Journal of Chromatography, 471 (1989) 429–436
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 385

CAPILLARY ZONE ELECTROPHORETIC SEPARATIONS OF PROTEINS IN POLYETHYLENE GLYCOL-MODIFIED CAPILLARIES

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SUMMARY

Fused-silica capillaries were wall modified with γ-glycidoxypropyltrimethoxysilane and polyethylene glycol 600 in order to decrease the influence of wall adsorption in capillary zone electrophoretic separations of proteins. It is shown that a significant decrease in adsorption is obtained and electro-osmotic flow is also diminished. For the proteins studied, symmetrical peaks were obtained in the pH range 3–5. However, some adsorption still occurs as the plate numbers are below theoretical expectations. At higher pH values appreciable peak deformations and drastic decreases in resolving power are observed. The procedure allows the rapid and efficient separation of protein mixtures suitable for separation in the indicated pH range, and the coating shows a good stability.

INTRODUCTION

Capillary zone electrophoresis (CZE) has been shown to be a separation technique with a very high resolving power for charged substances. The technique is less laborious than conventional gel electrophoresis with respect to quantitation because the preparation of the gel and staining/destaining procedures for band detection are absent in CZE.

Although CZE has frequently been applied to the separation of small molecules such as amino acids[1], peptides[2] and organic[3] and inorganic ions[4], a main interest is in its application to the separation and characterization of proteins. Especially proteins with molecular weights between 5000 and hundreds of thousands are suitable for analysis by CZE. In liquid chromatography (LC), highly efficient separations of proteins are very limited owing to the small diffusion coefficients, which lead to slow mass transfer. In contrast to LC, small diffusion coefficients are desirable in CZE as the efficiency (N) increases according to[2]

$$N = \mu_{0,i} V / 2D_i \quad (1)$$

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0021-9673/89/$03.50

where V is the applied voltage and $\mu_{0,i}$ is the overall mobility and D_i the molecular diffusion coefficient of a sample component i. This simple relationship was derived by Jorgenson and DeArman Lukacs[2] under the assumption of diffusion-limited band broadening. However, in practice, it will be difficult to realize this ideal situation, owing to the occurrence of additional band broadening caused by thermal gradients in the capillary system and perturbations in the local electric field within a sample zone due to conductivity changes. However, these additional band-broadening effects are small compared with the band broadening that occurs when large molecules distribute between the liquid and the wall, which will destroy the efficiency. As has been shown by Martin and Guiochon[5], the slightest adsorption will cause an appreciable decrease in efficiency, while moderate adsorption can lead to completely destroyed peaks shapes and poor reproducibility. Therefore, it is of paramount importance that adsorption is absent in order to exploit the full benefit of the separation of CZE.

Several approaches have been employed to eliminate wall adsorption of proteins. One of these, described by Lauer *et al.*[6], is based on adjustment of the pH of the buffer system to a value above the iso electric point (p*I*) of the proteins. Under such circumstances, both proteins and the silanol groups on the wall are negatively charged and repulsive forces result in strongly diminished adsorption. Highly efficient separations have been demonstrated using this approach[6]. Also, very low pH values, where electrical wall charges are small, have been applied[7]. However, in biopolymer separations it is of great importance to retain the pH as a freely adjustable parameter for various reasons: the stability of the sample materials may be involved; the pH is the predominant parameter governing the separation properties of the solutes; high pH values lead to a high electro-osmotic flow, which impairs the resolving power; and too large pH–p*I* differences may cause structural changes in the proteins.

An approach of more general scope is to bond chemically a neutral, hydrophilic coating to the capillary surface in order to shield the silanol groups so that a broad range of proteins can be separated with high resolution at various pH values. Moreover, the shielding of the silanol groups reduces the ζ-potential. It will be shown that the coating has a strong benificial influence on the electro-osmotic flow and the extent of adsorption.

