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Performance evaluation of a diol-modified capillary and its hydride precursor for the HPCE separation of proteins

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San Jose State University, 1993

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**PERFORMANCE EVALUATION OF A DIOL-MODIFIED CAPILLARY AND
ITS HYDRIDE PRECURSOR FOR THE HPCE SEPARATION OF PROTEINS**

A Thesis

Presented to

the Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Xiaoli Liu

December, 1993

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ABSTRACT

PERFORMANCE EVALUATION OF A DIOL-MODIFIED CAPILLARY AND ITS HYDRIDE PRECURSOR FOR THE HPCE SEPARATION OF PROTEIN

by Xiaoli Liu

A diol-modified capillary prepared by a novel approach provides a hydrolytically more stable Si-C linkage between the organic moiety and the silica surface. The modification procedure involves the catalytic reaction of allylglycidylether with a surface silicon hydride intermediate, followed by opening of the epoxy-ring in an aqueous acidic medium. When compared to the hydride capillary intermediate, the diol-modified capillary appears to give higher separation efficiency and more precise solute migration probably because its anchored chains shield the unreacted silanols on the capillary walls from interacting with the solute. The hydrolytic stability of the diol-modified capillary is evidenced by its stable electroosmotic characteristics. This capillary is useful for basic proteins in the buffer pH range of 4-6. The lifetime of the capillary is at least 25 injections with good reproducibility of migration time. When compared to a conventional Si-O-Si-C (siloxane) bonded diol-modified capillary, the novel one has enhanced hydrolytic stability and insured better reproducibility of solute migration.

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1. INTRODUCTION

1.1. Background

High performance capillary electrophoresis (HPCE) has received much attention in recent years for its potential utility in the separation of macromolecules such as proteins and nucleic acids; this is often difficult to achieve by other techniques like high performance liquid chromatography (HPLC). The low diffusion coefficient of proteins, that leads to limited separation efficiency in HPLC, however, is desirable for both high efficiency and good resolution on HPCE. This can be documented by means of the following equations:

$$N = (\mu_e + \mu_{eo}) V / 2D \quad (1)$$

$$R = (1 / 4\sqrt{2})(\mu_{e1} - \mu_{e2}) \sqrt{V / D(\mu_e - \mu_{eo})} \quad (2)$$

where N is the efficiency in terms of number of theoretical plates, μ_e is the analyte's electrophoretic mobility, μ_{eo} is the electroosmotic mobility, V is the applied voltage, D is the analyte's diffusion coefficient, R is the resolution, and the subscripts 1 and 2 refer to two adjacent protein bands (1). The HPCE technique has found its widest application in the fields of biosciences, biotechnology and pharmacology, although its use for inorganic analysis is currently under expansion.

There are several advantages offered by HPCE when compared to other analytical separations. One of them, the small sample consumption (volumes in the order of nL), is a desirable feature in the analysis of biological macromolecules because these samples are frequently difficult to obtain and more expensive. Others, such as high efficiency, high

speed, low operation cost and simple operation are also attractive advantages in the separation field. There are, however, some problems associated with HPCE separations including solute adsorption, column heating, and detection volume. Among them, peak tailing arising from solute adsorption on the inner capillary wall is one of the most serious problems associated with protein separations. This problem can cause decreased efficiency, degraded peak shapes and poor reproducibility of protein migration times.

Many attempts have been made recently to avoid protein adsorption on the inner capillary surface during HPCE separation. Efforts have been aimed to adjust the electrolyte's pH relative to the isoelectric point (pI) of a protein in order to change its net charge as well as that of the capillary wall and thus manipulate Coulombic interactions (2). Addition of Zwitterions or ionic salts to the electrolyte for reducing protein adsorption has also been suggested in the literature, although their effectiveness is still limited (3, 4). Modifying the inner wall of a fused silica capillary with organic material is perhaps the most effective approach to minimize the number of adsorption sites (5-7).

In the case of variation of the electrolyte's pH, the silica surface in contact with the aqueous solution develops a negative charge at pH values above 2 (2). If the pH of the electrolyte is adjusted so high that both proteins and the surface of the fused silica capillary become negatively charged, then, Coulombic repulsion occurs and a significant decrease of protein adsorption on the capillary surface is achieved. It is also possible to obtain well-separated bands for proteins in a fused-silica capillary with low pH buffers. In this case, the surface silanol groups are largely neutralized and electrical wall charges are very small (8). However, at too high or too low pH (that is, at large pH-pI differences), proteins may not be stable and structural changes may occur.

In the case of addition of ionic and Zwitterionic salts to the electrolyte, protein ionic exchange interactions with the charged inner surface of a capillary can be eliminated by

increasing the concentration of competing ions in the buffer (4). The greater ionic strength can, however, cause Joule heat generation that may result in band broadening at the high voltages commonly used in HPCE. Zwitterions have little contribution to the conductivity of the electrolyte; they associate with both the negatively charged surface of the capillary and charged protein sites to avoid solut adsorption and to help break up protein-protein interactions (3). Although the addition of Zwitterions to the electrolyte has the advantage of using higher voltages and obtaining shorter migration times than those of ionic salts, some of them do not appear to work well with very basic proteins such as the lysozymes (3).

In the case of permanent modification of a capillary wall with organic material, several methods have been used over the past five years which are fairly successful in minimizing protein adsorption as well as eliminating electroosmotic flow. Covalently bonded organic coatings such as polyether (5,6), diol (7), poly(vinylpyrrolidone) (8) methyl cellulose and non-cross-linked polyacrylamide (9) have been reported. These coatings generally consist of hydrophilic moieties anchored to the wall of the fused-silica capillary through siloxane linkages resulting from organosilanization. They remove or mask the silanol groups either with sufficient thickness or relative long chains of the hydrophilic moieties so that the access of protein solutes to the adsorption sites on a fused-silica capillary are significantly reduced. Since extensive silanol removal occurs during siloxane formation, electroosmosis is drastically reduced upon capillary wall modification.

