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Journal of Chromatography A, 695 (1995) 287–296

JOURNAL OF CHROMATOGRAPHY A

Cellulose acetate-coated fused-silica capillaries for the separation of proteins by capillary zone electrophoresis

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Received 12 October 1994; accepted 30 November 1994

Abstract

Thin-film coatings of cellulose acetate on the surface of fused-silica capillaries were tested for the separation of proteins by capillary zone electrophoresis. The coating procedure is very simple and only involves the filling of the capillary with cellulose acetate solution, followed by flushing the capillary with helium. The coating appears to mask the underlying silanol groups towards basic proteins effectively; column efficiencies up to 10^6 plates per metre were achieved for ribonuclease A. The high efficiency, the batch-to-batch and the run-to-run reproducibility and the long-term stability of the coating are advantageous features of the method. The coating procedure provides a simple, stable and easy to reproduce method of surface deactivation and can be applied with other cellulose derivatives such as cellulose triacetate or cross-linked hydroxypropylcellulose. The films can also be applied to shield the surface of hollow polypropylene fibres. Unfortunately, this skin coating is destroyed above pH 7.5 and therefore cannot be recommended for zone electrophoresis at alkaline pH or for isoelectric focusing.

1. Introduction

There is a great demand for rapid, high-resolution analytical techniques to monitor the isolation and purification of proteins. Capillary zone electrophoresis (CZE) is a method that potentially offers rapid and quantitative protein analyses of high resolution and efficiency. However, satisfactory results are often difficult to obtain in protein separations with CZE. This is mainly due to the inherent tendency of these macromolecules to interact with the silanol groups present on the inside surface of the fused-silica capillary. These undesirable phenomena impede the full adoption of CZE as a routine bioanalytical technique. In order to exploit CZE fully for the analysis of proteins, the interaction between the silanol groups and proteins has to be eliminated.

Four different approaches have been reported and involve, briefly, (i) elimination of the interaction by adjusting the pH of the buffer to such a value that the silanol groups are non-charged [1]; (ii) adjusting the pH of the buffer of such a value that the charges of the silanol groups and protein have the same sign [2,3]; (iii) dynamic modification of the surface by neutral and cationic additives in the buffer [4–9]; and (iv) chemical modification using covalent bonding of the fused-silica surface [10–18].

The first three approaches are simple but are less attractive for the following reasons. To

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0021-9673/95/$09.50
SSDI 0021-9673(94)01185-0

diminish the interaction, the pH of the running buffer must be low or high and this increases the risk of hydrolysis [19] or denaturation [20] of the proteins. Also, one wants to retain the pH as an adjustable parameter in the CE of proteins. Dynamic modification by neutral and cationic additives in the background electrolyte has been shown to be an effective way to deactivate the silica surface. However, additives may change the buffer properties and can cause detection problems. Therefore, of the four approaches, chemical modification of the surface looks the most promising, also because of the flexibility to vary the pH to a certain extent. Unfortunately, the reported methods are laborious, except for the method of Malik et al. [21]. Moreover, some of the coatings show poor long-term stability.

Recently Gilges et al. [22] reported on an extremely efficient poly(vinyl alcohol) (PVA) coating by transferring a PVA film into a water-insoluble, permanently adhering layer by thermal treatment. This approach looks very attractive from the point of view of simplicity to prepare coatings.

In this paper, we report on another very simple, rapid and reproducible method to shield surface silanol groups by physically adhering a thin film of cellulose acetate on the capillary wall; we propose the term "skin coating" for this type of shielding. The physically adhered films were tested as a coating for the separation of proteins by CZE.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 50 μm I.D. (365 μm O.D.) was purchased from Polymicro Technologies (Phoenix, AZ, USA). The 56 μm I.D. (350 μm O.D.) hollow polypropylene (PP) fibre, manufactured by the melt-spinning department of AKZO, was a kind gift from AKZO Research Laboratories (Arnhem, Netherlands). Cellulose acetate (CA), M_r 30 000, and cellulose triacetate (CTA) were obtained from Aldrich Chimie (Steinheim, Germany), Tris–HCl from BDH (Poole, UK), sodium dihydrogenphosphate, sodium hydroxide, hydrochloric acid and paraformaldehyde from Merck (Darmstadt, Germany), acetone and hydroxypropylcellulose (average M_r 100 000) from Janssen Chimica (Geel, Belgium) and methylene chloride from Rathburn Chemicals (Walkerburn, UK). All protein samples were purchased from Sigma (St. Louis, MO, USA): cytochrome *c* (horse heart), lysozyme (chicken egg-white), ribonuclease A (bovine pancreas), trypsinogen (bovine pancreas), α-chymotrypsinogen A (bovine pancreas) and β-lactoglobulin A and B (bovine milk). Table 1 gives the molecular masses (M_r) and p*I* values of these proteins. Deionized water for the preparation of solutions was obtained from a Milli-Q water-purification system (Millipore, Milford, MA, USA).

