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Preparation and characterization of a stable polyacrylamide sieving matrix-filled capillary for high-performance capillary electrophoresis

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ABSTRACT

A stable sieving matrix of polyacrylamide filled in a capillary was developed. The inner wall of a fused-silica capillary was covalently bonded with a linear polyacrylamide through Si–C linkages, in which cross-linked polyacrylamide gel or linear polyacrylamide solution was filled. The stability of the coating was examined by exposure of the capillaries to alkaline buffer (pH 8) for up to 30 days. Compared with the coatings with linear polyacrylamide bonded through siloxane linkages, the present capillary markedly reduced the electroosmotic flow. Thus, the sieving matrix in the capillary was stabilized, resulting in a prolonged lifetime of the capillary and good reproducibility of separations. The migration behaviours of oligonucleotides were compared for the cross-linked gel and linear polyacrylamide solution at the same concentration.

INTRODUCTION

High-performance capillary electrophoresis is a rapidly growing technique for the separation of a wide range of analytes, especially biopolymers such as proteins and polynucleotides. However, good reproducibility of the separation of proteins is difficult because of the instability of the gel and the considerable adsorption on the capillary wall. Many reports [1–6] have described the techniques to overcome this difficulty by coating the inner wall of the capillary with hydrophilic polymers through siloxane (Si–O–Si–C) bonds. However, these bonds are prone to nucleophilic cleavage under basic conditions [7,8]. Coating with stable Si–C bonds is a more successful approach to this problem [9].

When polyacrylamide gel was directly bonded to the capillary through siloxane bonds [10–14], it was often found that the bubbles are generated inside the capillary during polymerization and electrophoretic separation. In order to prepare bubble-free gels, the procedure was modified by applying high pressures of up to 400 bar during polymerization [15]. Another modification is that the capillary wall is first covalently bonded with linear polyacrylamide through siloxane linkages to eliminate electroosmotic flow [16], then filled with polyacrylamide gel under moderate pressure.

A gel-filled capillary without a coating is not stable. The separation efficiency decreases rapidly because a small part of the gel is slowly extruded out of the capillary by the electroosmotic pressure generated by the zeta potential on the untreated capillary surface. We solved this problem by employing the Si–C bond coating

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technique mentioned above [9], but this needed to be improved because it was time consuming. Cross-linked or linear polyacrylamide was formed in a fused-silica capillary which was covalently bonded with linear polyacrylamide through Si–C linkages. This type of capillary suppressed the electroosmotic flow so that the sieving matrix in the capillary became sufficiently stable.

EXPERIMENTAL

Reagents

3-Methacryloxypropyltrimethoxysilane (MAP-S) was purchased from Shin-etsu Chemicals (Tokyo, Japan), vinylmagnesium bromide (1 *M* solution in tetrahydrofuran) from Aldrich (Milwaukee, WI, USA) and thionyl chloride, acrylamide (AA), N,N′-methylenebis(acrylamide) (bisAA), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate (APS), tris(hydroxymethyl)aminomethane (Tris), tetrahydrofuran (THF), boric acid, urea and Orange G from Wako (Osaka, Japan). Polydeoxyadenylic acids $[p(dA)_{12-18}]$, polydeoxycytidylic acids $[p(dC)_{12-18}]$, polydeoxyguanylic acids $[p(dG)_{12-18}]$ and polydeoxythymidylic acids $[p(dT)_{12-18}]$ were purchased from Pharmacia (Piscataway, NJ, USA). Crude oligonucleotide products (S-1, 5′-TTCAGCAGCCACGCCC-AG-3′; S-2, 5′-TGATCTTCCCCTACTACG-3′; A-1, 5′-TAGGCGTATGGACGGCTG-3′; and A-2, 5′-TGGAGCAGGTGAAGAAGC-3′) were prepared by an oligonucleotide synthesizer.

Apparatus

Fused-silica capillaries (50 μm × 75 μm I.D. × 375 μm O.D.) were purchased from GL Sciences (Tokyo, Japan). Separations were performed on a CE-800 capillary electrophoresis system (Jasco, Tokyo, Japan). The operating conditions are given in the figure captions.

