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High-performance capillary electrophoresis of SDS-proteins using pullulan solution as separation matrix

Manabu Nakatani*[,a], Akimasa Shibukawa[b], Terumichi Nakagawa[b]

[a]*Pharmaceutical Research Department, Nippon Boehringer Ingelheim Co. Ltd., 3-10-1, Yato Kawanishi, Hyogo 666-01, Japan*
[b]*Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan*

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Abstract

Sodium dodecyl sulphate (SDS) capillary electrophoresis using pullulan solution as a separation matrix was developed for the separation and molecular mass determination of proteins. The silanol functions on the inner surface of a fused-silica capillary were deactivated by coating with linear polyacrylamide through Si–C linkages, into which the pullulan solution was filled. The stability of the coating was examined by exposure to an alkaline buffer solution (pH 9.2) for up to 30 days. Compared with conventional coatings with linear polyacrylamide through siloxane linkages, the present capillary was more stable even under alkaline conditions and markedly reduced electroosmotic flow. Thus, polymer solutions of low viscosity such as pullulan solution could be stabilized in the capillary, resulting in a prolonged life-span of the capillary and improved reproducibility of separations. An excellent linear relationship was obtained between the mobility and the logarithm of the molecular mass of SDS-proteins. The relative standard deviation of migration times was below 0.5% when the pullulan solution was refilled in each analysis ($n = 10$). The calibration plots of the integrated peak areas at 214 nm *vs.* concentration of standard proteins were linear in the range 5 μg/ml–0.1 mg/ml.

1. Introduction

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) has been used for the separation and molecular mass determination of polypeptides and proteins [1]. However, it is time consuming, detection is tedious and there are difficulties in quantification. SDS capillary gel electrophoresis (SDS-CGE) has several advantages over slab gel electrophoresis in that a high-intensity electric field can be applied, resulting in high resolution, and on-column detection can be employed, thus permitting high-speed separations and real-time detection.

SDS-CGE has been achieved using cross-linked polyacrylamide [2,3], linear polyacrylamide [4–7] and other polymers [8–10]. Although proteins can be detected at a wavelength of 200 nm where they have strong UV absorption, cross-linked and/or linear polyacrylamide also has strong UV absorption below 230 nm. Therefore, UV-transparent polymer matrices such as dextran and poly(ethylene glycol) have been used [9]. In addition, these water-soluble polymer solutions can be easily refilled into the capillary because of the low viscosity. However, the low viscosity causes gradual extrusion of the matrices out of the capillary by the electroosmotic pressure generated on the untreated capillary surface. To overcome these difficulties, efforts

* Corresponding author.

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have been made to inactivate silanol functions by coating the capillary with hydrophilic polymers, which were covalently bonded to silanol groups through siloxane linkages (Si–O–Si–C) [11–17]. This treatment was effective in reducing the zeta potential and in preventing the adsorption of proteins. However, the siloxane linkages are prone to hydrolysis under alkaline conditions [19]. This paper describes the durability of coatings under alkaline conditions, and demonstrates the separation of SDS-proteins in a hydrolytically stable capillary using pullulan solution as a sieving matrix.

2. Experimental

2.1. Materials

Acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium peroxodisulphate (APS), tris(hydroxymethyl)aminomethane (Tris) of electrophoretic grade, thionyl chloride, tetrahydrofuran (THF), pullulan (claimed molecular mass $M_r = 50\,000$–$100\,000$), which is a branched polysaccharide composed of α-(1–6)-linked maltotriose (Fig. 1), 2-(cyclohexylamino)ethanesulphonic acid (CHES), 2-(N-morpholino)ethanesulphonic acid (MES), sodium dodecyl sulphate (SDS) and 2-mercaptoethanol of analytical-reagent grade were obtained from Wako (Osaka, Japan). Mesityl chloride and vinylmagnesium bromide (1 *M* solution in THF) were obtained from Tokyo Kasei (Tokyo, Japan). 3-Methacryloxypropyltrimethoxysilane (MAPS) was purchased from Shin-etsu Chemicals (Tokyo, Japan). The buffer solution for SDS-CGE consisted of 0.1 *M* Tris–CHES and 0.1% (w/v) SDS (pH 8.7). Standard proteins were obtained from Sigma (St. Louis, MO, USA).

