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PERFORMANCE OF CARBOHYDRATE-MODIFIED FUSED-SILICA CAPILLARIES FOR THE SEPARATION OF PROTEINS BY ZONE ELECTROPHORESIS

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SUMMARY

The walls of fused-silica capillaries were chemically modified with small carbohydrate moieties in order to diminish the wall adsorption of proteins in capillary zone electrophoresis. A diol-type coating, prepared by bonding of γ-glycidoxypropyltrimethoxysilane to the wall followed by acidic hydrolysis, shows for proteins a similar electrophoretic behaviour at various pH values to a polyethylene glycol (PEG) coating tested previously. Although good peak shapes were obtained for proteins in the pH range 3–5, the efficiency on the diol coating is worse than that on the PEG coating. At higher pH values the peaks are deformed and the efficiency is lost. A maltose coating appears to shield the silica surface well for proteins up to pH 7. The peak shapes of proteins are acceptable, but the efficiency of the maltose coating is smaller than that on the diol coating. The diol coating is stable in the indicated pH range. However, with the maltose coating good stabilily is obtained only on adding an antimicrobial agent to the buffers.

INTRODUCTION

Capillary zone electrophoresis (CZE) has been shown to be an attractive technique for the separation of ionizable substances[1–3]. In contrast to liquid chromatography, in CZE low diffusion coefficients favour the realization of a very high resolving power[2,4] and this makes the technique extremely suitable for separations of proteins[2–5,7]. However, a high resolving power can only be attained when no adsorption of the solutes on the wall occurs, as was extensively discussed by Martin and Guiochon[6]. The slightest extent of adsorption (*i.e.*, on the silanol groups) will cause an appreciable decrease in efficiency, and moderate adsorption can lead to completely destroyed peak shapes and poor reproducibility[7]. Therefore, it is of paramount importance to eliminate adsorption in order to exploit the full potential of the separation power of CZE. Several approaches have been employed to eliminate the wall adsorption of proteins: adjustment of the pH of the buffer to a value such that both the silanol groups and the proteins are negatively charged and repulsive forces result in strongly diminished adsorption[4]; applying very low pH values so that the silanol

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groups are largely protonated and result in a small electrical charge on the wall[5]; and chemical modification of the wall with a neutral, hydrophilic moiety in order to shield the silanol groups[5,7–9].

Although manipulation of the pH to eliminate the adsorption of proteins can result in highly efficient separations of certain proteins[4,5], in biopolymer separations it is desired to have the pH available as a freely adjustable parameter, preferably in the range 5–8, for various reasons: the pH is the predominant parameter influencing the migration and selectivity in protein separations; the stability of the sample may depend on the pH; structural changes in proteins may be induced at too large pH-p*I* differences; and at high pH values the electroosmotic flow is high, which may impair the resolving power. Therefore, shielding of the silanol groups by chemical bonding of a neutral, hydrophilic moiety to the capillary surface is more attractive. The shielding of the silanol groups diminishes the adsorption. An additional advantage may be that the electroosmotic flow is reduced. Under such conditions good separations may be obtained over a wide pH range.

The chemical modification of silanol groups with neutral, hydrophilic moieties is well documented in high-performance liquid chromatography[10]. The same techniques can be used to modify the capillary surface in CZE. So far the following modifications have been employed in CZE: polyacrylamide[8], methylcellulose[8], glycol[9], polysiloxanes[5], glycerol-glycidoxypropyl[5] and polyethylene glycol (PEG)[7]. The separation of proteins in polyacrylamide- and methylcellulose-coated columns showed a good performance at higher pH but the coatings in refs. 5 and 7 were only useful in the low pH range between 1.5 and 5. The shielding properties of the PEG coating for proteins were good up to about pH 5, but at higher pH significant adsorption occurred. Therefore, there is still a need for reproducible wall modifications which are stable and suppress adsorption over a wide pH range.

In liquid chromatography, neutral, hydrophilic carbohydrate packings (*e.g.*, Sephadex) have been successfully applied to the separation of biopolymers[11]. These packings appear to exhibit substantial inertness towards proteins. This property makes it attractive to modify the capillaries in CZE by chemically bonding of carbohydrate moieties to the silanol groups on the surface, as has been demonstrated by Hjerten[8] with methylcellulose. In this study we tested the chemical modifications of fused-silica walls with a small carbohydrate moiety, maltose and epoxy-diol, for the separation of proteins by CZE.