Preparation of linear polyacrylamide-coated capillary through siloxane linkages

A detection window was prepared by removing the polyimide coating from a small section of a fused-silica capillary before coating. The inner surface of the capillary was first treated with 1 *M* NaOH for 3 h at room temperature, then reacted with MAPS by the method described by Hjerten [2].

Preparation of linear polyacrylamide-coated capillary through Si–C linkages

A non-cross-linked monolayer of polyacrylamide was bonded to the inner surface of the capillary through Si–C linkages by the reaction shown schematically in Fig. 1, where the procedure employed was the improved version of the reported method [9].

The capillary with a detection window was treated with 1 *M* NaOH, washed with distilled water for 1 h and dried at 110°C under nitrogen overnight. Thionyl chloride was introduced into the capillary using a suction pump for several minutes. After both ends of the capillary had been sealed with a small torch, it was kept at 70°C for 6 h. After reaction, both ends of the capillary were opened and excess thionyl chloride was blown out with nitrogen. A solution of 0.25 *M* vinylmagnesium bromide–THF was introduced into the chlorinated capillary by suction for several minutes, then both ends were sealed and the capillary was kept at 70°C for 6 h for the Grignard reaction to proceed. The capillary was opened and rinsed with THF and distilled water. An aqueous solution containing 3% AA, 0.1% APS and 0.1% TEMED was filled into the capillary, which was kept for 1 h at 28 ± 2°C for polymerization to take place. The capillary was then washed with distilled water by suction until small drops of water appeared rapidly at the end of the suction side.

Preparation of cross-linked polyacrylamide gel-filled capillary

A 5-ml volume of aqueous solution containing 0.1 *M* Tris, 0.25 *M* boric acid, 7 *M* urea and AA–bisAA (7%T, 5%C) was degassed in an ultrasonic bath. A 30-μl volume of 10% TEMED and 10 μl of 10% APS were added to the solution and mixed thoroughly. The mixed solution was immediately filled into the above-mentioned coated capillary. The polymerization proceeded under a pressure of 200 bar to prevent bubbling. After polymerization, the gel-filled

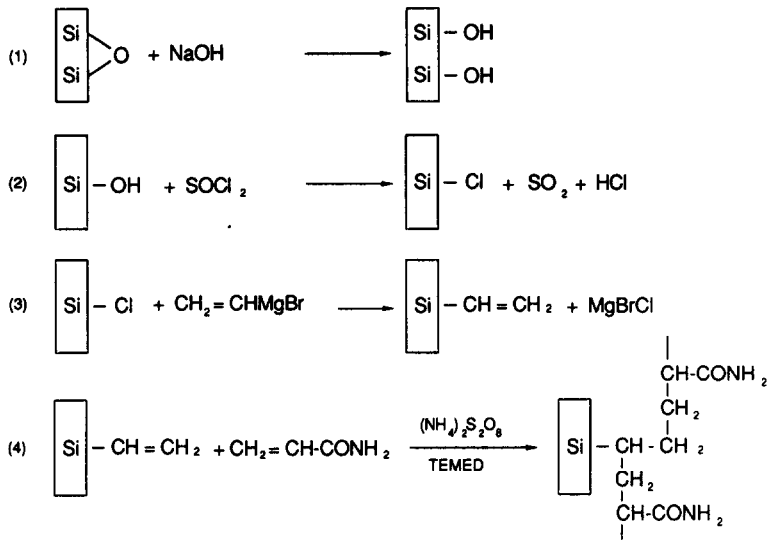

Fig. 1. Reaction scheme for coating a capillary with linear polyacrylamide through Si–C bonds.

capillary was conditioned at −100 V/cm for 30 min before electrophoretic runs.

