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Prevention of protein adsorption on surfaces by polyethylene oxide–polypropylene oxide–polyethylene oxide triblock copolymers in capillary electrophoresis

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ABSTRACT

Protein adsorption to untreated fused-silica capillaries in capillary electrophoresis was prevented by a chemical modification of the capillary surface. This was achieved through the coating of Pluronic polymers onto capillaries which were pre-derivatised with silylating agents. The polymers were made up of polyethylene oxide–polypropylene oxide–polyethylene oxide triblock copolymers. The coatings prevent protein adsorption and provide high-efficiency separations. Two different types of silylating agents were used and the effects of the concentrations on the stability and efficiency of the columns were examined. The application of these columns for capillary electrophoretic separation of protein samples was demonstrated.

INTRODUCTION

Recent developments in capillary electrophoresis (CE) have been remarkable and its applications in the analysis of physiological substances have been widely explored [1–7]. There is, however, an inherent problem in the use of untreated fused silica for solutes such as proteins: their inevitable adsorption onto the inner walls of the capillaries and the hysteresis [8] of electroosmotic flow (EOF) with changes in pH. The consequences of these phenomena are irreproducible separations and unacceptable variations in migration times.

Much work has been done to develop methods for preventing adsorption, as well as eliminating EOF, particularly for protein analysis [9–20]. Strategies to control wall effects include the use of buffers that have extremely high or low pH values [9,10], metal ion additives [11,12] and high ionic strengths [13]. These methods generally require the use of extreme conditions and therefore may only be useful for certain specific groups of proteins. Permanent surface wall silanol modifications is more practical in eliminating EOF and protein adsorption. Briefly, surface wall modification includes coating the columns with non-ionic surfactants [14], polyethylene glycol [15,16], polyacrylamide [17], linear polyacrylamide anchored by a silicon–carbon bond [18] and more recently, cross-linked polyacrylamide [19] and hydrogel polymers [20]. Although these coated columns are able to achieve the objective of preventing adsorption and eliminating EOF for CE and capillary isoelectric focusing applications, the coating procedures involve tedious silanization and elaborate coating chemistries. This is disadvantageous if coated columns are to be used for routine analysis.

In this work, a procedure was developed for the coating of columns using Pluronics as the coating materials. Pluronics, consisting of polyethylene oxide–polypropylene oxide–poly-

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ethylene oxide (PEO–PPO–PEO) triblock copolymers, have been widely used in the chemical, agricultural and pharmaceutical industries as non-ionic polymer surfactants [21]. Their commercial availability, stability, low toxicity [21] and low cost are attractive properties for them to be considered as coating materials for CE.

In this study, a simple and versatile approach for preparing protein-resistant coated columns is described. Alkylsilane-treated capillary columns were coated with Pluronic triblock copolymers. The relative importance of PEO and PPO chains of Pluronic copolymers in preventing protein adsorption and on the EOF was examined. The use of different silylating agents and their concentrations on the stability and efficiency of the columns were evaluated. Finally, the usefulness of the coated columns in preventing protein adsorption was demonstrated.

EXPERIMENTAL

Instrumental

CE was conducted in a laboratory-built system. A Spellman Model RM30P10KD (Plainview, NY, USA) high-voltage power supply capable of delivering up to 30 kV was used. Detection of peaks was accomplished through the use of a ISCO Model CV^4 UV detector (Lincoln, NE, USA) with wavelength set at 200 nm. Untreated fused-silica capillaries of 50 μm I.D. and effective lengths of 31 to 33 cm and overall lengths of 43–45 cm were obtained from Polymicro Technologies (Phoenix, AZ, USA). Chromatographic data were recorded on a Shimadzu Model R6A (Kyoto, Japan) integrator. Samples were hydrodynamically injected for 5 s at a height of 8 cm. N,N-Dimethylformamide (DMF)–water (50:50) was used as the neutral marker for EOF measurements.