2.2. Electrophoretic conditions

Electrophoresis was performed at 25°C with a laboratory-built system consisting of a high-voltage power supply (HCN 35-35.000; FUG Electronic, Rosenheim, Germany) and a UV detector, adapted for on-column detection (Model 757; Applied Biosystems, Foster City, CA, USA). The total set-up was placed in a Plexiglas box; opening the door automatically shut off the high voltage. The current in the system was measured over a 1-kΩ resistance in the return circuit of the power supply. The operating voltage was 20 kV unless indicated otherwise. Electromigration sample introduction was used (2 s,

Table 1
Isoelectric points (p*I*) and molecular masses (M_r) of the proteins [23]

Protein	Abbreviation	M_r	p*I*
Cytochrome *c*	Cyt *c*	12 200	10.8
Lysozyme	Lys	14 000	10.0
Ribonuclease A	Ribo A	13 500	8.7
α-Chymotrypsinogen A	α-Chym	21 600	8.7
Trypsinogen	Tryp	24 500	8.7
β-Lactoglobulin A	Lacto A	35 000	5.1
β-Lactoglubulin B	Lacto B	35 000	5.2

20 kV unless indicated otherwise). Before each run we rinsed the column and the collection and source vials with fresh background electrolyte solution. The optical window was prepared by burning off the outer polyimide coating before column preparation. The wavelength used for detection of the proteins was 210 nm, 0.02 AUFS (unless indicated otherwise). Electropherograms were recorded with a BD41 chart recorder (Kipp and Zonen, Delft, Netherlands) at a chart speed of 10 mm/min.

2.3. Column preparation

The flushing and coating solutions used to prepare the columns were delivered via a reservoir which can be pressurized by helium, as shown in Fig. 1. The coating procedure involves the following consecutive steps: a 75-cm long untreated fused-silica capillary is first rinsed with acetone for 15 min at 4 bar, then flushed for 15 min at 4 bar with the selected cellulose acetate solution in acetone. Next, the helium pressure is released and the capillary is pulled out of the coating solution and within 30 s the solution in the capillary is flushed out by restoring the helium pressure quickly to 4 bar, leaving a thin liquid film on the silica surface. The final immobilization is achieved by drying the coating with a gentle stream of helium for 30 min at 4 bar. The capillary is then ready for installation in the CZE system.

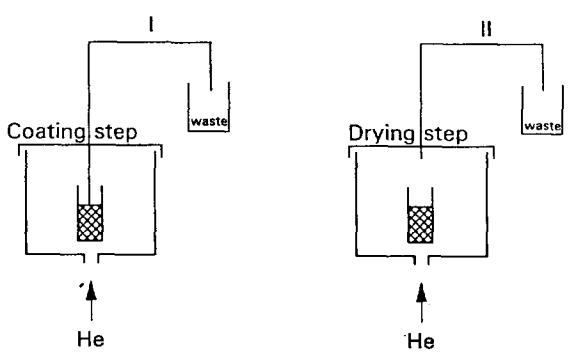

Fig. 1. Schematic representation of the coating and flushing reservoir.

2.4. Cross-linking procedure for hydroxypropylcellulose

The preparation of cross-linked hydroxypropylcellulose (HPC^c) was performed according to Ref. [24] and was as follows: hydroxypropylcellulose (4.5%, w/v) was dissolved in methanol and mixed with a cross-linking agent [3.5% (w/v) paraformaldehyde] and a catalyst [3.5% (w/v) hydrochloric acid] by using a glass rod; this solution should be used directly after its preparation.

3. Results and discussion

Our aim was to develop a simpler method to prepare an efficient coating for the separation of proteins by CZE than the laborious methods used so far. The choice to apply a film of cellulose diacetate, commonly referred to as cellulose acetate (CA), as a coating for the separation of basic proteins was inspired by the application of such films as a permselective layer, e.g., on electrodes to build size-exclusion selectivity into electrochemical detection [25–29] and from application of CA in slab gel electrophoresis [30]. Cellulose acetates are prepared by treating cellulose with a mixture of acetic acid and acetic anhydride in the presence of sulphuric acid as a catalyst. The reaction is generally allowed to proceed to substitute all three hydroxyl groups. The fully substituted triester derivative is then hydrolysed to give the desired level of substitution. Both cellulose acetate (CA) and cellulose triacetate (CTA) are good film-forming agents and have been used in a variety of pharmaceutical applications. The applications of cellulose triacetate include its use in dialysis membranes. Hydroxypropylcellulose (HPC) is a cellulose ether; it is water soluble and therefore has to be chemically cross-linked before it can be applied as a coating in CZE experiments [31].

The developed coating procedure is very simple, but the success rate and quality of the layer turned out to be largely dependent on some important parameters. Therefore, these aspects will be discussed below in more detail.

