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Short Communication

Improved capillary zone electrophoretic separation of basic proteins, using a fluorosurfactant buffer additive[a]

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ABSTRACT

In this paper, a new method to reduce the adsorption of basic proteins in capillary zone electrophoresis is described. Small amounts of a cationic fluorosurfactant are added to the running buffer. This leads to a surface charge reversal. Consequently, proteins at a pH below their p*I* are repelled from the wall. High efficiencies and symmetrical peaks were obtained for a number of model proteins, even when running buffer solutions with a low ionic strength were employed. Reproducibility was excellent. It is believed that the extreme hydrophobic nature of the fluorocarbon chain of the surfactant is a significant factor for the improved performance.

INTRODUCTION

Capillary zone electrophoresis (CZE) is rapidly becoming an important tool for the separation of biomolecules like peptides and proteins. During the last few years, intensified research has scored impressive results in terms of speed, resolution and detectability. A key problem in protein separation, which was already pointed out by Jorgenson *et al.* in one of their early reports [1], is the tendency of these molecules to adsorb on the surface of the capillary tubing. The often employed fused-silica tubing has a negatively charged surface due to the presence of silanol groups, which attract the positively charged sites of proteins by electrostatic force. This seriously impairs the separation efficiency.

Several solutions have been proposed to circumvent this problem. Lauer and McManigill [2] suggested the use of a carrier medium with a pH above the p*I* of the proteins. Under such conditions, proteins have a net negative charge and are then repulsed from the wall by coulombic forces. Other approaches have been to increase

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the ionic strength of the buffer, using conventional salts [3] or zwitterions [4]. A different route to reduce the adsorption of proteins is by the action of suitable hydrophilic surface modifiers, such as polyacrylamide [5], polyvinylpyrrolidone [6], polyethylene glycol or maltose [7], which are covalently linked to the surface silanol groups via an organosilane coupling agent. Also a modifier with a terminal arylpentafluoro ligand [8] has been reported for surface deactivation. Recently Gordon *et al.* [9] have proposed an alternative method involving adding ethylene glycol to the protein samples.

Although some workers have demonstrated that it is possible to obtain high efficiencies (in excess of 500 000 theoretical plates/m), such results have been obtained under restrained conditions (such as extreme pH or by using a separation media with a high ionic strength). Swedberg for example [8] used high buffer concentrations, (in some cases also with additional salt). This leads to an extended analysis time (decreased electroosmotic flow) and an increased joule heat generation. The latter necessitates the use of very narrow bore tubing, which, in its turn, demands high analyte concentrations, due to a decreased detectability.

The possibility of being able to use buffers with a low ionic strength, at a freely adjusted pH, is very desirable since this would allow us to match the running conditions to the practical situation, and thus enable us to reduce the risk of protein denaturation, while keeping the necessary degree of freedom to tune selectivity. In order to achieve the best possible result, the coulombic interactions with the surface must be reduced to an absolute minimum.

In the present report, we describe a simple method, where we propose the use of a fluorinated cationic surfactant as an additive. Due to a surface charge reversal as well as a charge interaction with the analytes, basic proteins are repelled by the silica surface, even in neutral pH solutions of low ionic strength. This is demonstrated with some model compounds.

EXPERIMENTAL

Apparatus

The apparatus used consisted of a high voltage d.c. power supply (Model 225, Bertan Assoc., Hicksville, NY, USA) delivering up to +/− 30 kV, and a UV detector (Model 206, Linear Instruments, Reno, NV, USA). The other parts were custom made: a plexiglass box with a safety interlock and an injection device, comprising an electronic timer for controlled electromigration injection. Provisions were also made to allow an *in situ* flushing of the capillaries by action of pressurized air (0.5–1.0 bar). Electropherograms were recorded on a Chromatointegrator (Hitachi, Tokyo, Japan).