Reagents and materials

Sodium dihydrogenphosphate, disodium hydrogenphosphate, sodium tetraborate and orthophosphoric acid used for preparing the buffer solutions were purchased from Fluka (Buchs, Switzerland). All protein samples were purchased from Sigma (St. Louis, MO, USA). Six different types of Pluronic polymers depicted in Table I were obtained from BASF (Parsipanny, NJ, USA). Silylating agents, octyldecyltrichlorosilane and dichlorodimethylsilane were obtained from Aldrich (Milwaukee, WI, USA).

TABLE I

PROPERTIES OF PLURONIC TRIBLOCK COPOLYMERS USED IN THIS INVESTIGATION

Pluronic	Physical state	Average M_r	PEO–PPO–PEO
P-103	Paste	4 950	19:56:19
P-104	Paste	5 850	28:56:28
P-105	Paste	6 500	37:56:37
F-108	Solid	14 000	129:56:129
P-84	Paste	4 200	22:38:22
P-123	Paste	5 750	19:69:19

Capillary coating procedure

An untreated fused-silica capillary was first rinsed with 0.1 *M* sodium hydroxide for 15 min followed by 15 min of water and finally with 5 min of methanol. It was dried by flushing nitrogen through it in a GC oven at 110°C for 3 h. Two different methods for silylating the capillary were investigated. The procedure for derivatising the capillary column with octyldecyltrichlorosilane with 5% methylene chloride was similar to that described by Towns and Regnier [14]. An alternative method of derivatising the capillary column developed in this laboratory involved passing a solution containing 5% of dichlorodimethylsilane (DDS) or octyldecyltrichlorosilane (ODTC) in toluene through a capillary for 5 min at room temperature. Subsequently, the capillary was statically coated with the silylating agent for 3 h, with a 5-min rinse using the silylating agent at the 1.5-h interval. This silylating procedure was modified from a method for treating glass coverslips [22] which involved silylating for 2 h at room temperature. Excess silylating agent was flushed out with methanol and the capillary was again dried in the oven for 2 h. Subsequently, Pluronic polymers dissolved in phosphate-buffered saline (PBS, pH 7.0) of varying concentrations (0.1–5 mg/ml) were introduced into the capillary with a syringe for 2 min and the capillary was then statically coated

with the polymer solution for 2 h at room temperature. The capillary was then rinsed with PBS, water and finally, buffer solutions before use.

RESULTS AND DISCUSSION

To determine whether a coating material is suitable to be used for coating capillary columns, several criteria have to be met:

(1) It should be able to give a smooth and even layer of coating to prevent protein adsorption.

(2) It should provide reproducibility in migration time over a wide pH range.

(3) It should provide reproducible separations for different columns coated with the same material.

(4) It should be stable over a wide pH range and over a wide potential difference across the capillary.

(5) It should be able to provide highly efficient separations.

The triblock copolymer used in this work consists of varying lengths of PEO–PPO–PEO as shown in Table I. It was adsorbed onto alkylsilane-derivatised surfaces and their utility was examined in terms of their ability to prevent protein adsorption and elimination of EOF. The various chain lengths of PEO and PPO may play an important role in preventing adsorption of proteins and reduction of EOF. Two types of alkylsilane were used, DDS and ODTC. They alone, when used to derivatise the capillary column, failed to prevent adsorption of proteins. This could be due to their high hydrophobicity and insufficient surface coverage.

It was found that the alkylsilane used for the derivatisation of capillary surfaces had an effect on the efficiency of the capillary. This is shown in Table II for two model proteins, cytochrome *c* and lysozyme. The number of theoretical plates per meter is calculated using the formula

$$N = 5.54(t_R/w)^2 \cdot (100/L)$$

where t_R is the migration time of the protein, w is the width at half height of the peak and L is the effective length of the column in cm. It was

TABLE II

EFFECT OF DIFFERENT SILYLATING AGENTS AND THEIR COMPOSITIONS ON EFFICIENCY

Conditions: 5 mg/ml F-108 modified column; 44 cm (32 cm effective length) × 50 μm I.D.; buffer: 0.01 *M* phosphate at pH 3; voltage: 10 kV.