3.1. Preparation of the cellulose acetate film

Cellulose acetate dissolves in various solvents such as tetrahydrofuran, dimethylsulphoxide and acetone. Acetone had our preference because of its low viscosity, high volatility and non-toxicity. In order to dissolve the cellulose acetate quickly, it was found crucial to add the CA to the acetone with vigorous shaking because it tends to agglomerate on wetting.

The coating method is similar to the dynamic coating technique as used in capillary gas chromatography, and generally consists of filling the column with a solution of the coating phase, followed by forcing this volume through the column with helium pressure, A thin film of this solution is left behind on the capillary wall. Continuous flushing with helium after coating evaporates the remaining solvent and leaves a thin film of the polymer.

Two ways of filling the capillaries with the coating solution were tested, one in which the CA solution is directly introduced in an empty capillary and the other in which the CA solution is introduced into a capillary previously filled with the solvent, acetone in the case of CA. With the former method we obtained less efficient columns. This may be attributed to the poor wettability of the glass surface by the coating solution itself, which can result in droplet formation and subsequent non-uniform films. It has been shown that treatment with pentane, methylene chloride or acetone decreases the critical surface tension of glass by up to 50% [32]. Further, it appears to be important to flush out the CA coating solution from the capillary with helium immediately after the capillary has been disconnected from the coating reservoir. Any delay in that step may cause plugging at the front edge of the capillary.

The film thickness depends on, amongst other parameters, the viscosity of the coating solution and the speed (pressure) with which the solution is flushed out of the capillary. In our experimental set-up, an inlet pressure of 4 ± 0.5 bar produced very efficient coatings. This inlet pressure was established as the experimental result of measuring the efficiency of coatings using the same 1% (w/v) CA coating solution with various inlet pressures (2–5 bar). The adjustment of the pressure in this range is important as larger deviations appeared to result in a considerable loss of efficiency. It is probably possible to find another optimum coating concentration when using another column diameter or pressure drop.

3.2. Column performance

So far, all the coatings developed for the separation of proteins have the purpose of masking the silanol groups on the capillary surface, and as a result the electroosmotic flow is decreased significantly. In that respect the cellulose acetate coating is different as the film is still permeable to small molecules but not for larger molecules such as proteins. It appears that the electroosmotic flow with a mobility of about $9.2 \cdot 10^{-9}$ $m^2/V \cdot s$ differs by only 3% from that of a capillary that was used as delivered. This is surprising as cellulose derivatives are often used as electroosmotic flow suppressors in capillary electrophoresis and isotachophoresis. Apparently, cellulose acetate behaves differently in this respect.

In order to determine the optimum CA concentration with our experimental set-up and conditions, the performances of films fabricated with 0.85–6.0% (w/v) cellulose acetate solutions were investigated with a set of three basic proteins. The effect of the CA concentration on the efficiency is given in Table 2. We expressed the efficiency as plates per metre for a fair comparison with coating procedures already described in literature. Although the highest efficiency was obtained with the 3 and 6% (w/v) CA solutions, the efficiencies differed only slightly.

Etching of the capillary with potassium hydroxide prior to the CA coating treatment appeared to have a negative effect on the separation efficiency, as can be seen in Fig. 2, showing the electropherograms of the test proteins on capillaries prepared with (Fig. 2A) and without (Fig. 2B) prior hydroxide etching.

The performance of the cellulose acetate-coated capillaries was also investigated with the

Table 2
Effect of CA concentration in the coating solution on the separation efficiency

CA (%, w/v)	Efficiency, $N \times 10^5$ (plates/m)		
	Cyt *c*	Ribo A	α-Chym
0.85	9.31	10.13	7.40
1.0	8.06	9.19	9.06
1.2	7.72	9.66	9.48
1.5	7.02	8.15	6.26
3.0	7.86	11.04	10.20
6.0	8.17	10.45	9.71

Tris–HCl (50 m*M*, pH 4.0); applied voltage 20 kV; electromigration injection, 2 s, 20 kV.

same set of basic proteins using phosphate and Tris–HCl as background electrolytes. Fig. 3 shows the electropherograms obtained with the same capillary using 30 m*M* phosphate (pH 4.0) (Fig. 3A) and 50 m*M* Tris–HCl (pH 4.0) (Fig. 3B) as the background electrolyte. As can be seen, efficient separations can be realized with both background electrolytes. Excellent column efficiencies (up to 10^6 plates per metre) were found when using 50 m*M* Tris–HCl in the pH range 3–4.5. A typical separation using 50 m*M* Tris–HCl (pH 4.5) is shown in Fig. 4. The CA coating is stable in the pH range 2–7.5 and shows good reproducibility of the migration

