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Improving the UV detection sensitivity of condensed polyacrylamide gel-filled capillaries using non-flow buffer-filled capillaries as a detection cell

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Abstract

Capillary coupling techniques are suggested for the connection of a non-flow buffer-filled capillary to gel-filled capillaries as a low-background detection cell. The UV detection sensitivity of the resulting capillaries is improved at least fivefold, as demonstrated by using polyamino acids as test samples. The detection sensitivity can be further improved by using a low-background running buffer. Several ways are suggested to overcome the problems of resolution decrease and baseline drift which were observed after coupling.

1. Introduction

Highly condensed polyacrylamide gel-filled capillaries are essential in the separation of polyamino acids [1] and oligosaccharides [2–4], but these capillaries yield very poor UV detection sensitivity because the gels show strong UV absorption which increases with decrease in the detection wavelength and increase in gel concentration. To improve the detection sensitivity, laser-induced fluorescence (LIF) methods have been suggested and demonstrated to be extremely sensitive [1–4]. However, the methods can only be used with samples that are fluorescent or can be labelled by fluorescent agents. In addition, the construction of the detector is expensive compared with UV detectors. It is therefore desirable to use UV detection as an inexpensive, universal detection method, but the background of the capillaries should be greatly reduced. The best way to reduce the background is to use a buffer-filled detection cell. Dovichi and co-workers [5,6] developed an off-line detection cell, a sheath flow cell, to improve further the detection sensitivity of LIF. It also seems possible to use a non-flow buffer-filled detection cell. This detection cell can be simply constructed by the capillary coupling technique, which is fairly frequently used in capillary free-solution electrophoresis.

In this paper, we discuss a method for the rapid connection of a low-background detection cell to gel-filled capillaries. The detection sensitivity and performance, including the problem

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of baseline drift, of the resulting capillaries were investigated using polyamino acids as test samples.

2. Experimental

2.1. Materials

Tricine [N-tris(hydroxymethyl)methylglycine], bicine [N,N-bis(2-hydroxyethyl)glycine], HEPES [N-(2-hydroxyethyl)-N′-(2-ethanesulfonic acid)], MES [2-(N-morpholino)ethanesulfonic acid], Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] and boric acid (biological buffers), γ-methacryloxypropyltrimethoxysilane, poly(Asp) [poly-(α,β)-D,L-aspartate sodium salt, M_r (average molecular mass detected by viscosity) = 6850], poly(Glu) [poly-L-glutamate sodium salt, M_r = 14 300] and poly(Lys) (poly-L-lysine, M_r = 3970) were purchased from Sigma (St. Louis, MO, USA). Acrylamide and Bis [N,N′-methylenebis(acrylamide)], electrophoretically pure, were obtained from Bio-Rad Labs. (Richmond, CA, USA). TEA (triethanolamine), Tris [tris(hydroxymethyl)aminomethane], TEMED (N,N,N′,N′-tetramethylethylenediamine), APS (Ammonium peroxodisulfate) and other chemicals were all of analytical-reagent grade from Merck (Darmstadt, Germany). All fused-silica capillaries were purchased from Composite Metal Services (Worcestershire, UK). Water purified with a Millipore Super Q system was used throughout.

2.2. Preparation of cross-linked gel-filled capillaries

All the capillaries with one-end immobilized cross-linked gels were prepared according to our previously developed method [7]. One end of a new capillary is dipped into a 0.5% (v/v) modification solution of γ-methacryloxypropyltrimethoxysilane in acetic acid–methanol (1:250) [5–8] until the solution reaches a height of 5 cm from the dipped end. The capillary is kept at room temperature for 10–20 min and then filled from the end without the silane solution with a degassed gelling solution [15–20%T + 5%C[1] prepared in 0.1 *M* tricine–0.05 *M* Tris containing 0.05% (v/v) TEMED and 0.05% (w/v) APS for the initiation of polymerization]. The polymerization is started from the silanized end of the capillary by dipping the tip in hot water of 50–70°C for about 1 min. The other end of the capillary is plugged into a sealed vial [7], which is then injected with 3–4 ml of ice-cooled water to build up pressure and also to slow the polymerization rate at this end. The capillary is hung vertically for about 4 h in a shockless and draught-free location at 15–25°C, with the pressurized end up and the heated end in water at 25–35°C. After hanging, about a 2-cm length of the cooled end of the capillary is cut off. The other (heated) end is checked under a microscope with fivefold magnification for possible voids, which should be cut off. The capillary is then stored at room temperature by dipping both ends into tricine–Tris buffer. When using these capillaries for electrophoresis, the end with the immobilized gel should be applied with a negative voltage to improve the running stability of the gels, that is, for the separation of anionic samples, the end of the capillaries with the gel not immobilized is used for sample injection, but for the separation of cationic samples, the immobilized end is used for sample injection.