EXPERIMENTAL

Apparatus

A 0–60-kV d.c. high voltage delivered by a power supply (Wallis, Worthing, U.K.) drove the electrophoretic separations. Platinum electrodes were used for the connection of the supply with the buffer reservoirs located at each end of the capillary. The total set-up was placed in a Plexiglas box; opening the box automatically shut off the high voltage. Fused-silica capillaries of 50–60 cm × 50 μm I.D. (SGE, North Melbourne, Australia) were used for the separations.

Detection was carried out at the cathodic side using a model 757 UV detector (Kratos, Ramsey, NJ, U.S.A.) with a modified cell arrangement for on-column work. This consisted of an arrangement to position the capillary and to adjust the slit in

front of the capillary for focusing the light beam at the inside part of the capillary. The wavelength was set at 205 nm. The detector signal was recorded by a chart recorder (Type BD40, Kipp and Zonen, Delft, The Netherlands). The current in the system was measured over a 10-kΩ resistance in the return circuit of the power supply by means of a battery-powered electrical service-meter. The temperature in the Plexiglas box was kept at 20-22°C during the separations. Care was taken to achieve hydrostatic equilibrium between the two buffer reservoirs.

Sample injection was done by means of electromigration[12] at constant voltage (5–10 kV) at the anodic side for a fixed period of time (10–30 s). The protein samples were freshly prepared each day by dissolving 1 mg/ml of protein in phosphate buffer of the appropriate pH. After each electropherogram the capillary was flushed with the buffer by vacuum suction for 30 s.

Chemicals

The proteins were obtained from Sigma (St. Louis, MO, U.S.A.). γ-Glycidoxypropyltrimethoxysilane was obtained from Serva (Heidelberg, F.R.G.), triethoxyaminopropylsilane from Janssen (Beerse, Belgium), sodium cyanoborohydride from Sigma (St. Louis, MO, U.S.A.) and maltose from Merck (Darmstadt, F.R.G.). The other reagents and solvents were of analytical-reagent grade. Doubly distilled water was used to prepare the buffers.

Procedures

The carbohydrate coating procedures are shown schematically in Fig. 1. All capillaries were first etched with 1 *M* potassium hydroxide solution for 3 h at room temperature and then rinsed with water for 10 min. Next, the capillaries were flushed with 0.1 *M* hydrochloric acid to remove K^+ ions from the silanol groups to generate free silanol groups at the surface of the wall. Finally, the capillaries were dried at 200°C for 3 h by gentle flushing with helium and were then ready for coating.

Epoxy-diol coating. The pretreated capillary was first coated with the coupling reagent γ-glycidoxypropyltrimethoxysilane by pumping through a 10% (v/v) solution of the reagent in dried toluene at 110°C for 3 h. at an inlet pressure of 0.5 MPa. The excess of reagent was removed by flushing the capillary with toluene, followed by helium purging. Next the epoxide group was opened by pumping through 0.1 *M* hydrochloric acid for 3 h. in order to produce what we refer to as an "epoxy-diol" coating. Finally, the capillary was rinsed with distilled water.

Maltose coating. For the coupling of maltose to fused-silica capillaries, use was made of the technique reported recently by Huisden *et al.*[13] for the modification of silica gel with maltose. The pretreated capillary was first coated with triethoxyaminopropylsilane by pumping through a 1% (v/v) solution of this reagent in dried toluene at 100°C for 4 h. The capillary was then flushed successively with toluene, acetone, methanol and then dried under a stream of helium. Next the capillary was placed in an oven, kept at 90°C and flushed for 4 h with a solution of 2% (w/v) of maltose and 0.3% (w/v) of sodium cyanoborohydride in methanol at 0.4 MPa. Finally, the capillary was rinsed successively with methanol and distilled water.