Various chemical modifications of the capillary have already been employed in CZE, such as with polyacrylamide[8], glycol groups[9], polysiloxanes[7] and a glycero-glycidoxypropyl coating[7]. However, especially for protein separations, there is still a need for wall modification schemes that satisfy the following requirements: effective suppression of adsorption; reproducibility in the preparation of capillaries; long lifetime of the capillaries and preservation of the inertness and efficiency over that lifetime; and applicability over a wide pH range.

This work involves the application of a simple procedure to modify fused-silica capillaries with polyethylene glycol (PEG) chains in order to create a suitable hydrophilic surface structure, while the chemical epoxy-based bonding used favours long-term stability.

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 (SGE, North Melbourne, Australia) of 50 or 100 μm I.D. were used for the separations.

Detection was carried out at the cathodic side partly using a fluorescence detector (RF-530, Shimadzu, Kyoto, Japan) slightly modified for on-column detection at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. For most of the measurements, a UV detector (Kratos 757) with a modified cell arrangement for on-column work was used. This new arrangement facilitated the positioning of the capillary and of an adjustable slit in front of the capillary for focusing the light beam on the inner part of the capillary. The wavelength was set at 205 nm. The signal from the detectors was fed to 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 constant to within 1°C (25–26°C) during the separations.

Samples were injected by means of the electromigration technique[10] at constant voltage (5–10 kV) at the positive side for a fixed period of time (10–30 s). The protein samples were dissolved at a concentration of 1 mg/ml in phosphate buffer of the appropriate pH and concentration and could be stored for a few days at 4°C.

Reagents

All proteins were obtained from Sigma (St. Louis, MO, U.S.A.). Polyethylene glycol was purchased from Merck (Darmstadt, F.R.G.) and γ-glycidoxypropyltrimethoxysilane from Serva (Heidelberg, F.R.G.). The other reagents and solvents used were of analytical-reagent grade. Distilled water was used to prepare the buffers.

Capillary modification

The coating procedure is shown schematically in Fig. 1. The capillary (50 or 100 μm I.D.) was first etched with 1 *M* potassium hydroxide solution for 3 h at room temperature and rinsed with water for 10 min. Next the capillary was flushed with 0.1

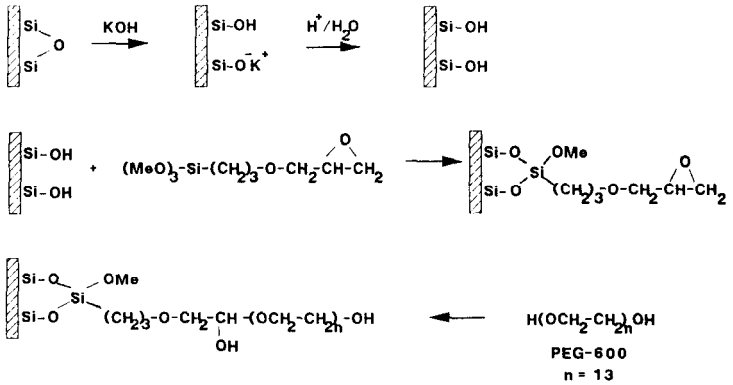


Fig. 1. Scheme of the procedure for the deactivation of the silica wall. Me = methyl.

M hydrochloric acid to remove K^+ ions from the wall and to produce free silanol groups at the surface of the wall. The capillary was dried at 200°C for 3 h with gentle flushing with helium. The dried capillary was then coated with the coupling reagent γ-glycidoxypropyltrimethoxysilane by pumping through a solution of the reagent in dried toluene (10%, v/v) at 110°C for 3 h at an inlet pressure of 0.5 MPa. Subsequently the unbound reagent was flushed from the capillary with toluene. Next the epoxide group was opened by a reaction carried out in the same manner with a solution of 20% polyethylene glycol 600 and 2% boron trifluoride etherate in dioxane for 1 h at 100°C. Finally, the capillary was rinsed with distilled water.

This standard procedure was applied to 50 and 100 μm I.D. capillaries with a length of approximately 1 m.