Preparation of linear polyacrylamide solution-filled capillary

A 5-ml volume of aqueous solution containing 0.1 *M* Tris, 0.25 *M* boric acid, 7 *M* urea and AA (7%T, 0%C) was degassed in an ultrasonic bath, then 30 μl of 10% TEMED and 10 μl of 10% APS were added and mixed thoroughly. The mixed solution was immediately filled into the coated capillary. After polymerization under atmospheric pressure, the gel-filled capillary was conditioned at −100 V/cm for 30 min before electrophoretic runs.

Assessment of durability of coatings under alkaline conditions

The uncoated capillary and the polyacrylamide-coated capillaries (50 μm I.D., total length 50 cm, effective length 30 cm) involving Si–O–Si–C and Si–C linkages prepared by the above methods were filled with 50 m*M* Tris–HCl buffer (pH 8) and kept at room temperature for up to 30 days. The electroosmotic flow was measured using mesityl oxide as a neutral marker and 50 m*M* Tris–HCl buffer (pH 8) as a running buffer, with an applied potential of 400 V/cm.

RESULTS AND DISCUSSION

Fig. 2 illustrates the electroosmotic mobilities observed in the uncoated and coated capillaries after exposure to alkaline conditions (pH 8) for up to 30 days. The coated capillaries involving Si–O–Si–C linkages showed a gradual increase in mobility, whereas those involving Si–C linkages showed a low and constant mobility unchanged from the initial value. The initial values of electroosmotic mobility in both types of coated capillary were very low, such that the

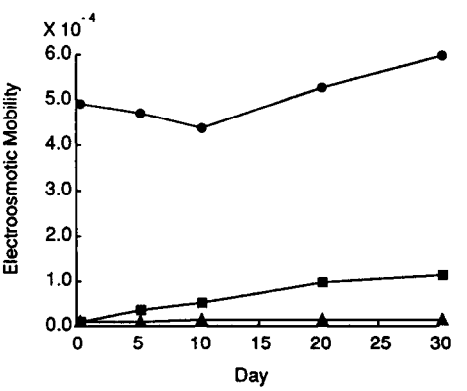

Fig. 2. Time course of electroosmotic mobility ($cm^2/V \cdot s$) as a function of number of days of exposure under alkaline conditions (50 m*M* Tris–HCl, pH 8.0). ● = Uncoated capillary; ■ = coated capillary through siloxane linkages; ▲ = coated capillary through Si–C linkages.

migration times of the marker substance exceeded 6 h. This indicates that the capillary wall has virtually no electric charge because of the almost complete conversion of silanol groups into AA derivatives. After exposure for 30 days, the electroosmotic mobilities in the coated capillaries were $5.21 \cdot 10^{-6}$ cm^2/V·s (Si–C) and $1.04 \cdot 10^{-4}$ cm^2/V·s (Si–O – Si–C), whereas the mobilities in the uncoated capillaries were as high as $4.61 \cdot 10^{-4}$–$5.89 \cdot 10^{-4}$ cm^2/V·s. This indicates that the Si–C bond is more stable than the siloxane bond which links silanol groups to AA. It is also suggested that the polyacrylamide network bonded through Si–C linkages can protect the siloxane bondings of the capillary wall itself from attack by hydroxyl anions. This effect may be stronger than that due to the polyacrylamide coatings through siloxane linkages.

The separations of p(dA) ranging from 12 to 18 bases [p(dA)$_{12-18}$] in the cross-linked polyacrylamide (7%T, 5%C) gel and the linear polyacrylamide (7%T, 0%C) solution-filled capillaries are shown in Figs. 3 and 4, respectively. The inner wall of both capillaries was coated

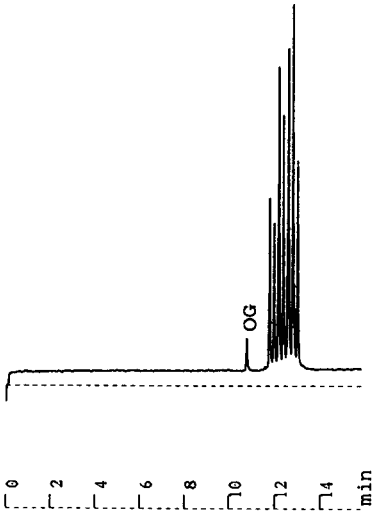

Fig. 3. Electropherogram of p(dA)$_{12-18}$ on cross-linked polyacrylamide gel-filled capillary. Capillary, 75 μm I.D. × 50 cm (30 cm to detector), inner surface coated with linear polyacrylamide through Si–C linkages; gel, polyacrylamide, 7%T, 5%C; buffer, 0.1 *M* Tris–0.25 *M* boric acid–7 *M* urea; electric field, −300 V/cm, 7 μA; injection, −5 kV, 2 s; detection, UV at 260 nm.