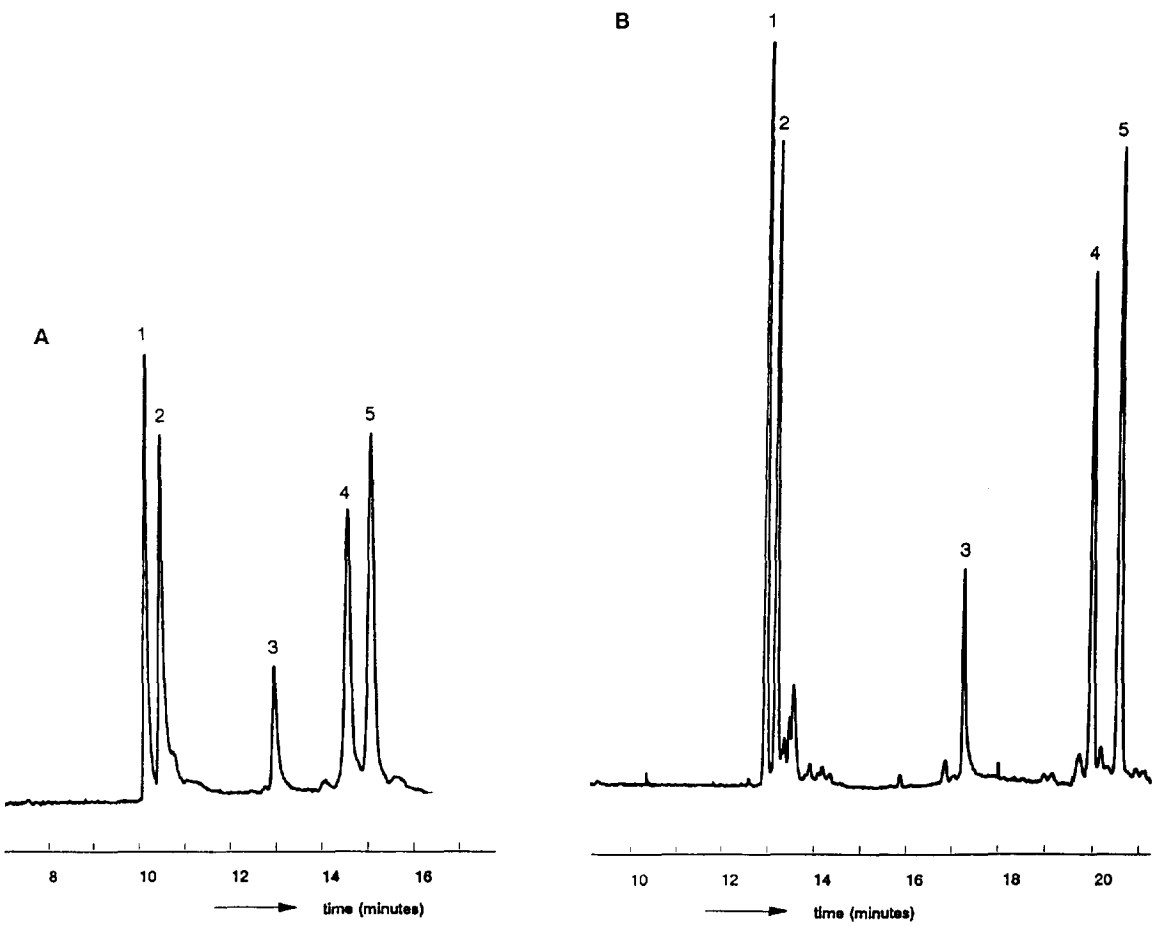

Fig. 2. CZE separation of the protein mixture on a CA-coated column. Total length of capillary, (A) 71.2 and (B) 75.2 cm; 50 μm I.D.; CA coating, 1% (w/v); effective length of capillary, (A) 47.5 and (B) 57.2 cm; electrolyte solution, 50 m*M* Tris–HCl (pH 4.3). Concentration of proteins, 0.2 mg/ml of (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, (4) α-chymotrypsinogen A and (5) trypsinogen. (A) Etched fused-silica capillary; (B) unetched fused-silica capillary.