RESULTS AND DISCUSSION

Fig. 2 shows separations of protein mixtures obtained with (A) an untreated and (B) a PEG-600 treated capillary in 0.01 *M* phosphate buffer at pH 6.8. In both separations the same mixture, *i.e.*, lysozyme, trypsin and chymotrypsinogen, was injected. Most of the constituents do not elute at all in the untreated capillary, whereas in the PEG-treated tube a good separation is obtained. Lysozyme and chymotrypsinogen are invisible in Fig. 2A. The one peak in Fig. 2A corresponds to trypsin, as was established in a separate experiment (not shown). It can be safely assumed that wall adsorption impedes the successful separation in Fig. 2A.

Fig. 2C shows a separation with a PEG-treated tube under the same conditions but with more optimized conditions in terms of pH and buffer concentration. This electropherogram is typical of the efficiency obtained during this work. Plate numbers between 80 000 and 150 000,, depending on the species, pH, buffer concentration and capillary diameter, can be achieved routinely.

In experiments such as that in Fig. 2B, the proteins elute in the order expected on the basis of their isoelectric points (see Table I). Lysozyme, which has the highest p*I*

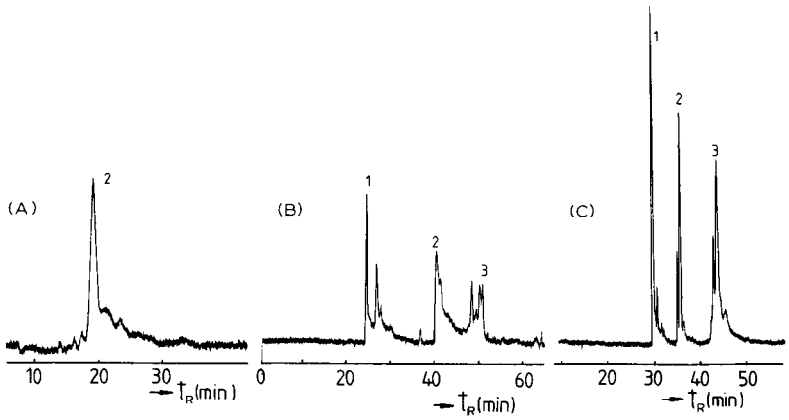


Fig. 2. Separation of a mixture of proteins (A) in an untreated and (B and C) in treated capillaries with fluorescence detection. Injection: (1) lysozyme, (2) trypsin and (3) chymotrypsinogen. (A) Untreated fused-silica capillary; buffer, 0.01 *M* KH_2PO_4, pH = 6.8. (B) PEG-coated capillary; buffer, 0.01 *M* KH_2PO_4, pH = 6.8. (C) PEG-coated capillary; buffer, 0.05 *M* KH_2PO_4, pH = 4.1.

TABLE I

ISOELECTRIC POINTS (p*I*) AND MOLECULAR WEIGHTS OF THE PROTEINS USED[11]

Protein	*pI*	*MW*
Cytochrome *c*	10.8	12 200
Lysozyme	10.0	14 000
Myoglobin	7.5	17 500
Trypsin	9.3	23 300
Ribonuclease	8.7	13 500
Trypsinogen	8.7	24 500
Chymotrypsinogen	8.7	21 600

value, has the highest charge of the three proteins at pH < 6 and elutes first. These results indicate that a substantial decrease in the adsorption activity of the wall for these proteins has been achieved.

Although the observed plate numbers are large, they are still low when compared with theoretical predictions. For instances, by means of eqn. 1 and using a value for the diffusion coefficient, D_i, of 10^{-10} m^2/s, a value for the mobility, $\mu_{0,i}$, of $2 \cdot 10^{-8}$ $m^2/V \cdot s$ and an applied voltage of 25 kV, a theoretical plate number of approximately $2.5 \cdot 10^6$ can be calculated, roughly a factor of 15 higher than the observed values. Although it was found to be possible to obtain higher plate numbers by improving the experimental set-up (cooling, injection), it can be assumed that the observed extra band broadening is still mainly caused by residual adsorption. As shown by Martin and Guiochon[5], a very small distribution towards the wall, such that it would not be noticed via deviations in the mobilities, can seriously impair the efficiency.