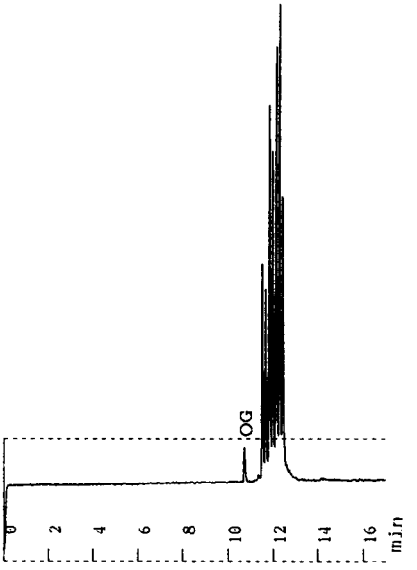

Fig. 4. Electropherogram of p(dA)$_{12-18}$ on linear polyacrylamide solution-filled capillary. Capillary, 75 μm I.D. × 50 cm (30 cm to detector), inner surface coated with linear polyacrylamide through Si–C linkages; gel, polyacrylamide, 7%T, 0%C; buffer, 0.1 *M* Tris–0.25 *M* boric acid–7 *M* urea; electric field, −300 V/cm, 7 μA; injection, −5 kV, 2 s; detection, UV at 260 nm.

with linear polyacrylamide through Si–C linkages in order to suppress electroosmotic flow and to stabilize the matrices. The peaks are clearly separated with baseline resolution. The peak marked OG corresponds to the internal standard (Orange G). The separation efficiency is almost the same as that found with conventional gel-filled capillaries [13,14,17].

The run-to-run reproducibilities of the migration times and relative migration times were determined by repeated injections of p(dA)$_{12-18}$ using the cross-linked polyacrylamide (7%T, 5%C) gel and the linear polyacrylamide (7%T, 0%C) solution-filled capillaries and are given in Table I. The relative migration time is given as the ratio of the migration time of the tested oligomer to that of Orange G. Although the reproducibility of the migration times obtained in the linear polyacrylamide solution-filled capillary is better than that in the cross-linked gel-filled capillary, there were no significant differences between relative migration times in the two capillaries.

TABLE I

REPRODUCIBILITY OF MIGRATION TIMES ($n = 10$)

Gel concentration	Sample	Migration time (min) (mean ± S.D.)	Relative migration time (mean ± S.D.)
7%T, 5%C	$P(dA)_{12}$	11.553 ± 0.21	1.087 ± 0.004
	$P(dA)_{15}$	12.135 ± 0.22	1.143 ± 0.004
	$P(dA)_{18}$	12.746 ± 0.23	1.201 ± 0.005
7%T, 0%C	$P(dA)_{12}$	11.453 ± 0.05	1.077 ± 0.003
	$P(dA)_{15}$	11.913 ± 0.05	1.122 ± 0.003
	$P(dA)_{18}$	12.359 ± 0.06	1.165 ± 0.003

The day-to-day reproducibilities (R.S.D.s) of the migration times ($n = 30$) using the cross-linked gel and the linear polyacrylamide solution-filled capillaries were *ca.* 3.5% and *ca.* 4.5%, respectively.