2.3. Preparation of linear gel-filled capillaries

A new capillary is filled with the above-mentioned silane solution and kept for 20 min. This capillary is then continuously filled with a degassed 5%T solution containing 0.05% TEMED and 0.05% APS, using a water vacuum pump, until the solution stops flowing (30–60 min). The capillary is stored as described in Section 2.2.

[1] %T is the total grams of acrylamide and Bis in 100 ml of solution and %C is the grams of Bis in 100 g of the total monomers; in the case of linear gels, %C is neglected.

2.4. Coupling of buffer-filled capillaries with gel-filled capillaries

Coating the capillaries [8]

A capillary is sequentially washed with 1 *M* NaOH, water and methanol for 30 min each. The silane solution mentioned above is filled into the capillary, which is kept for 1 h. The silanized capillary is washed with methanol and water for 20 min each and then filled with 2%T + 3%C solution containing 0.1% TEMED and 0.1% APS. After 30 min of polymerization, the cross-linked gelling solution is replaced with 3.5%T linear gelling solution (containing the same amount of TEMED and APS), and the capillary is kept for at least 1 h. The coated capillary is washed with water for 20 min and dried with an air flow for more than 20 min using a water vacuum pump. The coated capillary can be stored at room temperature for 6 months.

Linearly coupled capillaries

There are two possibilities for connecting linearly a piece of buffer-filled capillary to a gel-filled capillary: (1) a buffer-filled capillary is simply connected to the outlet of a gel-filled capillary (Fig. 1A, type A); (2) the buffer-filled capillary is inserted between gel-filled capillaries (Fig. 1A, type B). To prepare such capillaries with an O.D. of 375 μm, connectors are needed. For easy manipulation, a wide connector of 1 cm × 0.53 mm I.D. cut from a coated, flexible capillary is used. There is hence a wide gap between the capillary and the connector, which should be sealed. It is crucial, during coupling, to prevent air bubbles from being introduced into the connector and other parts of the buffer-filled capillaries. To avoid bubbles, the connector and the detection capillary are filled with a newly degassed buffer and then immersed in the buffer just before coupling [Fig. 1C, (b) and (c)]. It is also important to fix the connected part of the capillaries, which can easily be achieved by coating the connected parts with a quick-drying glue (a cyanoacrylate with UHU Vertrieb, Bühl, Germany, was used). The detailed coupling steps are as follows.

(1) To form a sealing membrane, one end of the capillaries to be connected is carefully coated with Pattex (Henkel, Düsseldorf, Germany) and kept for about 5 min. If the capillary has been filled with a gel or a buffer, the end should be covered with a drop of buffer to prevent it from drying out [Fig. 1C, (a) 3]. If the capillary is