Organosilanization is the most common approach by which a fully hydroxylated silica surface is coupled with an organic material, $(R'O)_3Si-(CH_2)_3-R$, to form a siloxane (Si-O-Si-C) linkage, as follows:

where R' may be a methyl (-CH₃), or ethyl (-C₂H₅) group, R contains the hydrophilic moiety and Y represents -H or -Si≡. This approach, which has been applied to surface modification for LC and, previously, GC stationary phases, borrows heavily from "silane coupling" technology (10). A major drawback of this approach is the unsatisfactory hydrolytic stability of the siloxane linkage under the extreme pH conditions frequently used in HPCE. Furthermore, the coverage of the organic moieties on the silica surface is limited by the steric hindrance of the organic group, which ultimately leads to higher concentration of unreacted surface silanols (11). These limitations result in significant protein interaction with the surface of the capillary, as evidenced by strong peak tailing. From an analytical standpoint, progressive loss of the coating during operation results in the degradation of separation efficiency and migration reproducibility. Silicon-carbon (Si-C) linkages have long been considered to be more stable toward hydrolysis than a siloxane (Si-O-Si-C) linkage, especially under relatively severe pH conditions (4, 11, 14). The formation of Si-C linkages on silica surfaces was initially reported for the preparation of chemically bonded chromatographic phases (11-13). The preparation involves the sequential reaction of silica with a chlorinating reagent (e.g., SOCl₂) as well as an organometallic compound (e.g., a Grignard reagent):

In addition to enhanced stability, a denser coverage of organic functionalities is thought to be formed onto the silica surface (12, 13). Recently, Cobb *et al.* have applied this approach to capillary electrophoresis (14). A polyacrylamide-coating, attached to the silica wall through a Si-C bond, was made by reacting a Grignard reagent containing a vinyl group with a surface-chlorinated silica on the capillary wall (14). The resulting vinyl-modified surface was then reacted with a polymerizing acrylamide solution. Despite the hydrolytic advantage provided by the direct Si-C linkage, bonded silica phases (including capillaries) prepared by this approach have not reached wide spread use because they are much more difficult to prepare than the organosilanized counterpart.

An alternative approach to produce Si-C linkages on silica surfaces has been reported recently by our research group (11, 15-17). The synthetic procedure involves the catalytic addition of surface silicon hydride to a terminal double bond:

The reaction, called hydrosilation, is catalyzed by complexes of transition metals from group VIII, in particular platinum. The preparation of the silica intermediate is accomplished by reaction of silica with a trisubstituted silane reagent such as triethoxy silane as follows (16):

The remarkable versatility of hydrosilation (eqn. 6) can be exploited to prepare a hydrophilic substrate useful in HPCE. A diol moiety has hydrophilicity and exhibits low protein adsorption, that has been reported in several publications (7, 18, 19). The diol-modified capillary can be prepared by catalytic reaction of allylglycidylether with a hydride intermediate, followed by opening of the epoxy-ring in an aqueous acidic medium. The Si-C linkage on the capillary surface is formed through hydrosilation as shown below:

Then, the surface epoxy-ring is opened:

The resulting hydrophilic moiety should provide minimal interactions with proteinic solutes. Thus, a capillary modified in this fashion can be used in a wider range of pH buffers, give improved reproducibility of protein migration and make the capillary lifetime

longer. These advantages are particularly important for the separation of proteins since the electrolyte's pH has a very strong influence on a protein's charge, and it is desirable to retain pH as a freely adjustable parameter for the separation.

1.2. Aims of This Work

A major goal of this research is to evaluate the electrophoretic performance of a diol-modified capillary and its precursor hydride-intermediate capillary in terms of some basic analytical parameters or merit such as separation efficiency, peak symmetry, and precision of protein migration. A comparison of the Si-C bonded diol-modified capillary with that obtained from the conventional Si-O-Si-C bonded one is also an aim of this research work. It is expected that under similar electrophoretic conditions the new diol-modified capillary will exhibit improved peak shape, higher efficiencies, better long-term precision and extended longevity when compared to the conventional organosilanization product.

2. EXPERIMENTAL

2.1. Reagents and Materials

Lysozyme (turkey and chicken egg whites), cytochrome c (horse and bovine hearts), ribonuclease A (bovine pancreas), α -chymotrypsinogen A (bovine pancreas), and trypsinogen (bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO). The molecular weight and pI values of these proteins are listed in Table I. Lactic acid, acetic acid, and ammonia were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). β -alanine, γ -amino-n-butyric acid (GABA), 4-morpholine ethane sulfonic acid (MES), α -amino- β -imidazolepropionic acid (histidine), 3-(N-morpholino)propane sulfonic acid (MOPS), 1,3-diaza-2,4-cyclopentadium (imidazole), N-tris-(hydroxymethyl)methyl glycine (tricine), tris(hydroxymethyl)amino methane (tris), and 3-(cyclohexylamino)-1 propanesulfonic acid (CAPS) were also purchased from Sigma Chemical Co. and were used as Zwitterion buffer components. De-ionized water obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA) was filtered through a 0.2- μ m Nylon-66 membrane filter (Alltech Associates, Deerfield, IL) and then used to prepare all buffer systems. All buffer solutions were similarly filtered before use. Allylglycidyl ether (AGE), 99+% (Aldrich Chemical Company, Inc., Milwaukee, WI) was used as the olefin reagent, while triethoxy silane (TES) (Petrarch Systems, Silanes & Silicons, Bristol, PA) was used for the preparation of the hydride intermediate. γ -Glycidoxypropyltrimethoxysilane (GPTMS) was used as the conventional organosilanization reagent for Si-O-Si-C bonded diol-modified capillaries. Tetrahydrofuran (THF) and p-dioxane, and (J. T. Baker Chemical CO., Phillipsburg, NJ), toluene (EM Industries, Inc.) were used as solvents for the preparation of both modified capillaries. Hexachloroplatinic acid (Aldrich) and 2-

Table 1. Model Proteins Used in This Work*

protein	organism	tissue	pI	molecular weight kD
lysozyme	turkey	egg white	10.5-11.0	13.9
lysozyme	chicken	egg	10.5-11.0	13.9
cytochrome c	horse	heart	9.0-9.4	13.4
cytochrome c	bovine	heart	10.3-10.8	12.2
ribonuclease A	bovine	pancreas	8.7-9.3	12.6
trypsinogen	bovine	pancreas	8.7	24.5
a-chymotrypsinogen A	bovine	pancreas	8.8-9.6	25.0
myoglobin	horse	skeletal musc	6.8-7.0	16.9

*Taken from references 20 and 21

propanol (J. T. Baker Chemical CO., Phillipsburg, NJ) were used for preparation of the "Speier's" catalyst solution.