Silylating agent	Efficiency (plates/m)	
	Cytochrome *c*	Lysozyme
5% DDS in toluene	613 922	767 870
5% ODTC in toluene	336 662	488 397
95% ODTC with 5% CH_2Cl_2	179 262	142 713

observed that 5% DDS in toluene offered the highest plate number compared to ODTC. This is surprising as stronger hydrophobic interaction between the longer alkyl chain of the ODTC and the Pluronic polymer would be expected. The PPO segment of the copolymer is known to adsorb onto hydrophobic surfaces by hydrophobic interactions while the water-soluble PEO chains would extend into the bulk aqueous medium [23,24]. One possible reason for this anomalous behaviour could be that DDS, being more symmetrical (two chloro and two methyl groups) than ODTC, is able to give a homogenous and smooth layer for the adsorption of the PPO segment. ODTC, on the other hand, tends to form polymeric layers which cannot be effectively shielded by the copolymeric Pluronic. Thus, effective anchoring of the PPO chain of the Pluronic polymer was inhibited. Consequently, the efficiency of the column was reduced in the case of ODTC. Another possible reason could be that DDS is less hydrophobic than ODTC, thus there is less chance for adsorption for the hydrophobic proteins. As evident from Table II, 5% of ODTC gave higher efficiencies compared to 95% ODTC. This shows that the higher the concentration of ODTC, the less efficient the column. The weak binding of the ODTC-derivatised columns may also account for the irreproducible migration times and unstable currents when high voltages (25 kV) were used. This irregular behaviour was not observed for the Pluronic–DDS-derivatised columns. Among

the various coatings studied, 5% DDS was able to offer the highest efficiencies. Therefore, it was subsequently used for silylating all the columns to be coated. It is worth noting that in the present work, the method of silylating the columns is simpler and does not involve heating as compared to other methods [14,15,25].

The EOF and efficiencies for the various Pluronic-coated capillaries is shown in Table III. The Pluronics (P-103, P-104, P-105 and F-108) chosen for the measurement of EOF were those in which the number of PO chains remained constant while the number of EO chains varied in length. With increasing molecular mass of the Pluronic polymers from P-103 to F-108, the adsorbed layer became thicker and more viscous. The increase in the viscous drag caused a reduction in EOF. F-108 is the most effective material in eliminating EOF compared to the other Pluronics. The EOF of F-108 coated capillary was approximately 1/60 that of the untreated capillary while that of P-103 was 1/6 at the conditions stated. Thus, moderation of EOF could be accomplished by using different Pluronic polymers, which at the same time also prevented protein adsorption.

The length of the PEO chain also has a significant effect on the column efficiency. As shown in Table III, when the length of the PEO chain became longer, the column efficiency increased. F-108, having the longest PEO chain (129 units), resulted in the highest efficiencies compared to the other Pluronics when used as coating material. This could be attributed to the long PEO chains which are able to intertwine to form a network extending into the aqueous medium, which prevents further interaction between the proteins and the surface.

The performance of the Pluronic-coated capillaries in terms of reproducibility and efficiency was evaluated at low and high pH. Fig. 1a and b show the separation of some basic model proteins at low pH using the coated and uncoated capillary columns, respectively. Improved resolution and high efficiencies were observed when coated columns were used. The molecular masses, isoelectric points and reproducibility of migration times for the model proteins separated at pH 3 are given in Table IV. The separation

TABLE III

EFFECT OF DIFFERENT PLURONIC POLYMERS ON EOF AND COLUMN EFFICIENCY FOR CYTOCHROME *c*

Conditions: 1 mg/ml Pluronic on capillary derivatised with DDS, 40 cm (31 cm effective length) × 50 μm I.D.; buffer: 0.01 *M* phosphate, pH 6; voltage: 10 kV.