Effect of pH of the buffer solution

Operation at pH values below the isoelectric points of the proteins ensures that the three model proteins all have a net positive charge and as a result they migrate in the direction of the negative electrode.

Fig. 3 shows the dependence of the mobility of three proteins, lysozyme, trypsin and chymotrypsinogen, on the pH of phosphate solutions. The mobilities reported are "overall" values, *i.e.*, they include the osmotic flow of the liquid, *i.e.* $\mu_{0,i} = \mu_{el,i} + \mu_{ec}$. It must be noted that the electrophoretic and electro-osmotic flow are both directed towards the negative electrode in this instance and thus have the same sign. There is a decrease in the mobility (except for lysozyme) on going from pH 3.0 to 5.1. Because the positive charge on the proteins decreases at higher pH, there is a reduction in electrophoretic mobility. This reduction is stronger for trypsin and chymotrypsinogen because of their lower iso-electric points (see Table I).

At a pH of *ca.* 6, peaks in the electropherogram with strong tailing were observed. This may be caused by increasing adsorption of the proteins on the wall, either by interaction with residual surface silanols or by interaction of the less protonated proteins with the neutral, hydrophilic PEG chains.

Effect of concentration of the buffer solution

Fig. 4 illustrates the relationship between the observed mobilities, $\mu_{0,i}$, of the three proteins and the phosphate buffer concentration over the range 0.01–0.08 *M* at pH 3.8.

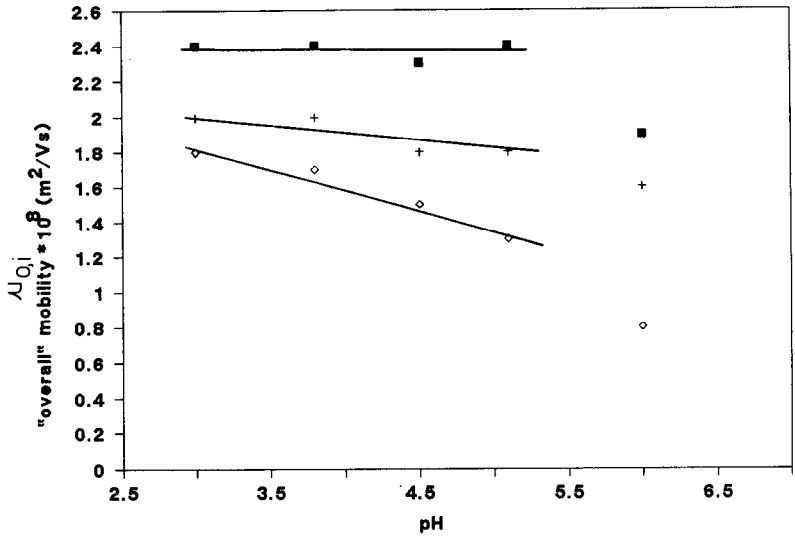


Fig. 3. Mobility, $\mu_{0,i}$, of the three model proteins as a function of pH at a KH_2PO_4 concentration of 0.05 *M*. Separation voltage, 20 kV; injection, 10 s, 10 kV. L = 0.59 m, $l_{inj.-det.}$ = 0.35 m. (■) Lysozyme; (+) trypsin; (◇) chymotrypsinogen.

It shows that the mobilities decrease on going from 0.01 *M* to 0.08 *M* KH_2PO_4 solution. This may be partly due to a changing effective charge (ζ-potential) of the proteins. However, the effect is mainly due to a strong reduction of the electro-osmotic flow.