2.2. Instrumentation

A vast majority of the results were obtained from a home-made HPCE instrument. An ABI Model 270A-HT system (Applied Biosystems, Foster City, CA) was used in some selected experiments. The home-made HPCE instrument consists of a high-voltage power supply Series 230 (Bertran Inc., Hicksville, NY), a Spectra 200 UV detector (Spectra-Physics, San Jose, CA) and a plexiglass box provided with automatic high-voltage cut off switch when opened. A Model 35900 D/A interface (Hewlett Packard, CA), an IBM-personal computer and a printout system completed the electrophoretic set up. Two platinum wires were used as electrodes. They were immersed in two buffer vials as shown in Fig. 1.

Modification of capillaries was performed on the apparatus shown in Fig. 2. A length of capillary (usually 10 m)(a) was installed in an oven reconditioned from a Model 5890 GC instrument (Hewlett Packard) (b) to control temperature during capillary modification. One end of the capillary is inserted into a reagent reservoir consisting of 15-mL heavy-wall glass tube (ACE glass, Vineland, NJ) (c), 1/8" "tee" Swagelok fitting (d), 1/8" to 0.4 mm Vespel/graphite ferrule (VG-2, 40% graphite, 60% Vespel, from Alltech Associated Inc.), and a threaded cap (e) with an "O" ring. The other end was inserted in a small vial (f) to collect the fluid and measure flow rate. Nitrogen pressure (20-30 psi) was applied to the side arm of the Swagelok fitting to force the liquid reagent through the capillary.

Fig. 1. Scheme of the home-made HPCE apparatus.

Fig. 2. Capillary modification apparatus: (a) capillary; (b) oven; (c) reagent reservoir; (d) Swagelok fitting; (e) threaded cap; (f) vial.

2.3. Procedures

2.3.1. Chemical Modification of Capillaries

2.3.1.1. Olefin Hydrosilation

2.3.1.1.1. Capillary Preconditioning

The capillary was first treated with a pH 10.0 (about 6 mM) ammonia solution for 20 h with a flow rate of 0.1-0.2 mL/h at room temperature. After rinsing for 1 h with deionized water, the capillary was flushed with 0.1 M HCl solution for 4 h to remove any ammonia adsorbed on the surface. After a 2-hour rinsing with deionized water, the tube was dried with N₂ for over 20 h at 100°C.

2.3.1.1.2. Preparation of the Hydride Intermediate

A TES treating solution was prepared by mixing 8.4 mL of dioxane, 1.2 mL of 1.0 M TES/dioxane solution and 435 µL of 2.3 M HCl with continuous stirring. The pre-conditioned capillary was first flushed with dioxane and then treated with the TES solution at 95±1°C for 90 min. The tube was then consecutively rinsed with THF/water (75/25 v/v) for 2 h and THF for 4 h at room temperature. The tube was finally dried under N₂ at 100±1°C for over 24 h.

2.3.1.1.3. Addition of Allyl-Glycidyl Ether

10 µL of 30 mM Speier's catalyst (hexachloroplatinic acid in 2-propanol) solution were added to 10 mL of 75 mM AGE/toluene solution. This mixture was allowed to equilibrate for 1 h at 65-70°C in a flask equipped with a condenser and a drying tube under a dry N₂

blanket. The hydride-modified capillary was first rinsed with dry toluene and then flushed with the AGE-Pt(II) equilibrated solution at $100\pm 1^\circ\text{C}$ under 30 psi of N_2 for 45 h. The capillary was rinsed with toluene (1 h) followed by THF (1 h), at room temperature and, finally, dried with N_2 (20 psi) at $100\pm 1^\circ\text{C}$ overnight.

2.3.1.1.4. Formation of the Diol

Hydrolysis of the bonded epoxide groups was carried out by treating the capillary with 0.05 M hydrochloric acid at $65\pm 1^\circ\text{C}$ for 4-6 h. The tube was then rinsed with THF/ H_2O (75/25, v/v) for 1-2 h and THF for 4 h at room temperature, and dried with N_2 at $90\pm 5^\circ\text{C}$ and 20 psi overnight.

2.3.1.2. Conventional Organosilanization

The pre-conditioned capillary (described in 2.3.1.1.1) was treated with 25.0 mL of a solution containing 0.8 mL of GPTMS, 15.0 mL of toluene and 9.2 μL of pyridine at $100\pm 1^\circ\text{C}$ for 20 h under 30 psi of N_2 . The capillary was then rinsed with toluene for 4 h and THF for 2 h. Drying was done with N_2 at $100\pm 1^\circ\text{C}$ overnight. Opening of the epoxy ring was carried out as described in 2.3.1.1.4.

2.3.2. Electrophoresis

Protein solutions of a concentration of 0.1-0.6 mg/mL were prepared daily by dissolving in a 10-fold dilution buffer (22). They were introduced into the capillary by hydrostatic (gravity) injection for a given time (1-4 s) at a given height (5-10 cm). Mesityl oxide was used as a neutral marker to measure electroosmosis of the capillaries. Zwitterion buffer solutions (23, 24) were used over the pH range of 4-10: 30 mM lactic acid/31 mM β -alanine at pH 4, 30 mM acetic acid/30 mM GABA at pH 5, 30 mM

MES/21 mM histidine at pH 6, 30 mM MOPS/24 mM imidazole at pH 7, 30 mM tricine/30 mM tris at pH 8, and 30 mM CAPS/52 mM ammonia at pH 10. Capillaries with 363-364 μm OD and 74-77 μm ID were used with a total length of 72-73 cm. The effective separation length (injection to detection distance) was 50 cm. The wavelength selected was either 230 and/or 214 nm. Currents through the capillaries were in the range of 1-17 μA at an applied voltage of 22 kV.