Materials and reagents

Fused-silica capillary tubing (360 μm O.D. and 50 μm I.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries used was 100 cm and the length from the injector to the detector was 90 cm. The fluorosurfactant Fluorad FC 134 was obtained from 3M Company (St. Paul, MN, USA). The chemical structure of this material is shown in Fig. 1. Buffer solutions were prepared from boiled, de-ionized water, that was passed through a Milli-Q system (Millipore, Bedford, MA, USA), and were filtered before use. Myoglobin, ribonuclease and

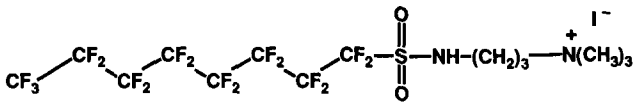

Fig. 1. General structure of Fluorad FC 134.

lysozyme were purchased from Sigma (St. Louis, MO, USA). Cytochrome *c*3 was a gift from the Department of Biochemistry and Biotechnology, Royal Institute of Technology (Stockholm, Sweden).

Stock protein solutions were prepared for each protein in 0.05 *M* phosphate buffer with a protein concentration of 10 mg/ml, except for cytochrome *c*3, which was prepared in 0.02 *M* (pH 7) 3-(N-morpholino)propanesulphonic acid (MOPS) buffer at a concentration of 1.0 mg/ml [10]. All solutions were stored in a freezer. The protein samples for the analyses were freshly prepared from the stock solutions in running buffer, containing FC 134 additive. Protein concentrations ranged from 0.12 to 0.22 mg/ml.

Sample injection was accomplished by electromigration. A reversed polarity setup was employed, where the capillary inlet was kept at negative potential, while the detector side of the capillary was grounded.

RESULTS AND DISCUSSION

When basic proteins are separated at a pH below their isoelectric points, they obtain a net positive charge, and are then strongly adsorbed onto the wall of silica tubing. When an anionic surfactant like sodium dodecyl sulphate (SDS), is added to the buffer solution, adsorption is suppressed due to a competing interaction with the cationic sites of the protein, and also by interaction between the hydrophobic parts of surfactant and the protein [11]. Unfortunately, the effectiveness of this approach seems to be rather modest. It has proven to be more suitable when dealing with smaller molecules like peptides, particularly in the form of micellar chromatography, where both charged and uncharged solutes can be separated, *e.g.* [12–14].

Also cationic surfactants like dodecyl and hexadecylmethylammonium salts have been used in micellar chromatography [14–16]. These compounds adsorb on the silicious surface and cause a drastic change in electroosmotic behavior [17–19]. This is initiated by an electrostatic attraction between the positively charged head groups and the negatively charged $Si-O^-$ groups [20]. Thus, the non-polar chains of the surfactant will form a hydrophobic layer. The chains will eventually be oriented in a perpendicular position at some saturation concentration, and the negative surface charge will be completely neutralized. The Stern potential is thereby reduced to zero and the electroosmotic flow is inhibited.

At still higher surfactant concentrations, an admicellar structure, consisting of a bilayer is formed by hydrophobic interaction between the non-polar chains. The cationic sites of the second adhering surfactant molecules are now faced towards the buffer solution, resulting in a positive surface charge, and a reversal of the electroosmotic flow direction (*cf.* Fig. 2). At the same time, electrophoretic migration of the proteins will occur in the opposite direction.