The four different homooligonucleotide mixtures, $p(dA)_{12-18}$, $p(dT)_{12-18}$, $p(dC)_{12-18}$ and $p(dG)_{12-18}$, were also applied to the cross-linked and the linear polyacrylamide solution-filled capillaries. The migration times relative to that of the internal standard (Orange G) showed linear relationships (correlation coefficient $r > 0.999$) with the base number of each homologous series, as shown in Figs. 5 and 6. The four lines in each figure are parallel to each other, but a comparison of the slopes in Figs. 5 and 6 suggests that the cross-linked polyacrylamide gel (Fig. 5) gives a slightly better resolution than linear polyacrylamide (Fig. 6). These oligonucleotides can be completely deformed by 7 *M* urea in the running buffer solution. If this is the case, p(dA) and p(dG) with purine bases are expected to have a larger molecular size than p(dT) and p(dC) with pyrimidine bases, and hence their migration times should be in the order p(dT), p(dC) < p(dA), p(dG). Further, if the total charge of each molecule can be regarded as the sum of independent dissociations of these bases and phosphate groups, the net charge in the running buffer solution, depending on the dissociation constant of the base, differs

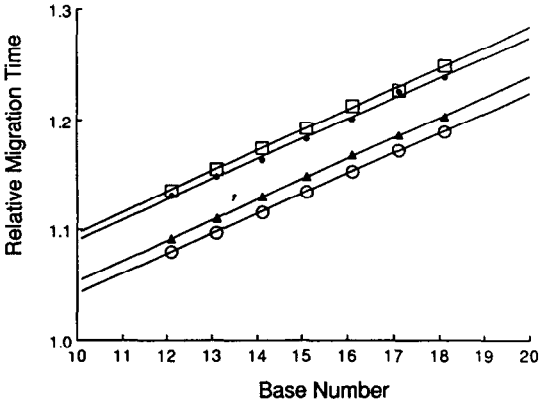

Fig. 5. Relationship between the base number and the relative migration time of the homooligonucleotide mixtures on a cross-linked polyacrylamide gel-filled capillary. Separation conditions as in Fig. 3. The calculation of relative migration times was based on the migration time of Orange G. ○ = $p(dA)_{12-18}$; □ = $p(dT)_{12-18}$; ▲ = $p(dC)_{12-18}$; ● = $p(dG)_{12-18}$.

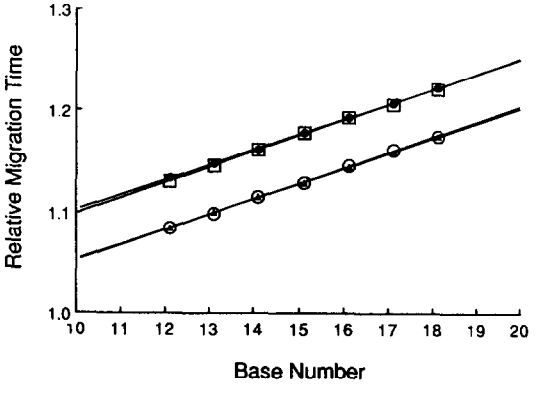

Fig. 6. Relationship between the base number and the relative migration time of the homooligonucleotide mixtures on a linear polyacrylamide solution-filled capillary. Separation conditions as in Fig. 4. The calculation of relative migration times was based on the migration time of Orange G. Symbols as in Fig. 5.

among homooligonucleotides having same number of base groups. The reported pK_a values [18–20] are *ca.* 4 for adenine and cytosine and 9.5 for guanine and thymine. These values suggest that only guanine and thymine nucleotides may have weak negative charges in the running buffer (pH 8.3), resulting in the migration time order p(dT), p(dG) < p(dC), p(dA). The experimental results in Figs. 5 and 6 indicate the order p(dA) < p(dC) < p(dG) < p(dT) in the linear polyacrylamide-filled capillaries. Further studies are required in order to resolve this discrepancy.