Column	EOF × 10^8 (m^2/V s)	Efficiency (plates/m)
Uncoated	6.0	103 355
P-103	1.0	73 686
P-104	0.9	85 148
P-105	0.8	371 798
F-108	0.1	630 853

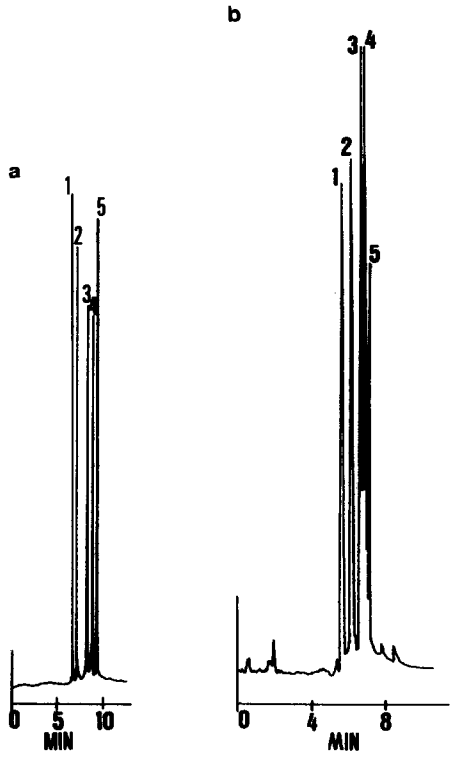

Fig. 1. Typical electropherograms showing the separation of a model protein mixture using (a) coated and (b) uncoated fused-silica capillary. For peak identification see Table IV. Electrophoretic conditions: 0.01 *M* phosphate buffer, pH 3.0 (adjusted with phosphoric acid) for coated column and 0.025 *M* phosphate for uncoated capillary; separation column: 43 cm (31 cm effective length) × 50 μm I.D.; voltage: 10 kV.

TABLE IV

REPRODUCIBILITY OF MIGRATION TIMES BETWEEN COATED AND UNCOATED COLUMN FOR SOME MODEL BASIC AND ACIDIC PROTEINS

Electrophoretic conditions for the basic proteins (peaks 1–5) are as in Fig. 1 and for the acidic proteins (peaks 6–8) are as in Fig. 2.

Peak No.	Protein	M_r [a]	pI [a]	R.S.D. (%)[b]	
				Coated	Uncoated
1	Cytochrome *c*	12 200	10.8	0.26	0.60
2	Lysozyme	14 000	10.0	0.24	0.51
3	Trypsin	23 300	9.3	0.24	0.57
4	Trypsinogen	24 500	8.7	0.24	0.59
5	α-Chymotrypsinogen A	21 600	8.7	0.24	0.61
6	Pepsin	34 700	3.1	0.11	0.76
7	Amyloglucosidase	89 000	3.6	0.26	0.77
8	Trypsin inhibitor	20 100	4.6	0.24	0.70

[a] M_r and pI values taken from ref. 26.
[b] $n = 7$ for basic proteins; $n = 5$ for acidic proteins.

conditions for both cases were identical except for the ionic strengths of the buffer system. McCormick [10] has found that the changes in EOF at low pH are minimal, accounting for the less than 1% R.S.D. for the migration times of the proteins when uncoated columns are used. However, the R.S.D. was further improved with the use of coated capillaries. It was noted that the migration order of the basic proteins was in accordance with their pI values, as expected. In addition, the stability of the coated column was tested using a buffer of pH 4.5 and no significant degradation of coating was observed even after 80 runs.

The coated column was also used to separate acidic proteins at alkaline (pH 8) conditions. The separation of three acidic proteins at pH 8 is shown in Fig. 2. The reproducibility in migration times for coated and uncoated columns is shown in Table IV. A three-fold improvement in the R.S.D. for the migration times was observed when coated columns were used. The polarity of the power supply was reversed for the coated column as the proteins would be negatively charged at this pH and the EOF would be too weak to elute them towards the detector at the cathodic end. The usefulness of a Pluronic-