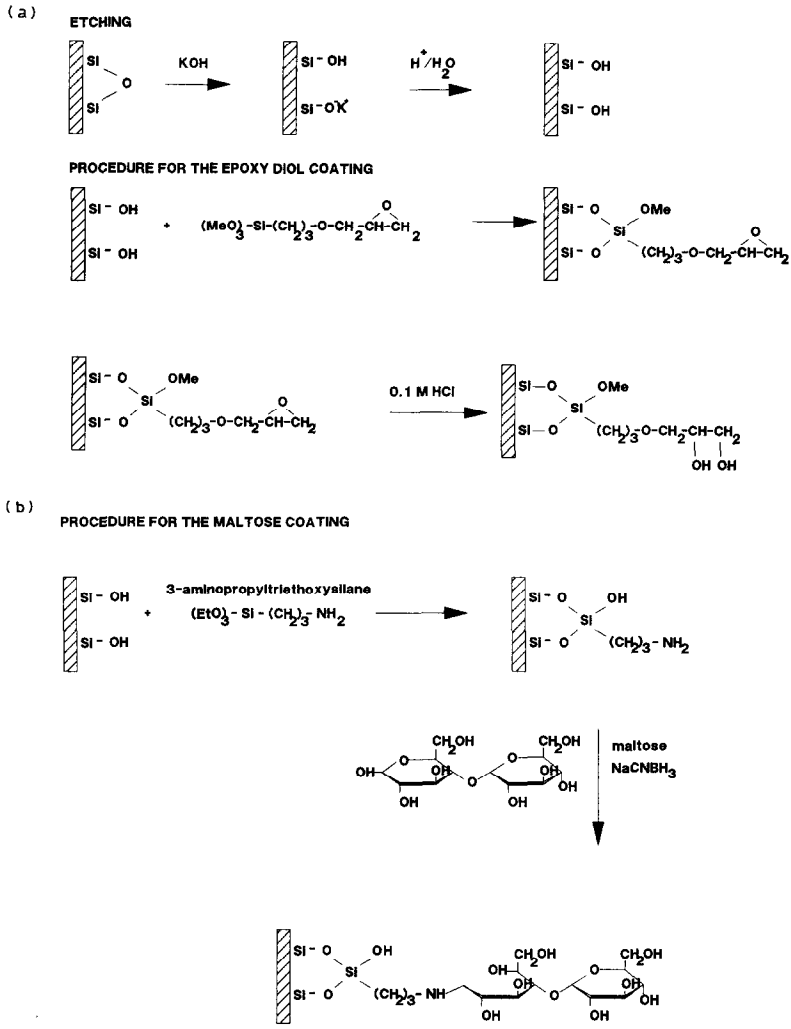

Fig. 1. Schemes of the procedures for the modification of fused-silica capillaries with (a) epoxy-diol and (b) maltose (Me = CH_3; Et = C_2H_5).

RESULTS AND DISCUSSION

During preliminary measurements with the maltose-modified capillaries it appeared that during the first few hours reproducible retention times and peak shapes of lysozyme and cytochrome *c* were obtained and then suddenly the coating collapsed. The reason was unclear, especially because the mobilities during the first few hours agreed well with those in the literature and indicated that the shielding of the silanol groups by the maltose moiety was effective. The problems with the stability of the maltose coating were probably caused by the action of bacteria developing in the buffer solution with time. The maltose coating is a very attractive matrix for these

bacteria and they can easily degrade the relatively small amount maltose on the capillary in a short time. The difficulties could be remedied by the addition of an antimicrobial agent such as sodium azide to the buffer. Therefore, in all further experiments with maltose and epoxy-diol as a precaution 0.01% (w/v) of sodium azide was always added to the buffer solutions.

Epoxy-diol coating

Fig. 2 shows the effect of the pH of the phosphate buffer on the overall mobility ($\mu_{o,i}$) of the test proteins in an epoxy-diol coated capillary. The overall mobility is the sum of the electrophoretic mobility ($\mu_{el,i}$) and the electroosmotic mobility (μ_{eo}), and these are both directed to the negative electrode. As can be seen from Fig. 2, the overall mobility increases with increasing pH, passes through a maximum at about pH 4 and then gradually decreases again at higher pH. This behaviour can be explained by the combined effect of the pH on the electroosmotic flow and on the net charge of the solutes. The electroosmotic mobility was determined from the retention time of a neutral marker (β-naphthol) and is shown in Fig. 3. The positive charge of the proteins decreases at higher pH and results in a reduction of the electrophoretic mobility, as can be seen in Fig. 4, showing the net electrophoretic mobility of the proteins ($\mu_{el,i}$) as function of pH.

