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Capillary electrochromatography of small molecules in polyacrylamide gels with electroosmotic flow

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Abstract

Traditionally, polyacrylamide gels have been used as sieving media for the separation of macromolecules such as proteins and polynucleotides. Since these analytes are electrically charged, they can migrate under an applied electric field. In an attempt to separate uncharged analytes, acrylamide (AA)–2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) copolymers and AA–N,N′-methylenebisacrylamide (BIS)–AMPS copolymers were prepared. These copolymers are clear, UV-transparent gels and give electroosmosis due to the dissociation of the sulfo group. The preparation of the new gels in capillary tubes yielded the separation of comparatively small molecules, which is probably based on a molecular sieving effect.

1. Introduction

Since the advent of capillary electrophoresis (CE, originally called capillary zone electrophoresis) in 1979–83 [1–3], several variants have emerged as powerful methods for analyzing complex mixtures in nanoliter volumes or less. At the HPCE '94 Symposium, Knox [4] gave recommended names for the various capillary electroseparation (CES) systems. According to this proposal, CES is grouped into two general classes: those whose separation process is primarily based on electrophoresis and those whose separation is primarily based on the difference in partitioning between two phases.

CE and capillary gel electrophoresis (CGE) fall in the former group and capillary electrochromatography (CEC) and capillary micellar electrochromatography (CMEC, originally termed micellar electrokinetic chromatography and often called micellar electrokinetic capillary chromatography) in the latter. It is recommended that the term CGE is used to describe the process whereby ionic analytes are separated in a gel-filled capillary [4]. CGE has been devoted primarily to the separation of large molecules such as proteins and DNA fragments. In CGE, separation is based on molecular size differences by restricted migration of analytes throughout the gel matrix. CMEC is capable of separating both neutral species and ions, unlike CE, which is a separation technique for ionic analytes only. The CMEC separation is based on the differential partitioning of analytes between the micelle (pseudo-stationary phase) and the aqueous phase.

The principle of CEC is the same as that of high-performance liquid chromatography, except that propulsion of a liquid through a column is effected by the application of an electric field

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rather than pressure. Columns can be either a tube packed with a conventional HPLC packing material (or sub-μm particles [5]) or an open-tubular column. Analytes are separated according to their different partitioning ratios between a mobile phase and a stationary phase. The term pressurized (flow) electrochromatography has also been used to describe an HPLC technique which applies pressure in combination with an electrical field [4,6].

Here, we report CEC separations of small organic compounds on columns containing non-particle packing materials. In preliminary studies [7,8], we have shown that the separation of charged, low-molecular mass compounds can be improved by using capillary columns filled with linear polyacrylamides without electroosmotic flow (EOF). In this work, we explored the variation of migration time with the concentration of polyacrylamide in detail since it seems to provide a starting-point to an understanding of the separation mechanism underlying the CEC separations of neutral compounds on charged columns which will be described here. Next, polyacrylamide gels of various composition were prepared using acrylamide (AA), N,N'-methylenebisacrylamide (BIS) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) through linear and cross-linking procedures. The dissociation of the AMPS built in the polymer makes the generation of EOF possible, which in turn allows the electrokinetic injection and separation of neutral compounds and ionic compounds. Finally, we draw the conclusion that restricted migration of analytes through pores in the polymer network (a physical gel or chemical gel) play a role in the separation mechanism, as in usual CGE.

2. Experimental

2.1. Apparatus

Separations were performed using a laboratory-made system, consisting of a Model HCZE-30PN0.25 (Matsusada Precision Devices, Kusatsu, Japan) high-voltage power supply, a Model 870-CE (Jasco, Tokyo, Japan) UV detector, 4-ml volume reservoirs and fused-silica tubing of 0.075 mm I.D. (or 0.10 mm) and 0.375 mm O.D. (GL Science, Tokyo, Japan).

The high-voltage end of the system was encased in a Plexiglas box equipped with an interlock system. Sample solutions were injected electrokinetically. All experiments were done at ambient temperature.