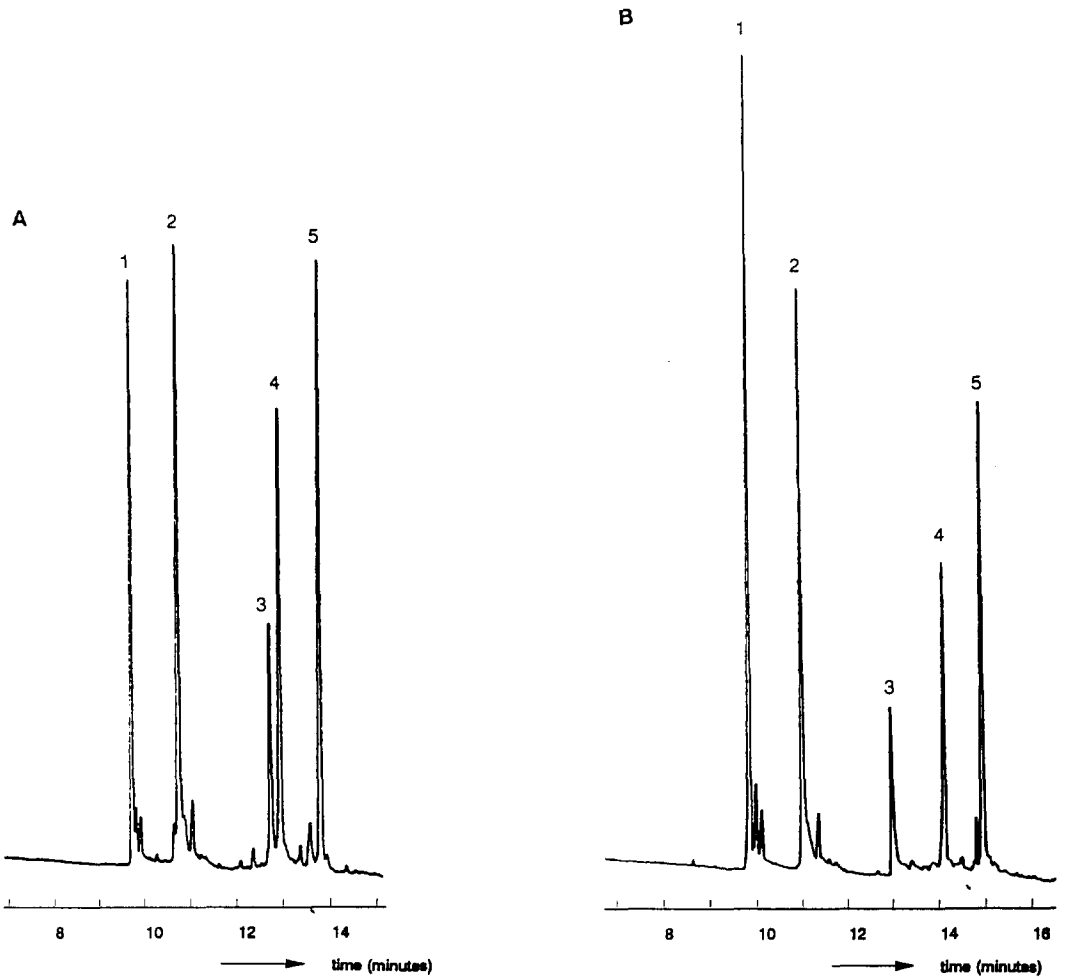

Fig. 3. CZE separation of the protein mixture on a CA-coated column. Total length of capillary, 74.9 cm; 50 μm I.D.; CA coating, 1% (w/v); effective length, 49.8 cm. Concentration of proteins, 0.2 mg/ml of (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, (4) α-chymotrypsinogen A and (5) trypsinogen. Electrolyte solution: (A) 30 m*M* phosphate buffer (pH 4.0); (B) 50 m*M* Tris–HCl (pH 4.0).

times as investigated for the basic proteins. The relative standard deviations (R.S.D.s) of migration times are reported in Table 3 and range between 0.2 and 0.4%.

The CA coating is in principle also suitable for acidic proteins, as is illustrated in Fig. 5, showing the separation of the two acidic proteins β-lactoglobulin A and B with theoretical plate numbers of 448 000/m for β-lactoglobulin A and 329 000/m for β-lactoglobulin B at pH 3.0.

3.3. Reproducibility and long-term stability of the CA coating

The reproducibility of the CA coating in terms of efficiency was investigated by measuring the plate number of the proteins on five columns prepared from the same 1.0% (w/v) CA coating solution. The variation in plate number was found to be less than 15%. A similar variation was found with columns produced with 1.0%