The dependence of osmotic flow on buffer concentration, as measured with β-naphthol as a neutral marker, is shown in Fig. 5. The contribution of electro-osmotic

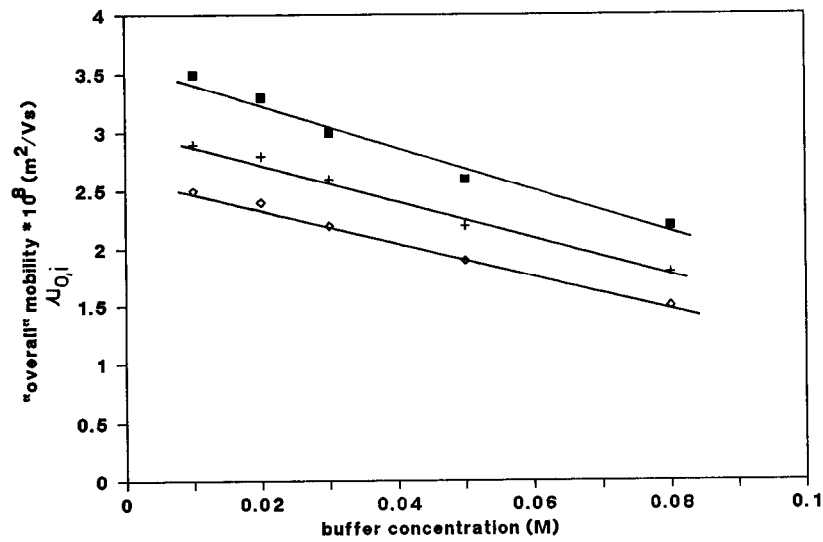


Fig. 4. Mobility, $\mu_{0,i}$, as a function of KH_2PO_4 concentration at pH 3.8. Separation voltage, 20 kV; injection, 10 s, 10 kV. L = 0.63 m, $l_{inj.-det.}$ = 0.37 m. (■) Lysozyme; (+) trypsin; (◇) chymotrypsinogen.

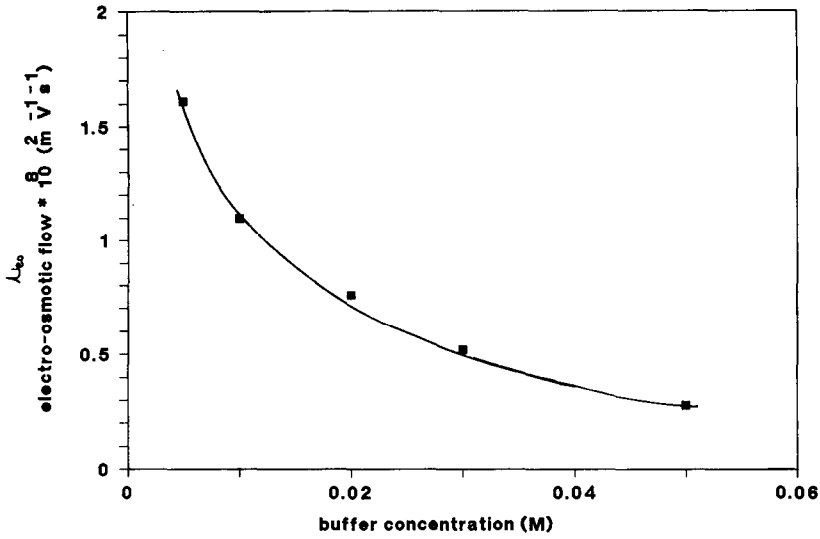


Fig. 5. Electro-osmotic mobility, μ_{eo}, as a function of KH_2PO_4 concentration at pH 3.8. L = 0.59 m, $l_{inj.-det.}$ = 0.35 m. Neutral marker: β-naphthol.

flow to the overall mobilities of the proteins is significant (10–70%), especially at buffer concentrations below 0.02 *M*. Working with buffer concentrations between 0.03 and 0.08 *M* resulted in a large reduction in the electro-osmotic flow.