2.2. Chemicals

Distilled, deionized water, purified with an Autosil WG 23 system (Yamato, Tokyo, Japan), was used in the preparation of buffers. AA, BIS, ammonium peroxodisulfate (APS) and N,N,N',N'-tetramethylenediamine (TEMED) were purchased from Nacalai Tesque (Kyoto, Japan), 3-trimethoxysilylpropyl ester, AMPS, alcohols and ketones from Tokyo Kasei Kogyo (Tokyo, Japan), tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany) and boric acid from Kishida Chemical (Osaka, Japan).

2.3. Column preparation

The capillaries were internally treated with 3-trimethoxysilylpropyl ester in the manner described by Hjertén [9]. Polymerization of acrylamide monomers was initiated by APS, catalyzed by TEMED and accomplished in the capillary with 100 m*M* Tris–boric acid. All columns were pre-electrophoresed until the baseline of the detector remained stable.

Throughout this work, the following definitions are used to represent the gel composition:

$$\%T = 100(a + b + c)/V \quad (1)$$

$$\%C = 100b/(a + b + c) \quad (2)$$

where a, b and c are the mass (g) of AA, BIS and AMPS, respectively, and V (ml) is the volume [10]. The AMPS content, %S, is defined here as

$$\%S = 100\gamma/(\alpha + \beta + \gamma) \quad (3)$$

where α, β, and γ are the molarity of AA, BIS and AMPS, respectively.

3. Results and discussion

3.1. Separation of charged molecules on linear polyacrylamide columns without EOF

Fig. 1A shows the ability of a linear polyacrylamide column to separate small organic compounds. Dansylated (Dns) amino acids were chosen as representatives of small molecules. The test solutes appears as a single peak in the electropherogram when separated in a free solution column [7,8]. For comparison, the separation of the test mixture was performed using a buffer solution which contained 10.0% (w/v) acrylamide monomer but no APS (and no TEMED). The result is shown in Fig. 1B, where the electropherogram remains the same in appearance as that in the free solution column. These results suggest the participation of a polymer network in the separation shown in Fig. 1A.

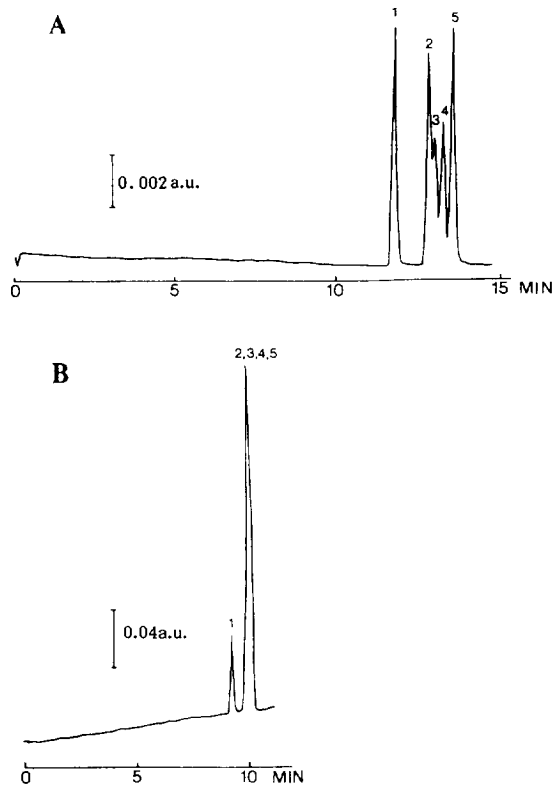

Fig. 1. Separation of dansylated amino acids (A) in linear polyacrylamide and (B) in acrylamide monomer solution capillaries. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary, 40.0 cm × 0.075 mm I.D. (15.0 cm effective length); applied voltage, 8.0 kV; detection wavelength, 254 nm. Peaks: 1 = Dns-Ala; 2 = Dns-Val; 3 = Dns-Leu, 4 = Dns-Ile; 5 = Dns-Phe. Note no EOF in either of these columns.

The size selectivity of polyacrylamide columns can be tested by means of a Ferguson plot [11,12]. Fig. 2 shows the Ferguson plots for dansylated amino acids on columns containing linear polyacrylamides between 0 and 15.0 %T. Slight deviations from linearity can be seen in the plots. Such a phenomenon has already been observed for DNA restriction fragments [13]. The slope for each component was calculated by least squares analysis in the range of 3.0–8.0 %T, and listed in Table 1. The slope is steeper for larger molecules, suggesting the size separation mechanism.