2.3.3. Calculation of Separation Efficiency and Peak Symmetry

The number of theoretical plates, as a measurement of efficiency of the HPCE separation, is calculated directly from the electropherogram (Fig. 3) by using the following equation for half peak height method:

$$N_{1/2} = 5.54 t_m^2 / (W_{1/2})^2 \quad (6)$$

where $N_{1/2}$ is the theoretical plate number calculated by the half peak height method, t_m is the protein migration time, $W_{1/2}$ is the peak's width at half peak height. The peak symmetry is evaluated by calculating the A/B ratio at 10 % of peak height (Fig. 4), where A and B are the distances at 10 % of the peak's height respectively (25).

Fig. 3. Measurement of separation efficiency (N) by using half peak height method.

Fig. 4. Measurement of peak symmetry (A/B ratio) at 10% of peak height.

3. RESULTS AND DISCUSSION

The main goal in developing the novel Si-C bound diol-modified capillary tube was to improve protein separations by minimizing interactions of the analyte with the fused-silica capillary surface. It is understood that, in the particular case of basic proteins, analyte-wall interactions are mainly of the "silanophilic" type; that is, those between the free amino-groups of the protein and the acidic silanols on the capillary wall. We are, therefore, interested in the effect of the surface modification on critical analytical data of merit such as migration reproducibility, band shape and peak efficiency. These parameters are directly measured on the electropherograms as migration time precision, asymmetry ratio (A/B, see Fig. 4) and plate count respectively.

3.1. Separation Efficiency and Peak Symmetry

In this research work, separation efficiency and peak symmetry were evaluated for the novel Si-C bound diol-modified capillary and its hydride precursor by using several basic proteins as model probes. These proteins are listed in Table 1 along with their relevant properties. Figs. 5 and 6 show typical electropherograms obtained on the diol-modified capillary and the hydride intermediate respectively with the home-made HPCE instrument for two mixtures of several basic proteins at pH 4. In both cases, a very good separation is obtained. These proteins, however, do not migrate in the order of their isoelectric points or molecular weights, a fact that has also been observed before (2).

Fig. 7 shows a comparison of separations of two cytochrome C's, horse and bovine, which only differ by three amino acids in a total sequence of 104 (8), by using the diol- and the hydride-modified capillaries. It seems clear that under the same conditions, the diol-modified capillary exhibits better efficiency and more reproducible behavior than the hydride intermediate, as shown in Table 2, while peak shapes are very similar.

Fig. 5. Electropherogram of basic proteins obtained on the Si-C bound diol-modified capillary with the home-made HPCE instrument: capillary, 50 cm to detection point, 73 cm total length, 75 μm ID, 363 μm OD; electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.72; applied voltage, 22.0 kV; current, 9 μA ; detection wavelength, 230 nm; injection, hydrodynamic 4 s at 10 cm height differential; protein concentration, 0.6 mg/mL each; proteins, 1 = lysozyme (turkey), 2 = cytochrome c (bovine), 3 = ribonuclease A, 4 = trypsinogen, 5 = α -chymotrypsinogen A.

Fig. 6. Electropherogram of basic proteins obtained on a hydride intermediate capillary with the home-made HPCE instrument: capillary, 50 cm to detection point, 72.9 cm total length, 75-76 μm ID. and 363 μm OD; electrolyte, 30 mM lactic acid/31 mM β -alanine at 3.72 pH; applied voltage, 20.0 kV; current, 8 μA ; detection wavelength, 230 nm; injection, hydrodynamic 3 s at 10 cm height differential; protein concentration, 0.25 mg/mL each; proteins, 1 = lysozyme (turkey), 2 = lysozyme (chicken), 3 = myoglobin, 4 = ribonuclease A.

Fig. 7. Electropherograms of two cytochrome c's (horse and bovine) obtained on a Si-C bound diol-modified capillary (A) and hydride modified capillary (B) with the home-made HPCE instrument: capillary, 50 cm to detection point, 73 cm total length, 75 μm ID and 363 μm OD; electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.72 in A and 3.69 pH in B; protein concentration, 0.2 mg/mL each; injection, hydrodynamic 1 s at 5 cm height; detection wavelength, 230 nm; applied voltage, 22.0 kV; current, 9 μA in A and 8 μA in B.

**Table 2. Separation Efficiencies and Peak Symmetries for Two Cytochrome c's
in the Novel Diol- and Hydride-Modified Capillaries***

cytochrome c	capillary	plate count,	plates/m	assymetry	A/B-value
		mean	% RSD	mean	% RSD
horse	diol	3.16E+05	2.7	0.97	9.7
bovine		2.93E+05	5.0	1.02	6.0
horse	hydride	5.26E+04	29.6	1.17	14.2
bovine		4.94E+04	22.1	1.06	16.2

* Based on five replicate measurements

That is probably due to the minimal interactions of proteins with the hydrophilic diol moiety on the capillary surface in comparison to those with the remaining silanol groups on the hydride surface. The average theoretical plate counts obtained with the diol-modified capillary are much higher (about one order of magnitude) than those with the hydride capillary although they are still lower than the plate count (about two million) predicted by the capillary electrophoresis theory (26). Although this indicates that protein-wall silanophilic interactions are significantly hindered by the hydrophilic diol moiety, there are still some silanols remaining on the capillary walls that cause a deterioration of the separation efficiency. Not surprisingly, since the diol-moiety is longer than hydrogen on the hydride capillary wall, it is much more efficient at shielding any silanol groups on the surface. This shielding necessarily diminishes the interaction of proteins with these groups so that a higher separation efficiency is achieved as compared to the hydride intermediate capillary. This is also illustrated by a slightly better average A/B ratio and overall precision obtained with the diol-modified capillary.