It should be noted that these electro-osmotic velocities were observed to be much smaller than those in untreated capillaries, in agreement with earlier observations on modified silica tubes[3,7,12]. This again is an indication of the good coverage of the active sites of the fused-silica wall by PEG. At concentrations higher than 0.08 *M*, one can observe strongly tailing peaks for the three proteins. This can be attributed to interactions between the PEG chains and the proteins, the charge of the latter being partly shielded by the phosphate groups at higher concentrations.

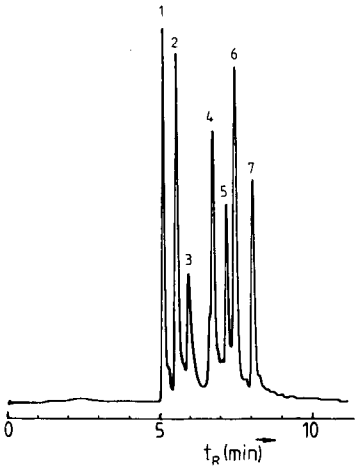
An example of the speed of this CZE system with the PEG-modified wall is shown in Fig. 6. Seven proteins are separated in less than 9 min.

Effect of applied voltage on the separations

As pointed out by Jorgenson and DeArman Lukacs[2], the reciprocal of the elution time should be linearly proportional to the applied voltage. In practice, the inverse elution time is not a linear function of voltage, as can be seen in Fig. 7. The increased temperature in the capillary at higher applied voltages reduces the viscosity of the buffer, which increases the lysozyme mobility.

Stability

The prepared wall-modified capillaries were found to be stable over several months. When not in use for a long period (months) they were stored after drying with a gentle stream of helium. They withstood treatment with dilute hydrochloric acid (0.01 *M*) applied after accidental irreversible adsorption of sample material, *e.g.*, at high pH.



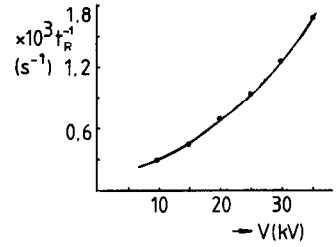


Fig. 6. Electropherogram of a mixture of seven proteins. Injection, 10 s, 10 kV. KH_2PO_4 concentration, 0.03 *M*, pH = 3.8. 1 = Cytochrome *c*; 2 = lysozyme; 3 = myoglobin; 4 = trypsin; 5 = ribonuclease; 6 = trypsinogen; 7 = chymotrypsinogen.

Fig. 7. Reciprocal of lysozyme retention time as a function of the applied voltage. $L = 0.89$ m, $l_{inj.-det.} = 0.65$ m. Buffer: concentration 0.05 *M*, pH = 3.8.

CONCLUSIONS

Deactivation of the wall of a fused-silica capillary with polyethylene glycol to avoid adsorptive interactions between the proteins and the wall is succesful at low pH. The capillaries can be used for several months without a noticable decrease in separation efficiency. However, it can be concluded that PEG-600-modified capillaries are not useful in the more interesting intermediate pH range between 5 and 8. Further work on fused-silica deactivation procedures, *e.g.*, with carbohydrate moieties, for the separation of proteins is in progress.

REFERENCES

1 E. Gassmann, J. E. Kuo and R. N. Zare, *Science (Washington, D.C.)*, 230 (1985) 813.
2 J. W. Jorgenson and K. DeArman Lukacs, *J. Chromatogr.*, 218 (1981) 209.
3 T. Tsuda, K. Nomura and G. J. Nakagawa, *J. Chromatogr.*, 248 (1982) 241.
4 T. Tsuda, K. Nomura and G. J. Nakagawa, *J. Chromatogr.*, 264 (1983) 385.
5 M. Martin and G. Guiochon, *Anal. Chem.*, 56 (1984) 614.
6 H. H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
7 R. M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
8 S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
9 J. W. Jorgenson, *Trends Anal. Chem.*, 3 (1984) 51.
10 D. J. Rose and J. W. Jorgenson, *Anal. Chem.*, 60 (1988) 642.
11 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.