozyme, β-lactoglobulin A and B, α-chymotrypsinogen.

Table 5
Separation efficiencies for proteins in ammonium acetate buffer, separation conditions see Fig. 8

Protein	N/m
Cytochrome *c*	353 000
Lysozyme	298 000
β-Lactoglobulin A	220 000
β-Lactoglobulin B	221 000
α-Chymotrypsinogen	306 000

4. Conclusion

It was possible to optimize the surface-initiated ATRP to create capillary coatings for CE. An endcapping with a less bulky silanyl reagent and a hydrolysis step were introduced, which enables reproducible migration times. A polymerization under ambient temperatures in 2 h was obtained through the change of DMF against a methanol/water mixture as solvent for ATRP. The quality of the coatings was judged by a comparison of protein separations at pH 3. The developed capillaries were investigated in a long-term test. They show reproducible migration times at pH 9 after 102 runs and excellent numbers of plates. The CZE with an MS compatible 100 mM ammonium acetate buffer results in complete separation of proteins with similar plate numbers still in the same range as found for a PVA coated capillary with 20 mM phosphate buffer.

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