3.1.1. Hydrolytic Stability

Other basic proteins, such as lysozymes, from turkey and chicken egg whites, were used to evaluate capillary performance in terms of separation efficiency and hydrolytic stability under basic conditions. The lysozymes were first separated (5 replicates) in a buffer containing 30 mM lactic acid/31 mM β -alanine (pH 4) in a diol-modified capillary on the ABI-270A-HT HPCE instrument. Then, the capillary was flushed with a buffer of 30 mM CAPS/52 mM ammonia (pH 10) for 1 h and the mixture was again separated in the same pH 4 buffer in quintuplicate. Fig. 8 shows a comparison of the separations of the two lysozymes before and after flushing with higher pH buffer. The electropherograms indicate a very good separation (Fig. 8A) before flushing with higher pH buffer and a base

Fig. 8. Electropherograms of two lysozymes (turkey and chicken) obtained on a Si-C bound diol-capillary with the ABI-270A-HT HPCE system at pH 3.69 before (A) and after (B) flushing with pH 10.36 buffer: capillary, 50 cm to detection point, 73 cm total length, 75 μm ID and 363 μm OD; electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.69, 30 mM CAPS/52 mM NH_3 buffer at pH 10.36; protein concentration, 0.1 mg/mL each; injection, 0.8 s at 5" vacuum; detection wavelength, 230 nm; applied voltage, 22.0 kV; current, 13 μA .

line separation (Fig. 8B) of the same mixture after flushing with higher pH buffer although separation B shows little but noticeable peak tailing. This suggests that the Si-C bonded modified capillary is fairly stable under basic conditions. Table 3 lists the average theoretical plate counts and A/B ratios respectively before and after flushing with the higher pH buffer. The average theoretical plate counts seem to be slightly higher after flushing with the higher pH buffer, however, the higher A/B ratio after flushing with the high pH buffer indicates that appreciable peak tailing resulted from the deterioration of the capillary during this process. Besides, the relative standard deviation before flushing with the higher pH buffer was slightly better than that after flushing with the buffer.

3.1.2. Effect of Separation Variables

It is a well-known fact that separation efficiency and peak symmetry in HPCE are affected by several factors. Factors considered in this research include applied voltage, sample concentration and injection volume, and the electrolyte pH. Another feature, the electroosmotic flow, a major contributor to the amount of time in which a protein resides in the fused-silica capillary, was also examined in this research.

3.1.2.1. Effect of Applied Voltage

Two lysozymes were separated in the diol-modified capillary and the effect of the applied voltage on the separation efficiency was examined. This was done in the range of 20 to 30 kV on a 73 cm length capillary tube. According to equation 1, changing the applied voltage should produce proportional changes in the separation efficiency. In practice, however, plate count per meter *versus* applied voltage (Fig. 9) exhibits a maximum at about 22 kV which corresponds to an electric field strength of 300 V/cm. This is the result of a second opposing effect, Joule heating generated by the system at

**Table 3. Separation Efficiencies and Peak Symmetries for Two
Lysozymes in the Novel Diol-Modified Capillary***

lysozyme	plate count,	plates/m	assymetry	A/B-value
	mean	% RSD	mean	% RSD
turkey	2.15E+05	3.1	1.15	9.1
chicken	2.12E+05	4.7	1.32	9.1

After being flushed with 30 mM CAPS/51.5 mM ammonia buffer (pH=10.48) for 1h:

turkey	3.29E+05	6.7	3.33	9.9
chicken	2.98E+05	3.9	3.37	9.8

* Measurements were performed on the ABI-270A-HT instrument.

Protein concentration 0.1 mg/mL in 10-fold diluted buffer.

Injection, 0.8 sec at 5" vacuum.

Fig. 9. Effect of applied voltage on peak efficiency for two lysozymes on a Si-C bound diol-modified capillary with the ABI-270A-HT HPCE system: electrolyte, 30 mM lactic acid/31 mM β -alanine at 3.69 pH; all other conditions as in Fig. 8 A.

higher voltages, leading to excessive band broadening and degraded efficiency. As a consequence, a compromise situation arises in which the highest possible voltage is used while maintaining heat generation to a minimum. This occurs at a field strength of 300 V/cm. Therefore, this value was chosen for the remainder of this research work.

3.1.2.2. Effects of Protein Concentration and Injection Volume

The separation efficiency and peak symmetry are influenced much by sample injection size and protein concentration. An "overloaded" capillary might be caused either by an excessive concentration of the analyte under a constant injection volume, called "concentration overload," or by excessive injection volume under a constant protein concentration, called "volume overload." Fig. 10 illustrates a typical case of concentration overload for lysozyme (turkey) in a diol-modified capillary on the ABI-270A-HT instrument. The peak shape of B is obviously "fronting" (A/B ratio of peak symmetry smaller than 1) and is broad due to the fact that the protein concentration is out of the linear range of the adsorption isotherm (27). As a result, the efficiency and A/B ratio of peak B are smaller than those of A. It should be pointed out that the fronting effect arising from concentration overload offsets the opposite effect, that is, tailing which originates from intense silanophilic interactions. When the effect of the latter on peak shape is under study, care should be taken to assure that concentration overload is not present. It should also be kept in mind that the combination of the two opposite effects is overall peak broadening. The injection volume overload case is shown in Fig. 11. The flat-topped profile of B (longer injection) results in significant degradation of the separation efficiency by a factor of about 5 while the symmetry remains practically unchanged. As expected, a fronting and flat band profile can be caused by both concentration and volume overloads, as shown in Fig. 12.

Fig. 10. Enlarged peak profiles of lysozyme (turkey) obtained on a Si-C bound capillary with the ABI-270A-HT HPCE system for injection concentration overload case: protein concentration, 0.1 mg/ mL in A and 0.5 mg/mL in B; injection, 1 s at 5 " vacuum; all other conditions as in Fig. 8A.