In CES, a higher electric field strength offers the advantages of enhanced efficiency and reduced analysis time unless an excessive Joule heating is reached. On the basis of the equations

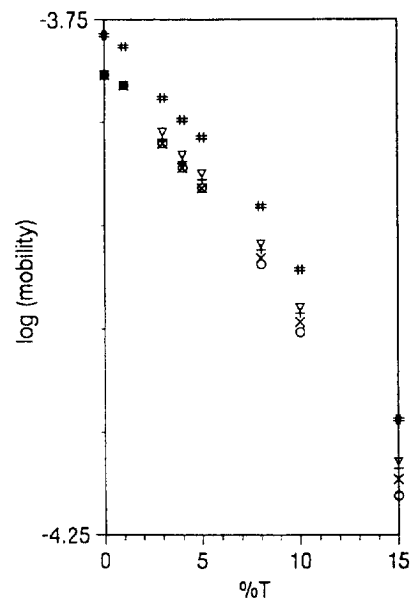

Fig. 2. Ferguson plot showing the dependence of the logarithm of the mobility of dansylated amino acids on %T. Solutes: # = Dns-Ala; ∇ = Dns-Val; + = Dns-Leu; × = Dns-Ile; ○ = Dns-Phe. Conditions as in Fig. 1 except the column I.D., 0.10 mm.

Table 1
Results of linear regression

Compound	Slope	Correlation coefficient
Dns-Ala	−0.0484	0.997
Dns-Val	−0.0499	0.999
Dns-Leu	−0.0500	0.999
Dns-Leu	−0.0505	0.999
Dns-Phe	−0.0541	0.999

Conditions: capillary, 40.0 cm × 0.10 mm I.D. (15.0 cm effective length); buffer, 100 m*M* Tris–150 m*M* boric acid (pH 8.2); applied voltage, 8.0 kV; detection wavelength, 220 nm.

derived by Wieme [14] and Liu et al. [15], the plate height (H) for CEC using the polyacrylamide gel columns may be described by

$$H = h^2/(12L) + l^2/(12L) + d/R + 2D/v + (1 - R)d^2v/D \quad (4)$$

where h is the initial width of a sample plug, L is the column length, l is the detector observation length, d is the distance of a channel between fibers, R is the relative zone velocity, D is the diffusivity of a solute in the buffer and v is the velocity of the mobile phase. This expression, if it is to be general, must include the thermal contributions [5,16]. If the mobile phase velocity is proportional to the applied field strength (E), the dependence of H on applied field strength can best seen by writing Eq. 4 as

$$H = A + B/E + CE \quad (5)$$

where A, B and C are constants with respect to field changes. Fig. 3 shows current and plate height of as a function of the applied field strength in both a column containing a straight buffer and a column containing 10.0 %T linear polyacrylamide. The current produced within the polyacrylamide column is significantly smaller than that produced within the straight buffer-containing column, probably owing to the higher

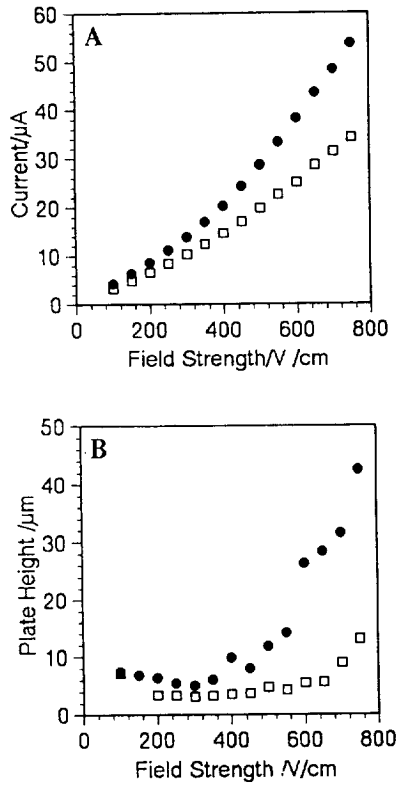

Fig. 3. Effect of field strength on current and plate height for (●) free solution and (□) 10 %T linear polyacrylamide columns. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary, 40.0 cm × 0.075 mm I.D. (15.0 cm effective length); detection wavelength, 254 nm; solute, Dns-Ala; injection, electromigration for 5 s at 2.0 kV.