The performance of the epoxy-diol coating at several pH values is shown in Fig. 5. The proteins elute in order of their isoelectric points and exhibit good peak shapes up to pH = 5 (see Fig. 5a). However, the plate numbers (35000–50000) are much smaller than the theoretically predicted values and also smaller than those measured

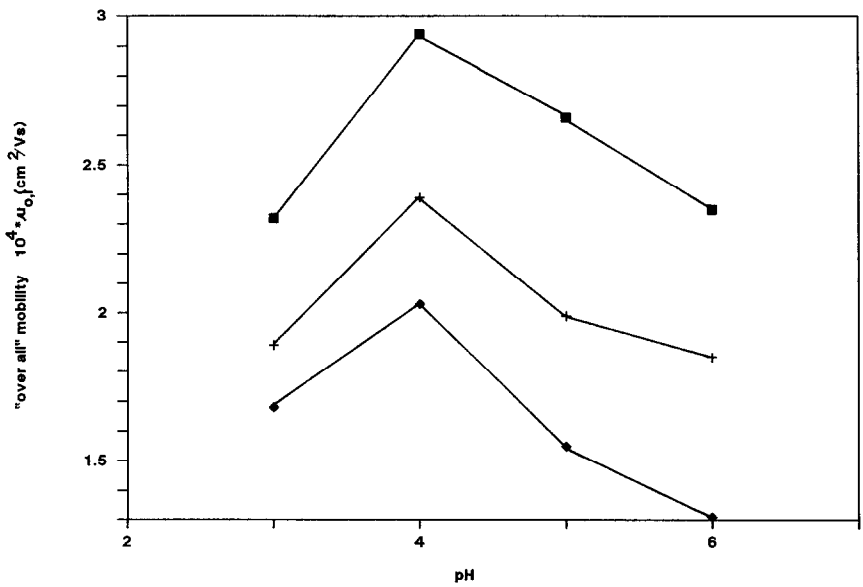

Fig. 2. Effect of the pH of the phosphate buffer on the overall mobility, $\mu_{o,i}$, of some test proteins on an epoxy-diol-modified fused-silica capillary. Voltage, 15 kV; injection, 10 s, 15 kV; total length of the detector (L) = 52.2 cm; length of the capillary from the injection end to the detector ($l_{inj\text{-}det}$) = 31.7 cm; Buffer = 0.05 M. (■) Lysozyme; (+) trypsin; (◆) chymotrypsinogen.

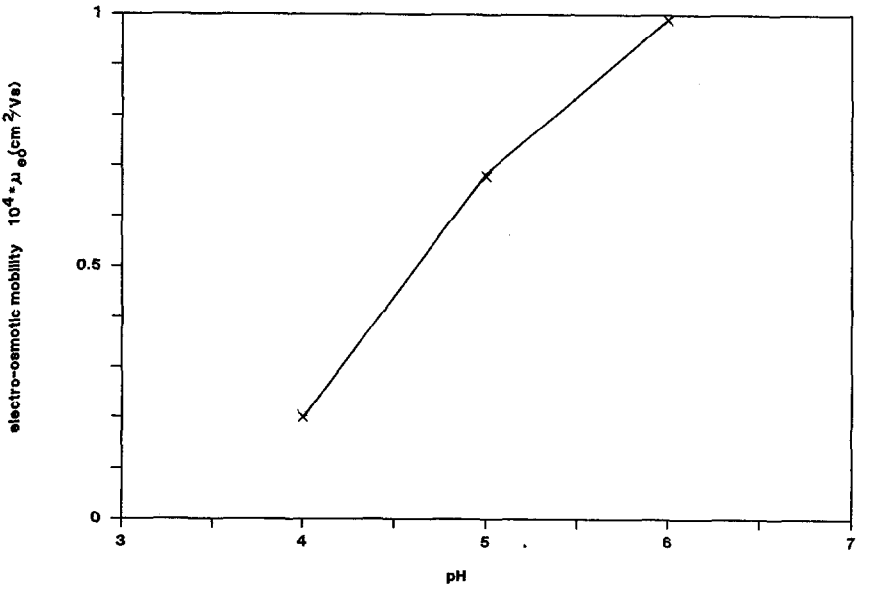

Fig. 3. Electroosmotic mobility, μ_{eo}, as a function of the pH of the phosphate buffer on a epoxy-diol-modified fused-silica capillary. Conditions in Fig. 2 Injection, 10–20 s, 15 kV; marker, β-naphthol.