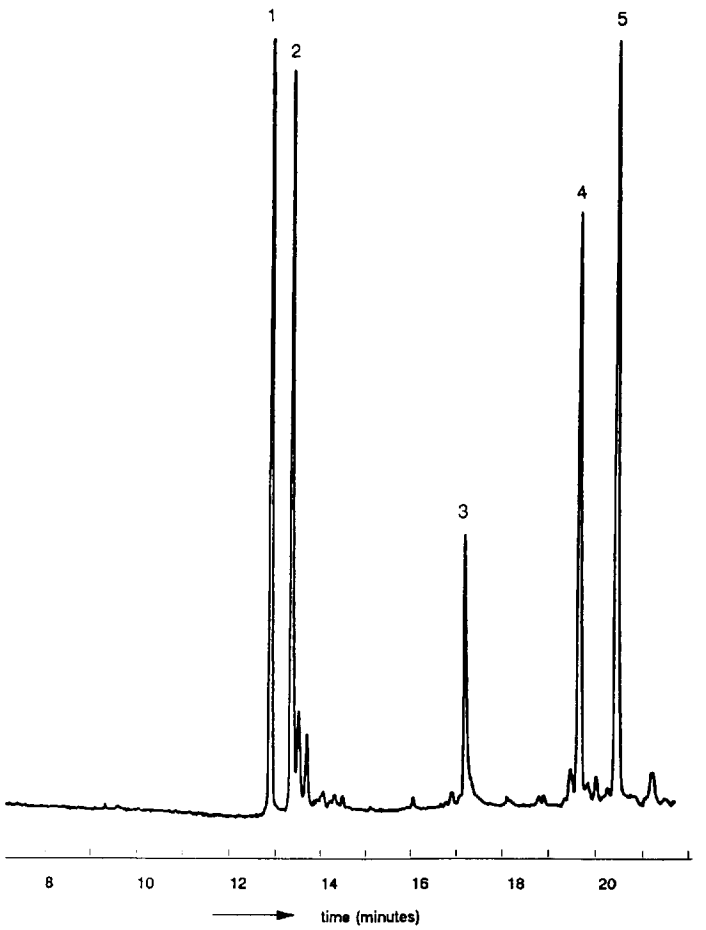

Fig. 4. CZE separation of the protein mixture on a CA-coated column. Total length of capillary, 78.8 cm; 50 μm I.D.; CA coating, 1% (w/v); effective length, 49.4 cm; electrolyte solution, 50 m*M* Tris–HCl (pH 4.5). Concentration of proteins, 0.2 mg/ml of (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, (4) α-chymotrypsinogen A and (5) trypsinogen.

Table 3
Relative standard deviations of migration times on a CA-coated capillary

Protein	R.S.D. (%)
Cytochrome *c*	0.3
Lysozyme	0.4
Ribonuclease A	0.3
Trypsinogen	0.2
α-Chymotrypsinogen	0.2

Tris–HCl (50 m*M*, pH 4.0); applied voltage, 20 kV; electromigration injection, 2 s, 20 kV.
[a] R.S.D. calculated from five experiments.

(w/v) CA solutions that were prepared separately. This variation is in our opinion acceptable, but a fair comparison with coating procedures described in the literature is impossible, because information regarding the reproducibility is scarce. In our hands, the success rate in preparing a good CA coating was almost 100%.

An influence of the surface properties of different batches of the fused-silica capillaries from our supplier on the efficiency of deactivation by CA was observed. A similar observation was made by Gilges et al. [22].

The long-term stability of the CA coating was

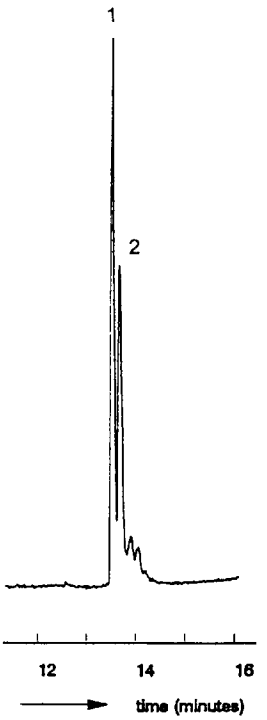

Fig. 5. CZE separation of β-lactoglobulin A and B on a CA-coated column. Total length of capillary, 74.4 cm; 50 μm I.D.; CA coating, 1% (w/v); effective length, 49.3 cm; electrolyte solution, 50 m*M* phosphate buffer (pH 3.0). Concentration of proteins, 0.2 mg/ml of (1) β-lactoglobulin B and (2) β-lactoglobulin A.

investigated by measuring the efficiency with time using Tris–HCl solutions of pH 4.3. No significant change in efficiency was noticed after continuously operating the column for more than 75 injections. Also, storage of columns in the electrolyte solution for 4 months did not alter the efficiency.

In the pH range 2–7.5, the CA coating remained intact. Above pH 7.5 the performance decreased abruptly and could not be regenerated.

3.4. Other cellulosic polymer coatings

The developed coating procedure for CA was also found to be applicable to other cellulosic polymers such as cellulose triacetate and cross-linked hydroxypropylcellulose. Methylene chloride was used to dissolve cellulose triacetate. Table 4 lists the polymers and the electrophoretic characteristics of the prepared capillaries. As can be seen, the prepared capillaries exhibit the same high separation efficiencies as found with the CA coating.

With cross-linked hydroxypropylcellulose (HPC^c), it was possible to modify the surface of a polypropylene hollow fibre, resulting in excellent efficiency characteristics for basic proteins. A typical separation of the selected test mixture of basic proteins on the HPC^c-coated hollow fibre is shown in Fig. 6A. Separations of this quality could not be obtained with dynamically coated PP fibres [33] or other coatings described in combination with hollow polymeric fibres [34].