viscosities in the former column. On the other hand, the column efficiencies are much higher with the polyacrylamide-filled column, indicating the anticonvective nature of the physical gel. The minimum plate height is obtained at ca. 300 V/cm. It is likely that beyond this field, the contribution to the plate height from Joule heating is sufficiently large to reduce the influence of the contributions from the others. Based on the success of these studies, we separated a mixture of 12 dansylated amino acids at the maximum field strength. The solutes were resolved and eluted within 17 min, as shown in Fig. 4.

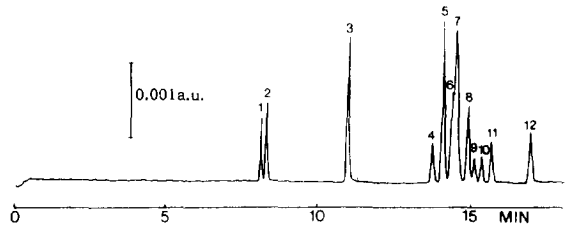

Fig. 4. Separations of 12 dansylated amino acids on a 10 %T linear polyacrylamide column. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary, 60.0 cm × 0.075 mm I.D. (30.0 cm effective length); applied voltage, 18.0 kV; detection wavelength, 254 nm; injection, electromigration for 5 s at 2.0 kV. Peaks: 1 = Dns-Asp; 2 = Dns-Glu; 3 = Dns-OH; 4 = Dns-Ala; 5 = Dns-Pro; 6 = Dns-Hyp; 7 = Dns-Thr; 8 = Dns-Val; 9 = Dns-Leu; 10 = Dns-Ile; 11 = Dns-Phe; 12 = Dns-Trp.

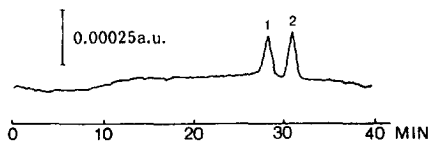

Fig. 6. Separation of a mixture of acetone and acetophenone on an AA–BIS–AMPS copolymer column. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary 65.0 cm × 0.075 mm I.D. (15.0 cm effective length); %T, 6.0; %C, 3.3; %S, 2.4; applied voltage, 13.0 kV; detection wavelength, 254 nm; injection, electromigration for 5 s at 13.0 kV. Peaks: 1 = acetone (0.5% in buffer), 2 = acetophenone (5 ppm in buffer).

3.2. Separation of uncharged molecules on linear and cross-linked polyacrylamide columns with EOF

We expected that the traveling of uncharged compounds through a polyacrylamide column would be allowed by the introduction of ionizable functional groups into the network. For this purpose, AMPS, which has a sulfo group, was copolymerized with AA or an AA–BIS mixture. The gels should be negatively charged in a wide range of pH. Under an applied electric field, the solvent (i.e. the mobile diffuse layer) moves since the charge is fixed into the gel. Figs. 5–8 show typical chromatograms of CEC separations of uncharged compounds on linear (10.0 %T, 2.4 %S) and cross-linked (6.0 %T, 3.3 %C, 2.4 %S and 12.0 %T, 5.0 %C, 7.8 %S) polyacrylamide columns with electro-osmotic flow (EOF). Since every solute is essentially neutral under the conditions used, these figures indicate that they are introduced into and move through the column by EOF.

It is not surprising that the coppolymerized acrylamide is not extruded from the capillary by the EOF, by which the migration of the solutes through the column is effected. One might

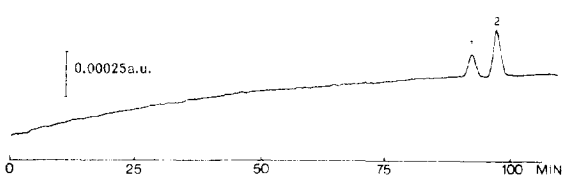

Fig. 5. Separation of a mixture of acetone and acetophenone on an AA–AMPS copolymer column. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary 65.0 cm × 0.075 mm I.D. (50.0 cm effective length); %T, 10.0; %S, 2.4; applied voltage, 13.0 kV; detection wavelength, 254 nm; injection, electromigration for 5 s at 13.0 kV. Peaks: 1 = acetone (0.5% in buffer), 2 = acetophenone (5 ppm in buffer).