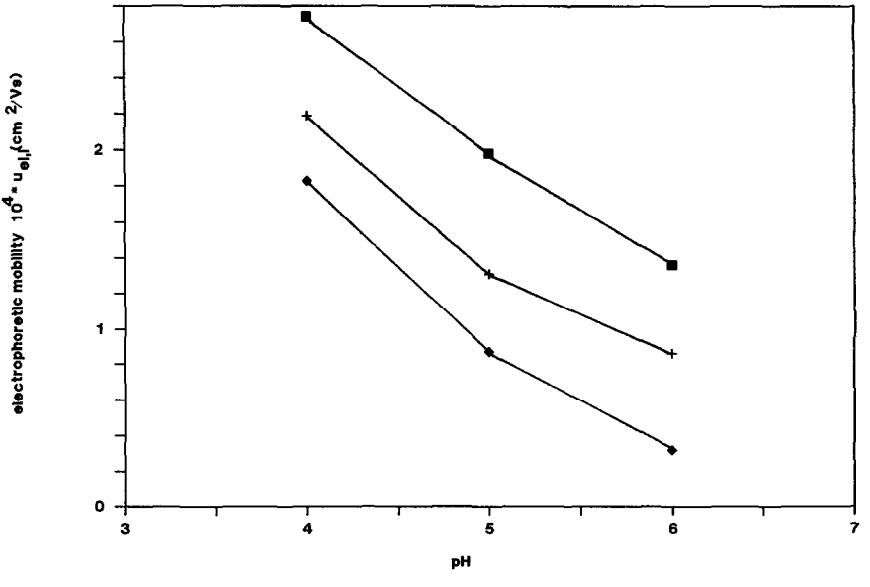

Fig. 4. Electrophoretic mobility, $\mu_{el,i}$, of some test proteins as a function of the pH of the phosphate buffer on an epoxy-diol-modified fused-silica capillary. Conditions and symbols as in Fig. 2.

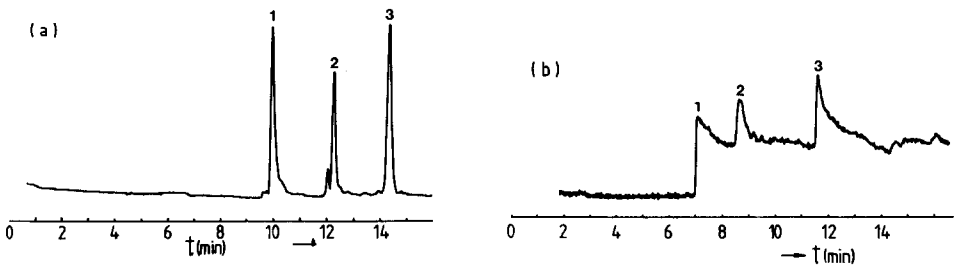

Fig. 5. Electropherograms of some test proteins on an epoxy-diol-modified fused-silica capillary at (a) pH 4.0 and (b) pH 6.0. (a) Voltage, 20 kV; l = 72.0 cm; $l_{inj\text{-}det}$ = 50.5 cm. (b) Voltage, 15 kV; l = 52.2 cm; $l_{inj\text{-}det}$ = 31.7 cm.1, Lysozyme; 2, trypsin; 3, chymotrypsinogen.

on a PEG-modified capillary (80000–150000).[7] This decrease in efficiency must be mainly attributed to the remaining weak adsorption of the solutes on the residual surface silanols. At pH > 5 the peak shapes are largely destroyed (see Fig. 5b) owing to strong interaction of the proteins with the residual silanols or the epoxy-diol layer. So far the performance of the epoxy-diol coating is worse than that of the PEG coating previously described.

The proper operation of the electrophoretic system can be checked by plotting the voltage against the reciprocal of the retention time of the solutes[2]. Such a plot should show a linear proportionality in the absence of the electroosmotic flow. Fig. 6 was obtained for the proteins under investigation at pH 3. The plot of inverse retention time *versus* applied field is linear up to 25 kV/m, but deviates from linearity at higher applied voltages. This deviation at higher voltages can be attributed either to the temperature increase caused by the heat dissipation in the capillary[14] or to persist-