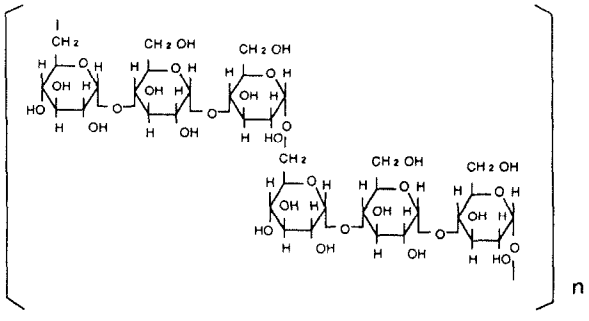

Fig. 1. Structure of pullulan.

2.2. Apparatus

A CE-800 capillary electrophoresis instrument (Jasco, Tokyo, Japan) was used with a fused-silica capillary of 50 μm I.D. for assessment of the durability of coatings and a fused-silica capillary of 75 μm I.D. for SDS-CGE (GL Sciences, Tokyo, Japan). Capillary electrophoresis was performed at room temperature without temperature control. The UV absorbance of polymer solutions was measured on a Shimadzu (Kyoto, Japan) UV-160A spectrophotometer.

2.3. Procedures

Preparation of linear polyacrylamide-coated capillary through siloxane linkage

A detection window was first opened by burning off about a 2 mm length of polyimide coating on a fused-silica capillary at an appropriate position, and the window was protected by covering it with a small plastic tube.

The inner surface of the capillary was treated with 1 *M* NaOH for 1 h at room temperature followed by washing with distilled water for 1 h, then reacted with MAPS as described by Hjertén [15]. After reaction, the capillary was washed with distilled water, filled with an aqueous solution containing 3% acrylamide, 0.1% (w/v) APS and 0.1% (v/v) TEMED, and kept at 28 ± 2°C for 1 h for polymerization. Water was then passed through the capillary by suction until only water came out.

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

The capillary with the detection window was coated with linear polyacrylamide through Si–C linkages by four-step reactions according to the following procedure, which was a simplified version of that employed by Cobb *et al.* [18].

The capillary was first treated with 1 *M* NaOH at room temperature for 1 h, rinsed with distilled water and dried at 110°C by flushing with nitrogen for 6 h. Thionyl chloride was passed through the dried capillary for several minutes using a suction pump. The capillary was sealed at both ends using a small propane torch and kept at 70°C for 6 h. After reaction, the seal was opened, and nitrogen was passed through the capillary for 5 min to remove excess thionyl chloride.

A 0.25 *M* vinylmagnesium bromide solution in THF was introduced into the capillary by suction, and the capillary was sealed again to complete the Grignard reaction. After the reaction at 70°C for 6 h, the seal was opened and the capillary was rinsed with THF for several minutes, then with distilled water. An aqueous solution containing 3% (w/v) acrylamide, 0.1% (w/v) APS and 0.1% (v/v) TEMED was filled into the capillary by suction and kept at 28 ± 2°C for polymerization. After 1 h, distilled water was passed through the capillary in the manner described above.

Preparation of pullulan matrix-filled capillary

A stock standard solution of pullulan was prepared by dissolving a weighed amount of pullulan in the running buffer containing 0.1 *M* Tris–CHES and 0.1% (w/v) SDS (pH 8.7). The solution was degassed by aspiration for 5 min and then filled into the capillary coated with linear polyacrylamide through Si–C linkages by means of suction or with a micro-syringe.