Fig. 11. Enlarged peak profiles of cytochrome c (horse) obtained on a Si-C bound capillary with the home-made HPCE instrument for injection volume overload case: hydrodynamic injection, approximately 0.5 s at 5 cm height differential in A and about 2 s at 5 cm height differential in B; protein concentration, 0.2 mg/ mL; all other conditions as in Fig. 7.

Fig. 12. Enlarged peak profile of cytochrome c (bovine) obtained on a Si-C bound diol-modified capillary with the home-made HPCE instrument for injection with both concentration and volume overload: protein concentration, 0.2 mg/mL; injection, hydrodynamic 2 s at 5 cm height differential; all other conditions as in Fig. 7.

3.1.2.3. Effect of Buffer pH

It has been known that buffer pH has a very strong influence on both the sign and magnitude of a protein's charge because of the amphoteric property of the amino acids that build up proteins. Similarly, pH influences the electrokinetic surface potential (zeta potential) of the capillary wall in the HPCE system; at pH values above 2, the surface becomes anionic. Although the interaction of proteins with the silanol groups of the capillary walls are almost eliminated by the presence of the hydrophilic diol moiety, at extreme pH conditions strong silanophilic interactions might develop if the bonded hydrophilic group is not stable enough towards hydrolytic cleavage. This is a particular problem at high pH. As a consequence, the electroosmotic flow in a hydrolytically unstable wall-modified capillary may increase dramatically as buffer pH increases, causing a large variation of migration times of proteins. Thus, hydrolytic instability of modified capillaries at higher pH values usually results in peak tailing as well as degradation of separation efficiency, and sometimes, in irreversible protein absorption.

The diol-modified capillary and its intermediate hydride capillary appear to work well up to pH 5. This is shown in Fig. 13 for the separation of two cytochrome C's in the diol-modified capillary and in Fig. 14 for four proteins in the hydride-modified one. When pH is increased to 6 using 30 mM MES/20.6 mM His buffer, the two lysozymes co-migrate and could not be separated probably due to the smaller difference between the protein pI's and the electrolyte's pH, which in turn results in a smaller charge developed on the proteins. The same situation occurred with the two cytochrome C's. Other proteins such as trypsinogen and ribonuclease A came out from the diol-modified capillary with very small peaks. This might be the result of protein adsorption on the capillary caused by increasing ionization of the unreacted silanols at higher pH or, by the formation of new ones under these conditions. In contrast, myoglobin came out as a well-shaped peak.

Fig. 13. Electropherogram of two cytochrome c's (horse and bovine) obtained on a Si-C bound diol-modified capillary at pH 5 with the home-made HPCE instrument: capillary, 49.6 cm to detection point, 72 cm total length, 75 μm ID and 363 μm OD; electrolyte, 30 mM acetic acid /99 mM GABA at pH 4.72; protein concentration, 0.5 mg/mL each; injection, hydrodynamic 2 s at 5 cm height differential; applied voltage 22.0 kV; current, 16 μA ; all other conditions as in Fig. 7.

Fig. 14. Electropherogram of basic proteins obtained on a hydride-modified capillary at pH 5 with the home-made HPCE instrument: applied voltage 22.0 kV; current, 16 μ A; electrolyte, 30 mM acetic acid /99 mM GABA at pH 4.82; protein concentration, 0.25 mg/mL each; injection, hydrodynamic 2 s at 5 cm height differential; proteins, 1 = lysozyme (turkey); 2 = lysozyme (chicken); 3 = ribonuclease A; 4 = myoglobin; all other conditions as in Fig. 7.

This might be because the lower pI for myoglobin produces a reduced net charge of the protein as the difference between buffer pH and myoglobin pI decreased. However, as the buffer pH value was further increased, proteins with higher pI values exhibited more extensive adsorption, as evidenced by badly tailing peaks or no peaks at all. The presence of an anionic capillary wall can also be demonstrated by the electroosmotic flow characteristics of the capillaries, as described below. In addition, lower pI proteins such as trypsin, pepsin, α -casein, albumin, etc. did not come out of the tube after more than 50 min in buffers with pH's 8 and 9. At these pH's, all proteins tested must be negatively charged and thus the samples were injected at the cathode. A possible explanation is that under these conditions, the direction of protein migration and the electroosmotic flow of the diol-modified capillary cancel each other and the net migration rate was not large enough to cause proteins to move out of the capillary in a reasonable amount of time.

3.2. Electroosmosis

Although electroosmosis is drastically reduced by the hydrophilic moiety bound on the capillary, it is not eliminated totally due to residual (unreacted) surface silanols left on the capillary during formation of the hydride. Ionization of these species and perhaps formation of new ones become extensive with high pH buffers. Fig. 15 illustrates the electroosmotic mobility, as measured with a neutral marker (mesityl oxide) as a function of buffer pH in the Si-C bound diol-modified capillary over the 4-10 pH range at 22 kV. At pH 4, the uncharged tracer did not show up after 1 hour, which is indicative of a very small electroosmosis ($< 9.2 \times 10^{-7}$ cm/sV). As pH increases to 6 and above, the electroosmotic flow increases rapidly as a result of increasing ionization of residual silanols and/or the formation of new ones. This explains the increased protein adsorption on the capillary with increasing pH.

Fig. 15. Electroosmotic mobility as a function of buffer pH for a Si-C bound diol-modified capillary on the home-made HPCE instrument: uncharged tracer, mesityl oxide at a concentration of 1 mM in the buffers; injection, hydrodynamic 10 s at 10 cm height differential; detection wavelength, 254 nm; applied voltage 22.0 kV; current, 9-10 μ A at pH 3.72, 14 μ A at pH 4.76, 4 μ A at pH 5.95, 8 μ A at pH 7.01, 7 μ A at pH 8.16, 6 μ A at pH 10.14, 4 μ A at pH 5.98 after exposure to higher pH buffer; all other conditions as in Fig. 7.