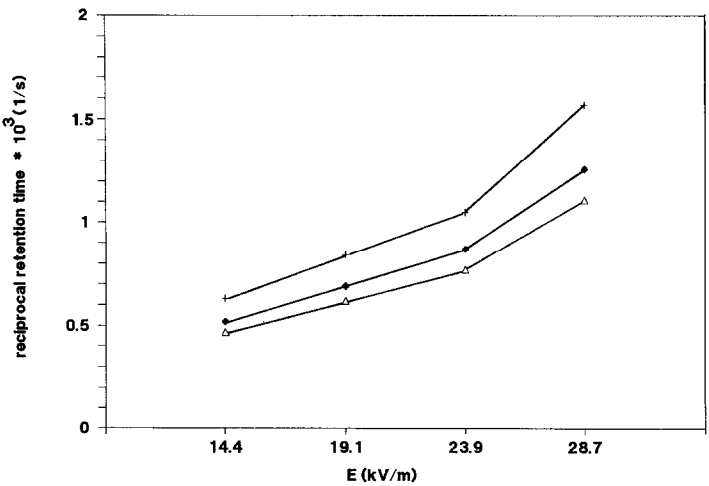

Fig. 6. Reciprocal of the retention times of some proteins as a function of the applied field on an epoxy-diol-modified fused-silica capillary. Voltage, 14–30 kV; buffer, 0.05 M; pH = 3.0; L = 52.2 cm; $l_{inj\text{-}det}$ = 31.7 cm; T = 18°C. (+) Lysozyme; (◆) trypsin; (△) chyriotrypsinogen.

ing electroosmotic flow. The higher temperature may reduce the viscosity of the buffer and result in a higher mobility of the solutes.

Maltose coating

The behaviour of the overall mobilities of lysozyme and cytochrome *c* on the maltose-modified capillary can be seen in Fig. 7. In contrast to the epoxy-diol coating (see Fig. 2), the overall mobilities observed with the maltose coating gradually increase with increasing pH. At pH 4–5 the overall mobilities of lysozyme appear to be significantly smaller than those determined on the epoxy-diol capillary under similar buffer conditions. This difference must be attributed to a deviating behaviour of the electroosmotic flow on the maltose coating at various pH values, as can be seen in Fig. 8. On the epoxy-diol capillary the electroendosmotic flow is always directed towards the negative electrode in the pH range 3–6 because the surface is negatively charged. However, with the maltose coating the net charge of the surface can be positive or negative, depending on the pH, because the maltose is coupled to aminopropyl groups bonded previously to the surface of the capillary. It can be expected that after the coupling, in addition to residual silanol groups, some free aminopropyl groups will be left. At low pH the aminopropyl groups are protonated and therefore the surface will have a net positive charge. This causes an electroosmotic flow towards the positive electrode. At higher pH the aminopropyl groups are deprotonated and the surface will have a net negative charge. This causes an electroosmotic flow towards the negative electrode. Fig. 9 shows the net electrophoretic mobilities of lysozyme and cytochrome *c* on the maltose capillary in the pH range 4.0–8.1 and the observed values agree reasonably well with those obtained on the epoxy-diol capillary

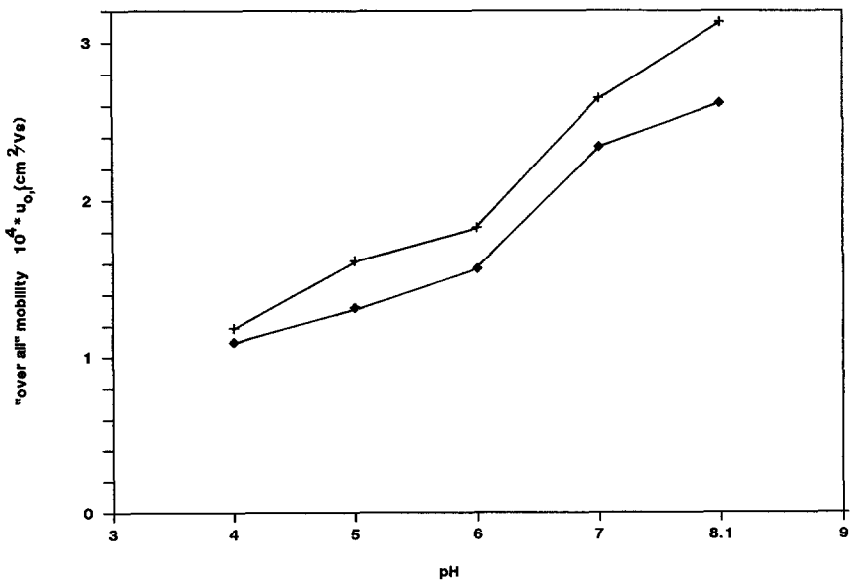

Fig. 7. Effect of the pH of the phosphate buffer on the overall mobility, $\mu_{o,i}$, of lysozyme and cytochrome *c* on a maltose-modified fused-silica capillary. Voltage, 10 kV; injection, 10 s, 10 kV; L = 39.5 cm; $l_{inj\text{-}det}$ = 20.0 cm; buffer, 0.05 *M*. (+) Lysozyme; (◆) cytochrome *c*.