Assessment of durability of coated capillaries under alkaline conditions

The capillaries (50 μm I.D. × 50 cm, effective length to the detector 30 cm) coated with linear polyacrylamide through Si–O–Si–C linkages or Si–C linkages were filled with 50 m*M* sodium tetraborate buffer (pH 9.2) and kept at room temperature for up to 30 days. After exposure to alkaline conditions for a given number of days, the electroosmotic flow-rates were measured in a running buffer of 20 m*M* sodium tetraborate (pH 9.2) using mesityl oxide as a neutral marker with an applied potential of 400 V/cm.

Protein samples for SDS electrophoresis

A mixture of standard proteins, containing *ca.* 0.1 mg/ml of the individual proteins, was dissolved in an aqueous solution containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol. The sample solution was incubated at 80°C for 15 min in a water-bath and submitted to analysis.

3. Results and discussion

3.1. Durability of coating under alkaline conditions

Fig. 2 illustrates the time courses of electroosmotic mobilities measured in the uncoated capillary and in the capillaries bonded with linear polyacrylamide through Si–O–Si–C linkages and through Si–C linkages, all of which had been kept under alkaline conditions (50 m*M* sodium tetraborate, pH 9.2). The coated capillary involving Si–C linkages showed a very low electroosmotic mobility even after exposure to alkaline conditions for 30 days, indicating high stability against hydrolysis. In contrast, the capillary

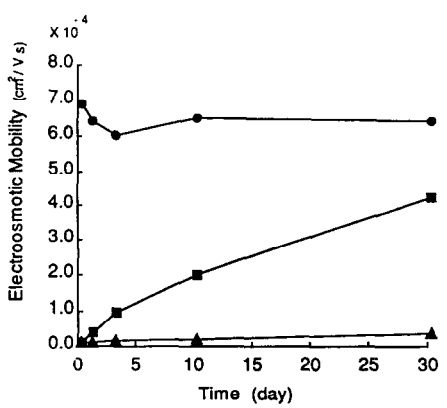

Fig. 2. Time course of electroosmotic mobility as a function of number of days of exposure under alkaline conditions [50 m*M* sodium tetraborate (pH 9.2)]. ● = Uncoated capillary; ■ = capillary coated with linear polyacrylamide through Si–O–Si–C linkages; ▲ = capillary coated with linear polyacrylamide through Si–C linkages. Capillary, 50 μm I.D. × 50 cm (30 cm to the detector); running buffer, 20 m*M* sodium tetraborate (pH 9.2); electric field, 20 kV 25 μA; injection, syphonic, 5 cm, 15 s; detection, UV at 250 nm; neutral marker, mesityl oxide (0.1%, v/v).

involving Si–O–Si–C linkages resulted in an increased electroosmotic mobility, although the initial value of the mobility was as low as that observed with the capillary involving Si–C linkages. After alkaline treatment for 30 days, the electroosmotic mobilities became $0.25 \cdot 10^{-4}$ $cm^2/V \cdot s$ (Si–C) and $4.13 \cdot 10^{-4}$ $cm^2/V \cdot s$ (Si–O–Si–C), while the mobility in the uncoated capillary remained as high as $6 \cdot 10^{-4}$–$7 \cdot 10^{-4}$ $cm^2/V \cdot s$ for 30 days. These results indicate that polyacrylamide coatings markedly decreased the zeta potential on the inner surface of the fused-silica capillary and that Si–C linkages are more stable than Si–O–Si–C linkages against hydrolysis. 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. It is important in capillary gel electrophoresis to reduce the electroosmotic flow in order to stabilize the gel. Therefore, the use of a coated capillary involving Si–C linkages is advantageous in optimizing separation conditions, because it widens the selection of separation media to soft gel or polymer solutions of low viscosity, and allows the use of running buffer solutions with a wide range pH.

3.2. UV absorbance of pullulan matrix

The UV absorbances of pullulan solution and linear polyacrylamide solution were measured using a spectrophotometer with a 1-cm light path length. The absorbance for 7% (w/v) pullulan in water was 0.399 at 214 nm and 0.136 at 280 nm, whereas the absorbance for the 3% (w/v) linear polyacrylamide in water was over 2.5 at 214 nm and 0.509 at 280 nm. These results indicate that pullulan is advantageous over linear polyacrylamide with detection in the lower wavelength region (below 230 nm), which gives a 20–50 times higher sensitivity for proteins than that at 280 nm [20]. Therefore, the use of a low wavelength is advantageous for the detection of low-concentration proteins, especially for the detection of small peptides lacking tyrosine and tryptophan residues.