An important advantage of the charge reversal is that proteins at a pH below

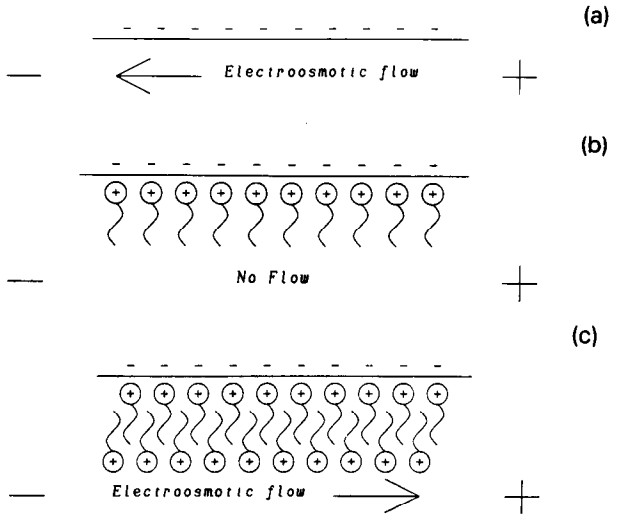

Fig. 2. (a) No surfactant added. Electroosmotic flow in normal direction. (b) Electrostatic adsorption of the positively charged surfactant headgroups to the negative silanol groups on the silica surface of the capillary inner wall. (c) Admicellar bilayer formation by hydrophobic interaction between the apolar chains, resulting in a reversal of the electroosmotic flow.

their p*I* will be repelled from the surface. Interactions between the surfactant and the proteins can enhance this effect. Addition of a suitable cationic surfactant to the running buffer may therefore be an effective approach to improve the separation of basic proteins at physiological pH values. This was experienced in our experiments. Fig. 3 shows an example of an electropherogram of four basic proteins. High efficiencies (Table I) were obtained for all compounds. Additionally, the reproducibility of the electrophoresis, in spite of the fact that a non-thermostated system was employed, is excellent (Table II), which is a further indication of the suppressed adsorption.

The migration order of the proteins is reversed compared to what is commonly observed. The most positively charged protein (lysozyme) appears as the last peak in the electropherogram. This is due to the fact that the transport rate of the proteins is dominated by the reversed electroosmotic flow towards the anode, while they migrate in the opposite direction by electrophoresis.

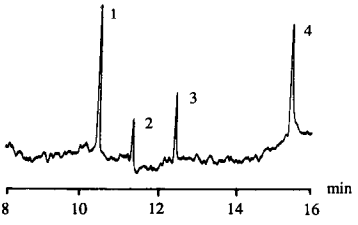

Fig. 3. Electropherogram of four model proteins. 1 = Myoglobin, 2 = ribonuclease A, 3 = cytochrome *c*3, 4 = lysozyme. Running buffer: 0.05 *M* phosphate (pH 7) with 100 μg/ml FC 134 added. Separation voltage: −30 kV. Current: 70 μA. Protein concentration: 0.22 mg/ml except for cytochrome *c*3 which is 0.13 mg/ml. Injection: electromigration at −15 kV for 10 s. UV detection: 230 nm.

TABLE I

EFFICIENCY FOR FOUR PROTEINS RUN IN BUFFER SOLUTION CONTAINING FC 134, BUFFER STRENGTH 0.05 *M*

Protein	Efficiency (plate number, *N*)	Mol. wt.	p*I*
Myoglobin (horse heart)	240 000	17 500	7.3
Ribonuclease A (bovine pancreas)	287 000	13 500	9.3
Cytochrome *c*3 (ref. 20)	542 000	14 300	9.5
Lysozyme (egg white)	372 000	14 000	11

The electroosmotic flow was rather large, as determined from the migration time of mesityl oxide, which passed the detector almost at the same time as myoglobin. The magnitude of the electroosmotic flow is of course dependent on several parameters, like pH, type of buffer, etc. We are presently investigating the influence of these parameters on the separation performance.

The moderate buffer strength and the surfactant concentration, used in the separation, shown in Fig. 3 can be further reduced, without any adverse effects on the separation and with maintained efficiency and excellent peak symmetry (Fig. 4 and Table III).

We believe that the observed effectiveness of deactivation is to an important extent due to the particular chemical nature of the surfactant. The hydrophobic chain of our additive has an extremely non-polar character, due to the presence of the fluorine atoms. This will enhance the stability of the bilayer. Compared to surfactants with a hydrocarbon chain, a more dense layer can be expected, since fluorinated chains pack more tightly [21].

A well known aspect of surfactants, when added to protein solutions, is their tendency to cause denaturation. In fact, a compound like SDS is used for this purpose in gel electrophoresis. Denaturing, induced by detergents is a complicated interplay, and is dependent on the character of the surfactant, the protein and the environment. The surfactants influence the protein conformation either by disrupting ion–ion bindings or by hydrophobic associations. This usually leads to a loss in biological activity, which is undesirable if subsequent bioassays are to be carried out with the separated materials.