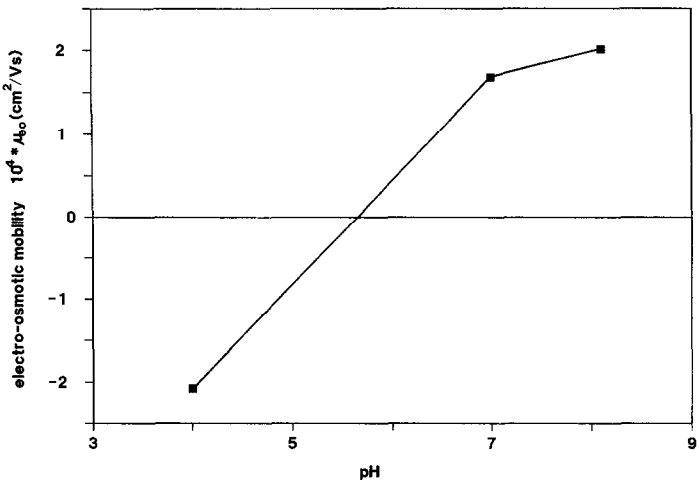

Fig. 8. Osmotic flow, μ_{eo}, as a function of the pH of the phophate buffer on a maltose-modified fused-silica capillary. Conditions as in Fig. 7. Marker, β-naphthol.

at a different set of pH values (see Fig. 4). The deviations that occur can be attributed to experimental errors, amplified by the subtraction involved in the calculation of these net mobilities. It should be noted that electroosmotic mobilities had to be determined in separate experiments, especially at pH 3, where injection had to be carried out at the cathodic side.

The performance of the maltose coating is shown in Fig. 10, which includes an electropherogram of lysozyme on an aminopropyl-modified capillary. It can be seen

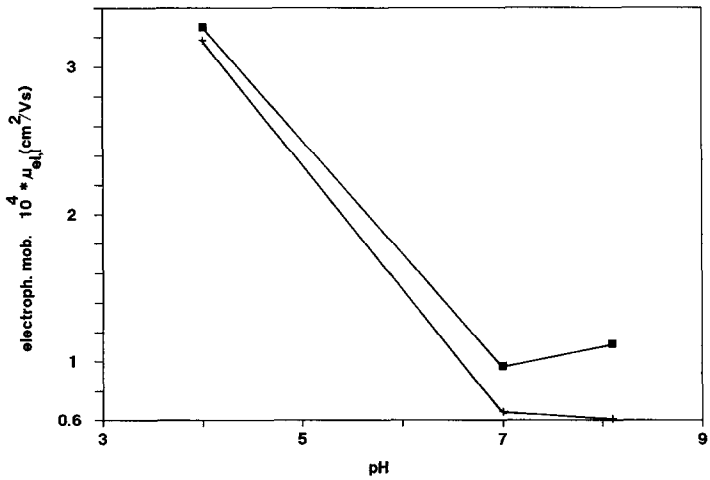

Fig. 9. Electrophoretic mobility, $\mu_{el,i}$, of lysozyme and cytochrome *c* as a function of the pH of the phosphate buffer on a maltose-modified fused-silica capillary. Conditions as in Fig. 7. (■) Lysozyme; (+) cytochrome *c*.