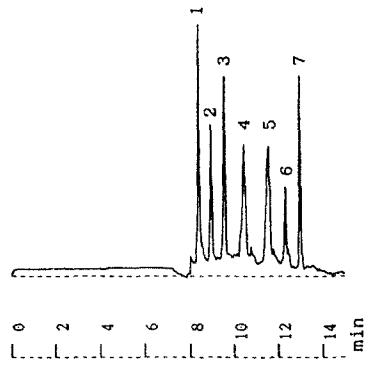

Fig. 3. Electropherograms of SDS-proteins on a 7% (w/v) pullulan solution-filled capillary. The inner wall of the capillary was coated with linear polyacrylamide through Si–C linkages. Capillary, 75 μm I.D. × 50 cm (30 cm to the detector); running buffer, 0.1 *M* Tris–CHES–0.1% (w/v) SDS (pH 8.7); electric field, −15 kV, 15 μA; injection, −5 kV, 5 μA, 5 s; detection, UV at 214 nm. Peaks: 1 = α-lactalbumin; 2 = trypsin inhibitor; 3 = carbonic anhydrase; 4 = ovalbumin; 5 = bovine serum albumin; 6 = phosphorylase b; 7 = β-galactosidase.

3.3. Separation of SDS-proteins

Fig. 3 shows the electropherogram of standard proteins separated in the 7% (w/v) pullulan solution-filled capillary which was coated with linear polyacrylamide through Si–C linkages, where the applied intensity of the electric field was −300 V/cm and UV detection at 214 nm was used. The proteins with molecular masses ranging from 14 400 to 116 000 were completely separated within 13 min with high efficiency; the theoretical plate number for peak 7 in Fig. 3, for example, was *ca.* 150 000. In contrast, no proteins were detected at the anodic end of the uncoated capillary filled with pullulan solution of the same concentration. This means that the electroosmotic flow overwhelmed the electrophoretic movement of SDS-proteins, which should be directed towards the anodic (detection) end. In fact, the peaks of proteins were observed when the polarity of the power supply was reversed, while the pullulan solution was gradually extruded because of electroosmotic flow.

Table 1 gives the statistical data for the run-to-run reproducibility of migration times deter-

Table 1
Reproducibility of migration times using 7% (w/v) pullulan

Peak No.	Protein	Molecular mass	Migration time (min)[a] (mean ± S.D., $n = 10$)
1	α-Lactalbumin	14 400	8.54 ± 0.031
2	Trypsin inhibitor	20 100	9.16 ± 0.024
3	Carbonic anhydrase	30 000	9.83 ± 0.023
4	Ovalbumin	43 000	10.70 ± 0.024
5	Bovine serum albumin	67 000	11.88 ± 0.041
6	Phosphorylase b	94 000	12.63 ± 0.036
7	β-Galactosidase	116 000	13.31 ± 0.035

[a] Separation conditions: the inner wall of the capillary was coated with linear polyacrylamide through Si–C linkages; capillary, 75 μm I.D. × 50 cm (30 cm to the detector); running buffer, 0.1 *M* Tris–CHES–0.1% (w/v) SDS (pH 8.7); electric field, −15 kV, 15 μA; injection, −5 kV, 5 μA, 5 s; detection, UV at 214 nm.

mined by the repeated injection of an SDS-protein into a 7% (w/v) pullulan solution-filled capillary which we had coated with linear polyacrylamide through Si–C linkages.