Table 4
Efficiencies of columns prepared with different cellulosic polymer coatings

Polymer	Concentration (%, w/v)	Efficiency, $N \times 10^5$ (plates/m)			
		Cyt *c*	Ribo A	Tryp	α-Chym
Cellulose acetate (fused silica)	1.0	7.38	10.40	8.21	8.09
Cellulose triacetate (fused silica)	1.0	7.17	10.26	–	8.28
HPC^c (fused silica)	4.5	7.85	9.66	8.98	9.04
HPC^c (hollow PP fibre)	4.5	7.44	–	8.76	7.00

Tris–HCl (50 m*M*, pH 4.0); applied voltage, 20 kV; electromigration injection, 2 s, 20 kV.

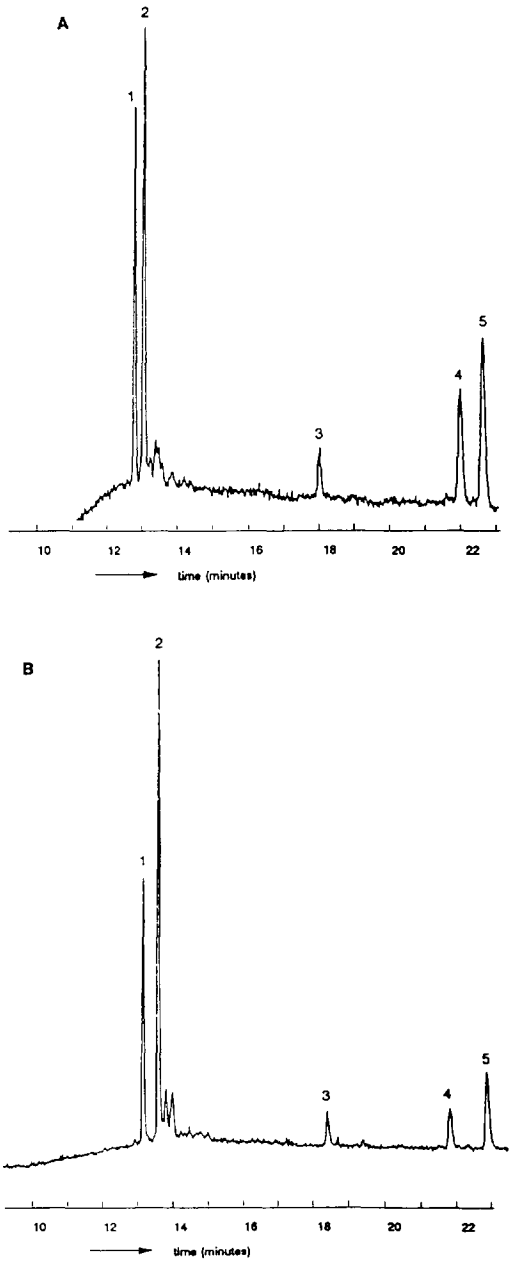

Fig. 6. CZE separation of the protein mixture on an HPC^c-coated hollow PP fibre. Total length of capillary, (A and B) 55.0 cm; HPC^c coating, 4.5% (w/v); effective length, 29.0 cm; electrolyte solution, 50 m*M* Tris–HCl (pH 4.3). Concentration of proteins, 1.5 mg/ml of (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, (4) α-chymotrypsinogen A and (5) trypsinogen. Separation voltage, 10 kV; electromigration injection, 2 s, 10 kV; detection wavelength, 280 nm (0.005 AUFS). (A) HPC^c-coated PP fibre; (B) HPC^c-coated fused-silica capillary.

For comparison, the same separation performed in a fused-silica capillary under otherwise identical conditions is shown in Fig. 6B. The R.S.D.s of the migration times and theoretical plate numbers *N* (per metre) on the fused-silica and the polypropylene capillaries coated with HPC^c are given in Table 5. This demonstrates that results of the same quality can be obtained with the PP tubes, which are more convenient and less fragile in practice. The main disadvantage of the polypropylene capillary is the limited UV transparency, which precludes the use of wavelengths below 240 nm, as can be inferred from a published UV absorbance spectrum [33]. Because of this limitation, the proteins were detected at 280 nm. At this wavelength only the aromatic amino acid residues in the proteins are detected, resulting in a decrease in detection sensitivity. The UV absorbance of the fibre material is assumed to be caused by (residues from) additives used in the production process. Further optimization of the production process and a reduction in the O.D.-to-I.D. ratio might improve this drawback in the future. Without coating the polypropylene fibre with cross-linked hydroxypropylcellulose the proteins are adsorbed on the capillary wall. The uncoated hollow polypropylene fibre is strongly hydrophobic and requires pre-wetting before introduction of an aqueous electrophoresis buffer. Methanol was used for this purpose in the

Table 5
Relative standard deviations of migration times and efficiencies with HPC^c surface modification

Protein	R.S.D. (%)[a]		Efficiency, $N \times 10^5$ (plates/m)	
	PP	Silica	PP	Silica
Cytochrome *c*	0.5	0.5	7.44	7.85
Lysozyme	0.6	0.5	6.43	8.03
Ribonuclease A	0.5	0.4	–	9.66
α-Chymotrypsinogen	0.7	0.4	8.76	8.98

Tris–HCl (50 m*M*, pH 4.0); applied voltage, 20 kV; electromigration injection, 2 s at 20 kV.
[a] R.S.D. calculated from ten experiments.

uncoated fibre. The pre-wetting agent can subsequently be simply exchanged with buffer solution.