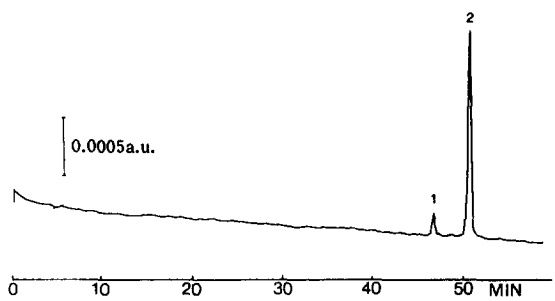

Fig. 7. Separation of a mixture of benzyl alcohol and cinnamyl alcohol on an AA–BIS–AMPS copolymer column. Conditions: buffer, 100 m*M* Tris–100 m*M* boric acid; capillary, 69.2 cm × 0.075 mm I.D. (59.5 cm effective length); %T, 12.0; %C, 5.8; %S, 7.8; applied voltage, 20.0 kV; detection wavelength, 254 nm; injection, electromigration for 5 s at 20.0 kV. Peaks: 1 = benzyl alcohol (0.23% in buffer); 2 = cynnamyl alcohol (0.015% in buffer).

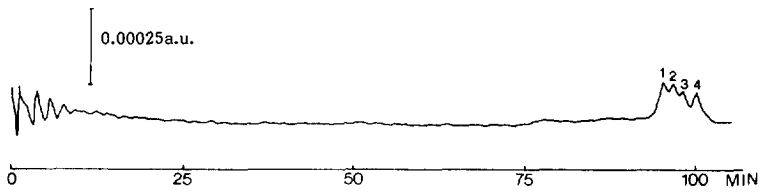

Fig. 8. Separation of a mixture of ketones on an AA–BIS–AMPS copolymer column. Conditions: buffer, 100 m*M* Tris–150 m*M* boric acid; capillary, 65.0 cm × 0.075 mm I.D. (50.0 cm effective length); %T, 6.0; %C, 3.3; %S, 2.4; applied voltage, 23.0 kV; detection wavelength, 254 nm; injection, electromigration for 5 s at 13.0 kV. Peaks: 1 = acetone; 2 = 2-butanone; 3 = methyl *n*-propyl ketone; 4 = 2-hexanone; 1% each in buffer.

Table 2
Reproducibility of migration times ($n = 5$)

Parameter	Linear polyacrylamide[a]		Cross-linked polyacrylamide[b]	
	Acetone	Acetophenone	Acetone	Acetophenone
Average time (min)	39.76	40.32	27.89	30.23
Standard deviation	0.09	0.10	0.07	0.09
R.S.D. (%)	0.23	0.25	0.24	0.29

[a] Conditions: capillary, 65.0 cm × 0.075 mm I.D. (50.0 cm effective length); %T, 10.0; %S, 4.9; buffer, 100 m*M* Tris–150 m*M* boric acid (pH 8.2); applied voltage, 13.0 kV; detection wavelength, 254 nm.
[b] Conditions: capillary, 65.0 cm × 0.075 mm I.D. (15.0 cm effective length); %T, 6.0; %C, 3.3; %S, 2.4; other conditions as for linear polyacrylamide.

question the stability of these columns. The reproducibilities of the migration times of acetone and acetophenone were examined on linear and cross-linked polyacrylamide columns. The reproducibility data are given in Table 2. It is apparent that both columns give good reproducibilities. However, we found that the migration times of the test solutes slowly decreased on linear polyacrylamide columns. Therefore, the cross-linked polyacrylamide columns are the more favorable choice.

In summary, the introduction of dissociative functional groups into polyacrylamide gels permitted the separation of uncharged molecules. The stability of the cross-linked polyacrylamide columns studied here is as good as that of common polyacrylamide gel columns without EOF. From data presented here and elsewhere [17], we suspect that the separation of such small molecules is made mainly according to their size.

Acknowledgements

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