TABLE II

REPRODUCIBILITY OF MIGRATION TIMES

$\bar{t}$ = mean time of migration; R.S.D. = relative standard deviation.

Protein	Within day		Day-to-day	
	$\bar{t}^a$ (min)	R.S.D. (%)	$\bar{t}^a$ (min)	R.S.D. (%)
Myoglobin	10.4	0.43	10.2	1.7
Ribonuclease A	11.2	0.45	11.1	1.5
Cytochrome *c*3	12.3	0.45	12.4	1.7
Lysozyme	15.1	0.67	15.5	2.4

[a] $n = 7$.

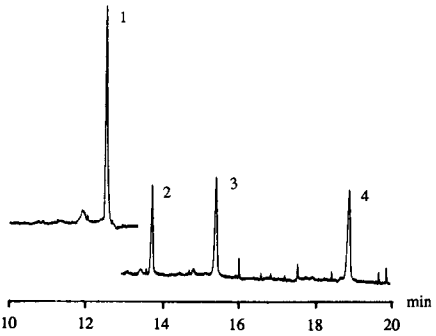

Fig. 4. Electropherogram of four model proteins (myoglobin run separately). 1 = Myoglobin, 2 = ribonuclease A, 3 = cytochrome *c*3, 4 = lysozyme. Running buffer: 0.01 *M* phosphate (pH 7) with 50 μg/ml FC 134 added. Separation voltage: −30 kV. Current: 11 μA. Protein concentration: 1.7 mg/ml for myoglobin, 0.20 mg/ml for ribonuclease A and lysozyme, 0.12 mg/ml for cytochrome *c*3. Injection: electromigration at −20 kV for 10 s and at −5 kV for 5 s for myoglobin. UV detection: 230 nm.

In this context, our additive has several favorable properties. It has been shown that the chemistry of the surfactant head group is very important for the ability to denaturate [22,23]. Some cationic surfactants do denature proteins [24], while surfactants with sterically hindered head groups, (like the nitrogen with the surrounding three methyl groups in FC 134) act by no means as potent as those with less bulky ligands. Also, on the whole, the denaturating behavior of cationic surfactants is usually lower than that of anionic compounds [24]. Finally, the fluorinated chain of the surfactant is very important. Fluorocarbons are well known for their biocompatibility. In fact, certain fluorocarbon liquids have been used as blood substitutes [25]. Due to the lipophobic behavior of the fluorinated chain, interaction with the hydrophobic part of proteins should be minimal. Instead, the excess surfactant will form non-interacting ("neutral") micelles. This occurs already at very low concentrations [the critical micellar concentration (CMC) in water is about 10 ppm according to the manufacturer]. It is therefore not unrealistic to assume, that the biological activity of proteins may be relatively unaffected in our proposed separation method. We are presently investigating this, as well as the applicability of other fluorinated surfactants.

TABLE III

EFFICIENCY FOR FOUR PROTEINS RUN IN BUFFER SOLUTION CONTAINING FC 134, BUFFER CONCENTRATION 0.01 *M*

Protein	Efficiency (*N*)
Myoglobin	352 000
Ribonuclease A	337 000
Cytochrome *c*3	283 000
Lysozyme	370 000

CONCLUSIONS

(1) Capillary zone electrophoretic separation of basic proteins, at a pH below their p*I*, can be significantly improved by adding a small amount of fluorinated cationic surfactant to the running buffer.

(2) A buffer, having a low ionic strength can be employed, while efficiency and resolution are maintained.

(3) Short and long term reproducibility is excellent.

(4) The method described is simple. Cheap, non-treated capillary tubing can be used (which can be rinsed with aggressive media if necessary). No complicated pre-treatments of the capillary tubing are necessary. Therefore, there should be a potential in clinical applications for the proposed concept.

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