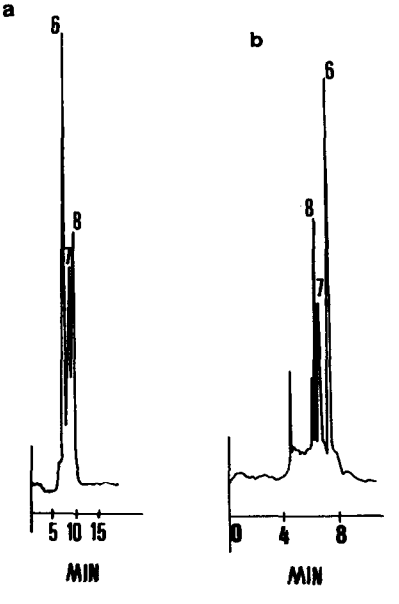

Fig. 2. Typical electropherograms showing the separation of some acidic proteins using (a) coated and (b) uncoated capillary at pH 8.0. For peak identification see Table IV. Electrophoretic conditions: (a) 5 mg/ml of F-108 modified column, 47 cm (35 cm effective length) × 50 μm I.D.; 0.01 *M* phosphate–borate buffer; voltage: − 18 kV. (b) 48 cm (40 cm effective length) × 50 μm I.D.; 0.025 *M* phosphate buffer; voltage: 12 kV.

coated capillary for the separation of some model acidic and basic proteins is evident from Fig. 3.

The separation efficiencies of the Pluronic-coated and of uncoated capillaries were also compared. The separation efficiencies at four different voltages for cytochrome *c* as an example are given in Table V. The separation efficiencies for all the basic proteins listed in Table IV at 10 kV are more than half a million theoretical plates. This figure is not only higher than that for the uncoated column (as indicated in Table V), but also considerably higher than the figures obtained by other coatings [8,14,18,25–27]. The high efficiency observed is due to the hydrophobic adsorption of the PPO chain to the evenly DDS-derivatised fused silica while the two ends of the hydrophillic PEO chain extend into the aqueous phase which further prevents the proteins from interacting with the wall of the fused-silica capillary.

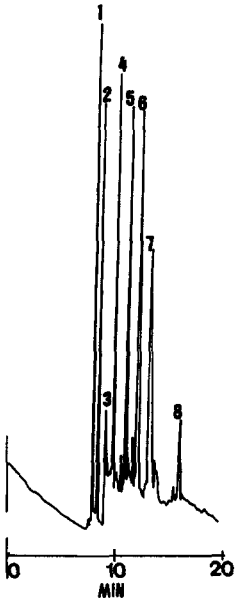

Fig. 3. Typical electropherogram showing the separation of a mixture of basic and acidic proteins. Peaks: 1 = cytochrome *c*; 2 = lysozyme; 3 = lactic dehydrogenase; 4 = trypsin; 5 = trypsinogen; 6 = α-chymotrypsinogen A; 7 = β-lactoglobulin A; 8 = carbonic anhydrase. Electrophoretic conditions: 0.1 mg/ml F-108 modified column, 43 cm (31 cm effective length) × 50 μm I.D.; 0.01 *M* phosphate, pH 4.5 (adjusted with phosphoric acid); voltage: 10 kV.

TABLE V

COMPARISON OF EFFICIENCIES OF COATED AND UNCOATED CAPILLARIES FOR CYTOCHROME *c* AT VARIOUS VOLTAGES

Condition: 5 mg/ml F-108 coated column, 43 cm (31 cm effective length) × 50 μm I.D.; buffer: 0.01 *M* phosphate, pH 3.

Voltage (kV)	Current (μA)	Efficiency (plates/m)	
		Coated	Uncoated
10	5	613 922	103 355
15	8	489 187	37 793
20	10	408 095	36 430
25	13	332 843	24 162

However, it should also be noted that the extremely high number of theoretical plates observed is only slightly more than half the number of theoretical plates predicted by theory [28]. The reason is that although electrostatic interaction of the proteins (in this case the proteins are positively charged) with the coated capillary wall has been reduced, hydrophobic interaction of protein with the coating is possible. Nevertheless, the prevention of adsorption and the increase in efficiencies achieved by using the Pluronics coating represent significant advantages for protein separations by CE.

The effect of separation voltage on efficiency is shown in Table V, both for coated and uncoated capillary columns. The number of theoretical plates was observed to decrease with increase in voltage. This observation is in accordance with the work of Cobb *et al.* [18]. Two possible reasons that would have caused this decline in efficiency are band broadening due to thermal effects at high voltages, and probable increased hydrophobic interaction with the coating material at higher temperatures. As the field is increased and the ions move faster, the possible desorption kinetics may cause band broadening. Since conditions at 10 kV offered the highest efficiency and reproducibility with reasonable migration times, subsequent separations were carried out at this voltage.