New ionizable silanols species can, in principle, be formed when the modified-capillary is exposed to high pH. Unreacted silicon hydride species lying underneath the anchored diol-moieties might react with water under the catalytic action of hydroxyl ions as shown by the following (16):

These newly formed silanols also undergo ionization at high pH and hence increase the anionic charge on the capillary wall. In order to test this hypothesis, after exposure to high pH buffer (up to 10.1), the same tube was again equilibrated with a lower pH buffer and the migration of the mesityl oxide marker measured again. If new silanols were formed upon exposure to high pH, the second measurement at lower pH should give a higher value when compared to the original one. The second measurements at pH's 6 (small circle indicated in Fig. 15) were found to be essentially equal to the original one, as shown in Table 4. This finding indicates that under the conditions used, the Si-C bound diol-moieties efficiently shields the underlying hydride from hydrolysis under moderately basic condition. It should be noticed that in the pH range 7-10, the migration times of mesityl oxide are in the 25-14 minute range which is in the range that is normally exhibited by low pI proteins. This observation may explain why the low pI proteins tested showed extremely long migration times under those conditions, as mentioned above.

Electroosmosis may affect not only efficiency but, more importantly, overall reproducibility of protein migration. While the presence of electroosmosis by itself does not cause degradation of reproducibility in protein migration at a given pH value, the zeta potential change caused by the adsorption of proteins on the walls might result in a

**Table 4. Migration of Uncharged Marker (Mesityl Oxide) at pH 6 Buffer
after Exposure to Higher pH Buffer**

capillary	No.	original migration time, min	after exposure to higher pH buffer migration time, min
novel diol	1	26.05	27.77
	2	26.01	27.87
	3	26.02	27.94
	mean	26.03	27.86
	stdv	0.02	0.09
	% rsd	0.1	0.3
conventional diol	1	26.88	13.45
	2	26.79	13.50
	3	26.70	13.56
	mean	26.79	13.50
	stdv	0.09	0.06
	% rsd	0.3	0.4

significant electroosmosis change so that the reproducibility of protein migration declines (14).

3.3. Precision of Protein Migration

Fig. 16 and 17 show a run-to-run comparison of the migration times of two cytochrome C's as a function of injection number on the diol-modified and its hydride intermediate capillaries respectively. Five consecutive injections were made daily over a period of five days. It should be recalled that a fresh buffer solution was prepared daily from the same stock. It seems clear that the diol capillary has much better precision than its hydride counterpart. This is very likely due to the decreased silanophilic interactions of proteins which result from an effective shielding of the hydrophilic diol moieties. Naturally, the relatively long hydrophilic chain of the diol-containing group ($-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2\text{OH}$) on the diol-modified capillary provides for much more effective shielding than that of the tiny hydrogen ($-\text{H}$) on the hydride-modified one. In the latter case, residual silanols are complete exposure to the proteins and thus cause the silanophilic-wall interaction evidenced by the adsorption on the capillary surface. As a result, the diol capillary exhibits shorter and more stable protein migration.

Long-term precision can also be examined by comparing similar plots for day-to-day data as illustrated in Fig. 18 and 19 for the same set of capillaries and proteins. Fig. 18 shows a slightly decreasing trend in migration times for the two cytochromes on the diol capillary. Such a trend is not apparent in the hydride capillary. The trend shown by the diol-capillary might be due to day-to-day environmental changes, particularly temperature. These factors, however, were not under control by using the home-made HPCE instrument. In any case, it seems obvious that the use of an internal standard should improve significantly the overall precision observed in both capillaries. In addition, the

Fig. 16. Migration times for cytochrome c's as a function of injection number obtained on a Si-C bound diol-modified capillary with the home-made HPCE instrument: electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.70 ± 0.03 ; current, 9 μ A; all other conditions as in Fig. 7.

Fig. 17. Plot of migration times as a function of injection number for cytochrome c's obtained on the hydride intermediate capillary with the home-made HPCE instrument: electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.70 ± 0.03 ; current, 7-8 μ A; all other conditions as in Fig. 7.

Fig. 18. Day-to-day variation in migration times for cytochrome c's obtained on a Si-C bound diol-modified capillary with the home-made HPCE instrument: electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.70 ± 0.03 ; current, 7-8 μ A; all other conditions as in Fig. 7.

Fig. 19. Day-to-day variation in migration times for cytochrome c's obtained on a hydride intermediate capillary with the home-made HPCE instrument: electrolyte, 30 mM lactic acid/31 mM β -alanine at pH 3.70 ± 0.03 ; current, 7-8 μ A; all other conditions as in Fig. 7.

diol-modified capillary used in this test was stored with the same buffer for 36 days and then used again for the same separations of cytochrome C's. It still gave good peak shapes, with precision in migration times of the proteins essentially equal to those obtained before. The life time of the capillary is, therefore, at least greater than one month under the conditions used.

3.4. Comparison of the Novel Diol-Modified Capillary to the Conventional One and Others

In order to compare the performance of the Si-C bound diol-modified capillary, a conventional Si-O-Si-C bound one was also evaluated. A good separation of the same five basic proteins was also obtained with the siloxane bound diol-modified capillary as shown in Fig. 20. The average theoretical plate counts as well as the A/B ratios for two cytochrome c's and lysozymes are shown respectively in Tables 5 and 6. Since the separations in the novel and the conventional capillaries for lysozymes were obtained on different instruments (the former on the commercial instrument and the latter on the home-made one), absolute comparisons are not, strictly speaking, possible. Nevertheless, general statements comparing both chemistries can be made. Although the average theoretical plate counts of the novel diol-modified capillary for cytochrome c's are lower than those of the conventional one, the overall precision of the former (2.7% and 5.0%) are much better than those of the latter (8.0% and 22.3%). The run-to-run precision of migration is also better for the novel diol-modified capillary than the conventional one, except for that of the first day as shown in Table 7. In addition, the conventional diol-modified capillary only works well at a pH 4. Above this pH value, low protein recoveries are obtained, as evidenced by small and/or broad peaks or simply no peaks at all. This observation was also described by Bruin *et al* (26). In comparison with the conventional diol-modified capillary, the better precisions of the results for separation efficiencies and

Fig. 20. Electropherogram of basic proteins obtained on a conventional siloxane bound diol-modified capillary with the home-made HPCE instrument: capillary, 50 cm to detection point, 73 cm total length, 77 μm ID and 364 μm OD; electrolyte, 30 mM lactic acid/31 mM β -alanine at 3.75 pH; applied voltage, 22.0 kV; current, 8-9 μA ; detection wavelength, 230 nm; injection, hydrodynamic 3 s at 10 cm height differential; protein concentration, 0.2 mg/mL each; proteins, 1 = lysozyme (turkey), 2 = cytochrome c (bovine), 3 = ribonuclease A, 4 = trypsinogen, 5 = α -chymotrypsinogen A.