4. Conclusions

Cellulosic polymer films, dynamically coated and physically adhering to the inside wall of fused-silica capillaries, appear to be an efficient coating for the separation of basic and some acidic proteins by CZE using Tris–HCl as electrolyte. The CA coating remains intact in the pH range 2–7.5.

Apart from the excellent efficiency, the simplicity and speed of fabricating the coating are important advantages compared with the laborious chemical modification methods used so far. Further, it was shown that this method of surface shielding can also be applied to hollow polypropylene fibres.

Acknowledgement

The authors thank Astra Hässle, Sweden, for financial support.

References

[1] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
[2] H.H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
[3] K.J. Lee and G.S. Heo, *J. Chromatogr.*, 559 (1991) 317.
[4] M.M. Bushey and J.W. Jorgenson, *J. Chromatogr.*, 480 (1989) 301.
[5] J.S. Green and J.W. Jorgenson, *J. Chromatogr.*, 478 (1978) 63.
[6] J.K. Towns and F.E. Regnier, *Anal. Chem.*, 63 (1991) 1126.
[7] A. Emmer, M. Jansson and J. Roeraade, *J. Chromatogr.*, 547 (1991) 544.
[8] M. Gilges, H. Husmann, M.-H. Kleemiss, S.R. Motsch and G. Schomburg, *J. High Resolut. Chrometry.*, 15 (1992) 452.
[9] J.A. Bullock and L.-C. Yuan, *J. Microcol. Sep.*, 3 (1991) 241.
[10] S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
[11] G.J.M. Bruin, R. Huisden, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 480 (1989) 339.
[12] M. Huang and M.L. Lee, *J. Microcol. Sep.*, 4 (1992) 491.
[13] J.W. Jorgenson and K.D. Lukacs, *Anal. Chem.*, 53 (1981) 1298.
[14] J.A. Lux, H. Yin and G. Schomburg, *J. High Resolut. Chromatogr.*, 13 (1990) 145.
[15] J.K. Towns and F.E. Regnier, *J. Chromatogr.*, 471 (1989) 429.
[16] B.J. Herren, S.G. Shafer, J. Alstine, J.M. Harris and R.S. Snyder, *J. Colloid Interface Sci.*, 115 (1987) 46.
[17] S.A. Swedberg, *Anal. Biochem.*, 185 (1990) 51.
[18] Y.-F. Maa, S.A. Swedberg and K.J. Hyver, *J. High Resolut. Chromatogr.*, 14 (1991) 63.
[19] B.L. Karger, A.S. Cohen and A.J. Guttman, *J. Chromatogr.*, 492 (1989) 585.
[20] S.A. Swedberg, *Anal. Biochem.*, 185 (1990) 51.
[21] A. Malik, Z. Zhao and M.L. Lee, *J. Microcol. Sep.*, 5 (1993) 119.
[22] M. Gilges, M.H. Kleemiss and G. Schomburg, *Anal. Chem.*, 66 (1994) 2038.
[23] P.G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
[24] S. Suto, H. Tashiro and M. Karasawa, *J. Appl. Polym. Sci.*, 45 (1992) 1569.
[25] L.S. Kuhn, S.G. Weber and K.Z. Ismail, *Anal. Chem.*, 61 (1989) 303.
[26] G. Sittampalam and G.S. Wilson, *Anal. Chem.*, 57 (1983) 1608.
[27] J. Wang and L.D. Hutchins, *Anal. Chem.*, 57 (1985) 1536.
[28] L.D. Hutchins, J. Wang and P. Tuzhi, *Anal. Chem.*, 58 (1986) 1019.
[29] C.-W. Wang and I.-C. Chen, *Anal. Chem.*, 64 (1992) 2461.
[30] J. Ambler, B. Janik and G. Walker, *Clin. Chem.*, 29 (1983) 340.
[31] V. Kumar and G.S. Banker, *Drug Dev. Ind. Pharm.*, 19 (1993) 1.
[32] M.L. Lee and B.W. Wright, *J. Chromatogr.*, 184 (1980) 235.
[33] M.W.F. Nielen, *J. High Resolut. Chromatogr.*, 16 (1993) 62.
[34] P.Z. Liu, A. Malik, M.C.V. Kuchar, W.P. Vorkink and M.L. Lee, *J. Microcol. Sep.*, 5 (1993) 245.