The electropherograms of a mixture of the four synthesized heterooligonucleotides using the cross-linked and the linear polyacrylamide-filled capillaries are shown in Figs. 7 and 8, respectively. These oligonucleotides have same chain length (18mer), but the sequence of base units differs. Both capillaries gave the same migration order, but the separation efficiency was slightly better with the cross-linked polymer than with the linear polymer. The relative migration times of these synthesized oligonucleotides were calculated by the following equation, which was

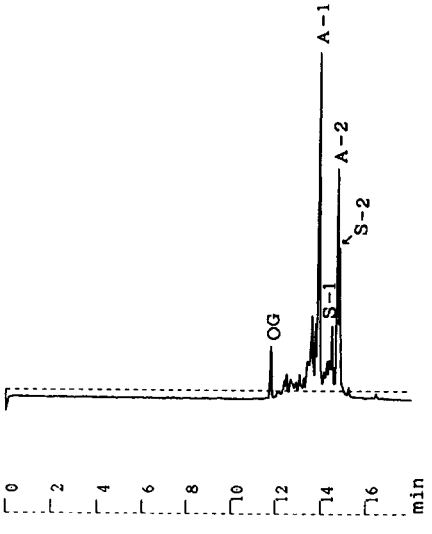

Fig. 7. Electropherogram of crude oligonucleotide products on a cross-linked polyacrylamide (7%T, 5%C) gel-filled capillary. Separation conditions as in Fig. 3. Sample: S-1 = 5′-TTCAGCAGCCACGCCCAG-3′; S-2 = 5′-TGATCTTCCC-CTACTACG-3′; A-1 = 5′-TAGGCGTATGGACGGCTG-3′; A-2 = 5′-TGGAGCAGGTGAAGAAGC-3′.

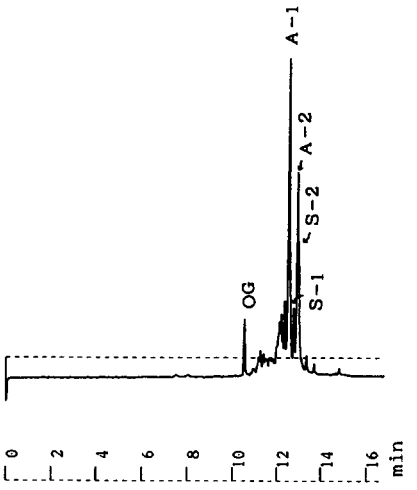

Fig. 8. Electropherogram of crude oligonucleotide products on a linear polyacrylamide (7%T, 0%C) solution-filled capillary. Separation conditions as in Fig. 4. Sample: S-1 = 5′-TTCAGCAGCCACGCCCAG-3′; S-2 = 5′-TGATCTTC-CCCTACTACG-3′; A-1 = 5′-TAGGCGTATGGACGGC-TG-3′; A-2 = 5′-TGGAGCAGGTGAAGAAGC-3′.

obtained from the linear relationship given in Figs. 5 and 6 according to Guttman *et al.*'s method [20]:

$$t'(A_aT_tC_cG_g) = a/nt'(A_n) + t/nt'(T_n) + c/nt'(C_n) + g/nt'(G_a)$$

where n is the oligonucleotide chain length ($n = a + t + c + g$) and a, t, c and g are numbers of the individual bases in the oligonucleotide, and $t'(A_n)$, $t'(T_n)$, $t'(C_n)$ and $t'(G_n)$ are the relative migration times of the n-mers of adenylic, thymidylic, cytidylic and guanylic acid homooligonucleotides, respectively. The results obtained were different from the observed values of relative migration time and migration order. This indicates that Guttman et al. equation, which was applied to three kinds of heterooligonucleotides having a different base only at the tenth residue from the 5′-terminus, cannot be applied to those having entirely different base arrangements. Therefore, not only the kind and number of bases but also their location and sequence in the chain need to taken into consideration in order to predict migration times.

CONCLUSION

As the capillary was coated with linear polyacrylamide which was covalently bonded to the silica wall through Si–C linkages, the cross-linked polyacrylamide gel or linear polyacrylamide solution in the capillary became stable even under alkaline conditions and could be used repeatedly owing to the easy refilling of sieving matrices. This type of the stable capillary will allow a wide range of soft gel or polymer solutions of low viscosity to be used for high-performance capillary electrophoresis.

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