Ten consecutive injections were performed by refilling the capillary with the matrix after every analysis. As a result, the relative standard deviations of the migration times averaged over seven proteins were less than 0.5%. This excellent reproducibility was obviously attributable to the suppression of electroosmotic flow and the stabilization of the separation matrix. Even after the coated capillary had been used for more than 100 injections during 1 month (the pullulan solution was replaced with water overnight), the electroosmotic mobility measured using 50 m*M* Tris–MES buffer (pH 7.0) remained constant at $1.04 \cdot 10^{-5}$ cm^2/V·s. This value is considerably lower than that measured with the uncoated capillary ($3.13 \cdot 10^{-4}$ cm^2/V·s).

The column-to-column reproducibility of migration times of seven proteins ($n = 3$) was below 1.3% (R.S.D.).

Fig. 4 shows the relationship between the electrophoretic mobility and the logarithm of the molecular mass of standard proteins on the coated capillary involving Si–C linkages at five different concentrations (1, 3, 5, 7 and 10%, w/v) of pullulan solutions. Excellent linearity (correlation coefficient $r^2 > 0.995$) was obtained in each relationship and the lines were almost parallel to each other in the range of pullulan concentrations examined. Ferguson plots [21] exhibited linear relationships ($r^2 > 0.993$) for each of seven proteins between log (mobility) and pullulan concentration. The slopes of the plots were found to be proportional to the molecular mass of the proteins ($r^2 = 0.960$). These results indicated that the separation was achieved by the difference in size or molecular mass of the proteins based on the sieving effect.

The linear range of the calibration lines for α-lactalbumin and carbonic anhydrase was examined using a 5% (w/v) pullulan solution-filled capillary which was coated with linear poly-

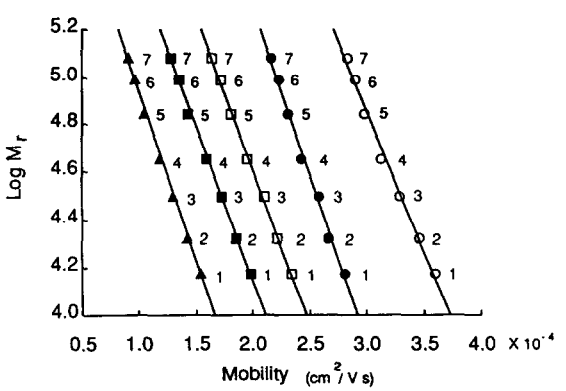

Fig. 4. Plots of log (molecular mass) *vs.* mobility of proteins measured in the capillary containing (○) 1, (●) 3, (□) 5, (■) 7 and (▲) 10% (w/v) pullulan solution. The capillary was coated with linear polyacrylamide through Si–C linkages. 1 = α-Lactalbumin; 2 = trypsin inhibitor; 3 = carbonic anhydrase; 4 = ovalbumin; 5 = bovine serum albumin; 6 = phosphorylase b; 7 = β-galactosidase.

acrylamide through Si–C linkages, the applied intensity of the electric field being −300 V/cm, the length of capillary 50 cm (30 cm to the detector) and UV detection at 214 nm was used. The concentration of the proteins in the buffer solution was varied, while electrokinetic injection was achieved at a constant electric field of −100 V/cm for 15 s. The plot of peak area *vs.* sample concentration was linear ($r > 0.999$) up to at least 5 μg/ml and passed through the origin. The precision for the integrated peak area was examined by repeated injection ($n = 3$). The R.S.D. of the peak area was less than 2.5% for α-lactalbumin ranging over 25 μg/ml and less than 5% for carbonic anhydrase ranging over 5 μg/ml. The minimum detectable concentration (signal-to-noise ratio = 4) for both proteins was 1 μg/ml under the given injection conditions.

4. Conclusions

The Si–C bondings which link linear polyacrylamide to silanol groups on a fused-silica capillary are more effective than siloxane bondings in stabilizing the inner surface of a fused-silica capillary. The capillary generated essentially no electroosmotic flow even under alkaline conditions, and hence the soft gel or a solution of pullulan can be used as a separation medium for the capillary electrophoresis of SDS-proteins. This serves to extend the applicability to the separation of a wider range of macromolecules.

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