**Table 5. Separation Efficiencies and Peak Symmetries for Two
Cytochrome c's in the Conventional Diol-Modified Capillary**

cytochrome c	plate count,	plates/m	asymmetry	A/B-value
	mean	% RSD	mean	% RSD
horse	5.44E+05	8.0	0.60	30.0
bovine	5.13E+05	22.3	0.71	37.1

**Table 6. Separation Efficiencies and Peak Symmetries for Two
Lysozymes in the Conventional Diol-Modified Capillary**

lysozyme	plate count,	plates/m	asymmetry	A/B-value
	mean	% RSD	mean	% RSD
turkey	6.72E+05	13.3	0.73	24.8
chicken	5.16E+05	38.5	0.99	23.8

After being flushed with 30 mM CAPS/51.5 mM ammonia buffer (pH=10.48) for 1h:

turkey	7.38E+05	7.1	1.39	56.3
chicken	5.84E+05	44.7	1.83	29.9

Table 7. Precision of Migration Times in the Novel and Conventional Diol-Modified Capillaries for Cytochrome c(horse)

capillary	day	migration time, mean	min %RSD	buffer's pH	current, uA
novel	1st	12.99	2.6	3.73	9
	2nd	11.88	0.4	3.71	9
	3rd	11.59	0.7	3.68	9
	4th	10.86	0.2	3.71	9
	5th	10.12	0.5	3.69	9
conventional	1st	14.40	1.7	3.71	8
	2nd	13.72	0.4	3.71	8
	3rd	13.23	0.9	3.70	8
	4th	12.86	2.3	3.69	8
	5th	12.44	0.9	3.69	8

migration times exhibited by the novel one can be attributed to a decreased exposure of remaining silanol groups to the protein probes. In contrast with the conventional capillary, the diol groups are anchored on a predominantly hydride surface and, thus, a decreased silanophilic interaction occurs. In the former case, only silanol species are lying underneath the diol-terminated chains. Silanophilic interactions with the basic probes are, therefore, more likely to occur. This is further evidenced by a plot of electroosmotic mobility (measured with mesityl oxide) as a function of electrolyte's pH for the siloxane-bound capillary (Fig. 21). Clearly, the conventional diol-modified capillary exhibits significantly higher electroosmotic flow for a given pH and applied potential. The small circle well above the curve illustrates that, unlike the Si-C bonded capillary, the electroosmosis is higher at buffer pH 6 after exposing the capillary to a pH 10 buffer. This means that under basic conditions, the Si-O-Si-C linkage hydrolyzes at least partially to regenerate silanols. However, it is surprising that the efficiency of the Si-C bound diol-capillary is lower than that of the siloxane bound one. One possibility is that these two modified capillaries were prepared from different fused-silica capillary sources (different lot number) although they have about the same inner and outer diameters. Since they have slightly different surface, surface coverage at different levels might result. Unfortunately, no direct measure of surface coverage can be obtained. Another possibility is that the two separations were performed in slightly different pH buffers, at different times, under different ambient temperature, etc.. This is also evidenced by a slightly but consistently higher current (9 μ A) for the Si-C bound capillary than that (8 μ A) for the Si-O-Si-C bound one at the same applied 22 kV voltage and same electrolyte. Additionally, compared to similar separations in other Si-O-Si-C bound diol-modified capillaries found in the literature and shown in Fig. 22 (18), it appears that our capillary exhibits better separation efficiency, resolution and peak shape than the others.

Fig. 21. Electroosmotic mobility as a function of electrolyte's pH for a conventional siloxane bound diol-modified capillary on the home-made HPCE instrument: uncharged tracer, mesityl oxide; concentration 1 mM in the buffers; injection, hydrodynamic 10 s at 10 cm height differential; detection wavelength, 254 nm; applied voltage, 22.0 kV; current, 8 μ A at pH 3.71, 13 μ A at pH 4.78, 4 μ A at pH 6.01, 8 μ A at pH 7.05, 6 μ A at pH 8.30, 6 μ A at pH 10.50, 4 μ A at pH 5.97 after exposure to higher pH buffer; all other conditions as in Fig. 20.

Fig. 22. 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; $l_{\text{inj-det}} = 50.5$ cm. (b) Voltage, 15 kV; $l = 52.2$ cm; $l_{\text{inj-det}} = 31.7$ cm. 1 = Lysozyme; 2 = trypsin; 3 = chymotrypsinogen (taken from reference 18).

4. CONCLUSIONS

The Si-C diol-modified capillary prepared by catalytic hydrosilation on a hydride intermediate appears to be well suited for the HPCE separations of basic proteins up to pH 6. The hydrophilic Si-C bound diol moiety substantially decreases the interactions of proteins with the silica capillary walls and thus reduces protein adsorption and peak tailing. The capillary's electroosmosis is also dramatically reduced by the chemical attachment of the diol moiety. Although separation efficiencies obtained with this capillary were lower than what the theory predicted, its higher stability toward hydrolysis provides improved precision of protein migration. Thus, better reproducibility of migration times, more stable separation and longer life time can be attained with this capillary. Optimum conditions for electrophoretic separations in the capillary include low concentration of proteins (about 0.2 mg/mL) with small injection volumes, at an electric field strength of 300 V/cm. Finally, it should be recalled that most of the results reported in this work were obtained on a home-made HPCE instrument, with manual sample injection and no temperature control. A more realistic evaluation of this capillary should be carried out with an automated system. It is believed that more precise analytical figures can be achieved with a commercial instrument.

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