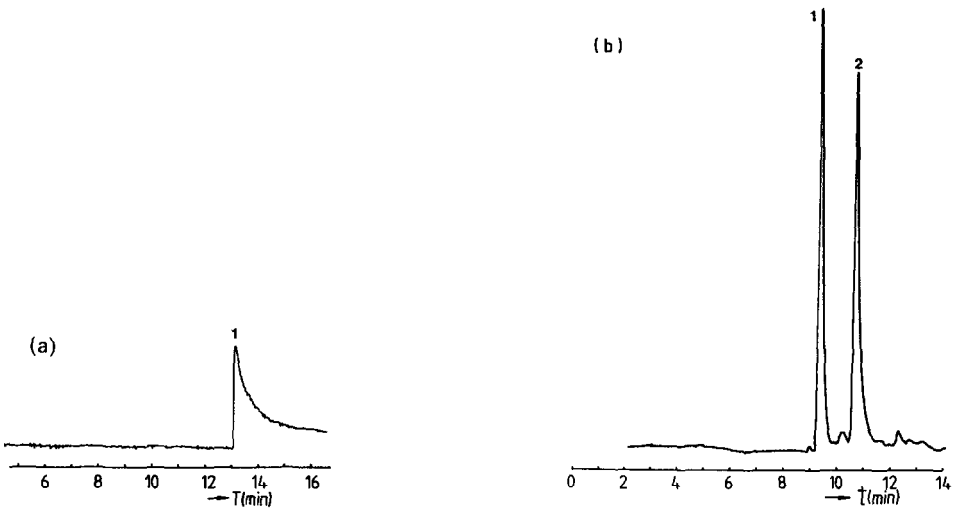

Fig. 10. Electropherograms of (a) lysozyme on an aminopropyl-modified capillary and (b) of lysozyme and cytochrome *c* on a maltose-modified fused-silica capillary at pH 6. Conditions in Fig. 7 (a) 1, lysozyme; (b) 1, lysozyme; 2, cytochrome *c*.

that the maltose moiety shields the underlying surface of the capillary reasonably well over the pH range 4–7. However, the plate number is significantly smaller (about 25000) on the maltose coating than on the epoxy-diol coating. This indicates that for proteins adsorption still occurs, although to a small extent. Despite these drawbacks, the applicability of the maltose coating in the pH range of 5–7 of interest with respect to proteins justifies further investigations with other types of carbohydrates.

Stability of the coatings

The stability of the maltose and epoxy-diol coatings during prolonged use at various pH values was checked by measuring the retention times of lysozyme and cytochrome *c* with a standard buffer of pH 4 and at a voltage of 15 kV. For both coatings the retention times were found to be constant within 5% during 1 week of continuous full operation. Although this operation time is still too short to be able to draw a final conclusion about the long-term stability of the coatings, the preliminary results are promising.

CONCLUSIONS

The chemical modification of the wall of a fused-silica capillary with epoxy-diol to reduce adsorptive interactions between proteins and the surface appear to be effective only at pH < 5. The coating is stable and behaves electrophoretically in a similar way to the PEG coating described previously; however, the efficiency on the epoxy-diol coating is worse than that on the PEG coating. More promising is modification of the wall of the capillary with maltose because it shows a good shielding of the surface at intermediate pH values up to about 7. The maltose coating appears to be stable provided that an antimicrobial agent is added to the buffers. Despite the fa-

vourable pH range, the present maltose coating must be improved because the efficiency is too low. Cross-linking of the maltose moieties by thermal treatment and application of the modification procedure to other carbohydrates are now under investigation.

REFERENCES

1 F. E. P. Mikkers, F. M. Everaerts and Th. P. E. M. Verheggen, *J. Chromatogr.*, 169 (1979) 11.
2 J. W. Jorgenson and K. D. Lukacs, *J. Chromatogr.*, 218 (1981) 209.
3 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, *Anal. Chem.*, 59 (1987) 1021.
4 H. H. Lauer and D. McManigill, *Anal. Chem.* 85, (1986) 166.
5 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
6 M. Martin and G. Guiochon, *Anal. Chem.*, 56 (1984) 614.
7 G. J. M. Bruin, J. P. Chang, R. H. Kuhlman, K. Zegers, J. C. Kraak and H. Poppe, *J. Chromatogr.*, 471 (1989) 429.
8 S. Hjerten, *J. Chromatogr.*, 347 (1985) 191.
9 J. W. Jorgenson, *Trends Anal. Chem.* 3 (1984) 51.
10 K. K. Unger, *Porous Silica*, Elsevier, Amsterdam, Oxford, New York, 1979.
11 P. Flodin, in *Dextran Gels and Their Application in Gel Filtration, Dissertation*, A.B. Pharmacia, Uppsala, 1962.
12 D. J. Rose and J. W. Jorgenson, *Anal. Chem.*, 60 (1988) 642.
13 R. Huisden, J. C. Kraak and H. Poppe, in preparation.
14 J. H. Knox, *Chromatographia*, 26 (1988) 329.