The effect of the concentration of coating

TABLE VI

COMPARISON OF THE EFFECT OF CONCENTRATION OF COATING SOLUTION ON EFFICIENCY AND MIGRATION TIME WITH RESPECT TO CYTOCHROME *c*

Conditions: 47 cm (35 cm effective length) × 50 μm I.D.; buffer: 0.01 *M* phosphate, pH 3.0; voltage: 10 kV.

Concentration of Pluronic F-108 (mg/ml)	Efficiency (plates/m)	Migration time (min)
0.1	527 577	7.245
1.0	630 853	7.418
3.0	735 628	7.759
5.0	613 922	7.645

Fig. 4. Electropherogram of a sample of protamine. Electrophoretic conditions: 5 mg/ml F-108 modified column, 43 cm (31 cm effective length) × 50 μm I.D.; 0.01 *M* phosphate–borate, pH 5.5; voltage: 15 kV; wavelength: 200 nm.

solution on the efficiency and migration time was investigated and the results are shown in Table VI. It was noted that there was no definite trend in column efficiency with changes in the concentration of Pluronic F-108. It is worthwhile to note that all concentrations gave theoretical plate numbers of at least half a million. Since coating thickness is expected to be proportional to the concentration of the coating solution [29], the EOF should decrease with an increase in concentration of the coating solution. This is indicated by the increase in the migration time of cytochrome *c*. The migration time was comparable at a coating concentration of 3 mg/ml and 5 mg/ml. This is an indication that the alkylsilane-derivatised fused silica has reached its coating saturation point. The migration time for the neutral marker, DMF, for all the concentrations investigated was longer than 2.5 h, corresponding to an EOF value of approximately $1.5 \cdot 10^{-9}$ $m^2/(V\ s)$ at pH values 3 and 6. In other words, for a particular copolymer such as F-108, a coating concentration of 0.1 mg/ml is as effective in eliminating EOF and protein adsorption as a 5 mg/ml coating solution.

Another advantage of using Pluronics as coating material is that there is no observable leaching of the polymer into the buffer as has been found for non-ionic surfactants by Towns and Regnier [14] with other coatings. It was therefore unnecessary to add small amounts of surfactants to the electrophoretic buffer. Since addition of surfactants into the buffer solution with a concentration greater than the critical micelle concentration may result in a reduction in efficiency [14], the present method is preferable from this viewpoint.

The application of the Pluronic-coated capillaries was demonstrated by the analysis of protamine, a basic protein found in the sperm of the Salmon fish. The electropherogram obtained is shown in Fig. 4. Protamine was eluted in a relatively short time (less than 6 min) and high efficiency could be obtained using the Pluronics-coated column. Another example is a sample of rabbit-anti-mouse antibodies, shown in Fig. 5. A single sharp peak corresponding to immunoglobulin G (IgG) was obtained.

CONCLUSIONS

In the present work, prevention of protein adsorption by PEO–PPO–PEO triblock copolymer in CE was demonstrated. A simple procedure developed for the silylation of fused-silica capillaries using 5% DDS in toluene has been described. Unlike other methods, extended heating and constant monitoring are not required. The use of DDS allows for a smooth layer for the adsorption of Pluronic polymers which consist of PEO–PPO–PEO triblock copolymers. Pluronic–DDS-derivatised capillary columns were found to be stable under high voltages and at a pH range of 3 to 8. Very high efficiencies

Fig. 5. Electropherogram of rabbit-anti-mouse antibodies, the major peak being IgG. Electrophoretic conditions: 5 mg/ml F-108 modified capillary, 43 cm length (31 cm effective length) × 50 μm I.D.; 0.01 *M* phosphate buffer, pH 3.0; voltage: 15 kV; wavelength: 200 nm.

(more than 500 000 plates) were obtained for some model basic proteins. In conclusion, the present study has shown that apart from their many industrial uses [21], the Pluronic polymers have yet another interesting and useful application, *i.e.* in the CE separation of proteins.

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