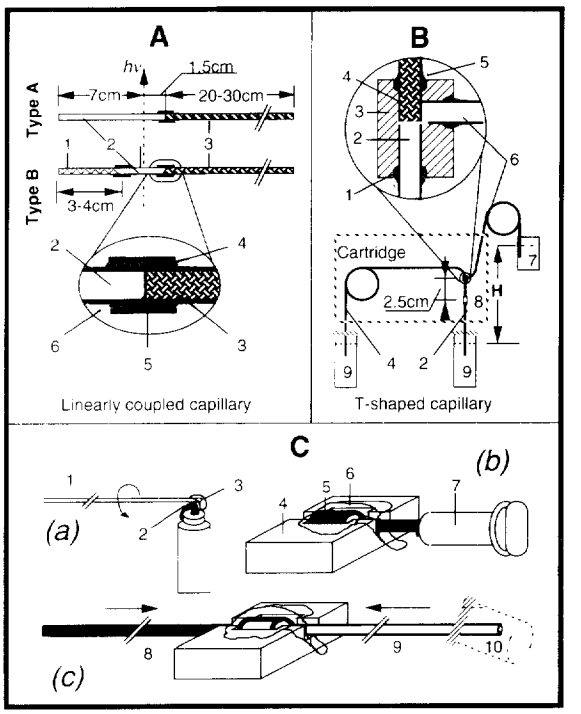

Fig. 1. (A) Structure of linearly coupled capillaries. 1 = Capillary filled with 5%T gel; 2 = buffer-filled capillary for detection; 3 = gel-filled capillary used for separation; 4 = Pattex (for sealing); 5 = connector; 6 = fixative. (B) Structure and the electrophoretic position of a T-shaped capillary. 1 = Pattex; 2 = detection capillary; 3 = connector; 4 = gel-filled capillary; 5 = fixative; 6 = buffer connection capillary (40 cm × 100 μm I.D.); 7 = buffer supply; 8 = detection window; 9 = electrode reservoirs. (C) Some important steps for connection of buffer-filled capillaries with gel-filled capillaries. (a) Coating the end of a gel- or buffer-filled capillary with Pattex; (b) holding and filling a connector; (c) a gel-filled capillary and a buffer-filled capillary are ready for plugging; the arrows show the plugging direction. 1 = Capillary; 2 = Pattex; 3 = buffer drop; 4 = holder made of glass or Perspex; 5 = connector; 6 = buffer (covering the connector totally); 7 = syringe; 8 = gel-filled capillary; 9 = buffer-filled capillary; 10 = sealed vial for filling, which can be removed just before the capillary is plugged into the connector.

empty, it is first coated with Pattex and then filled with a newly degassed buffer using a pressure method. We prefer to use a sealed vial [7]: the untreated end of the capillary is plugged into the sealed vial (with buffer), which is then pressurized by injection of 1–5 ml of air. The buffer is kept flowing until the coated end is immersed in a buffer or slipped into the connector [Fig. 1C, (c) 10].

(2) The connector is filled with the degassed buffer and immersed in the buffer. There are different ways to fill the connector and to protect or cover it with buffer. Fig. 1C, (b) shows a method used in our laboratory where the holder is made of glass or Perspex, with a chamfer for holding the connector. The buffer is injected into the connector until it flows out of the connector and covers the whole connector using a syringe or other tool.

(3) The Pattex-coated ends of the buffer-filled capillary and the gel-filled capillary are first plugged into the buffer outside the connector [the sealed vial at the end of the buffer-filled capillary, Fig. 1C, (c) 10, can now be taken off] and then plugged oppositely into the connector until the tips meet in the middle part of the connector. The connected capillaries are held manually, the buffer outside the connector is cleaned with filter-paper and the connected part is then coated with the quick-drying glue mentioned above. The fixative needs 2–5 min to dry out. It should be mentioned that the buffer used during coupling is generally 0.1 *M* tricine–0.05 *M* Tris. Some buffers, such as boric acid, dramatically decrease the adherence of the fixative.

(4) The detection window is opened at the buffer part, 1.5 cm from the tip of the separation gel (Fig. 1A). About 2 mm of the coating are manually removed using a scalpel and further cleaned with methanol.

T-shaped coupling

The construction and the electrophoretic position of a T-shaped capillary is shown in Fig. 1B. The connector (ca. $1.2 \times 0.7 \times 0.3$ cm) is made of Perspex with a T-tunnel of diameter 0.4 mm. Similarly to linear coupling, the connector is filled and covered with the degassed buffer and then mounted simultaneously with two pieces of buffer-filled capillaries and a piece of a gel-filled capillary. The capillary end is also coated with Pattex for sealing and the connected parts are fixed using the same quick-drying glue. The detection window is opened at the detection capillary, 2.5 cm from the gel tip (Fig. 1B, 8).

2.5. Electrophoresis

Before injection, all the capillaries were equilibrated with running buffer at 25°C and 200 V/cm for 60 min. When the running buffer was different from that used for the preparation of gelling solution (0.1 *M* tricine–0.05 *M* Tris), the capillaries were equilibrated with the running buffer for at least 2 h until the current became stable. The running buffer was renewed three times during equilibration, but during separation it was renewed after every run. Electrophoresis was performed using a Beckman P/ACE system 2100 controlled by an IBM SP/2 computer with System Gold software (version 7.0). The cartridges were laboratory-made so that different coupled capillaries could be mounted without breakage. The aperture mounted was 100×200 (axial direction) μm. The data sampling rate was 1 Hz and the rise time was 1 s. The sample was introduced into the capillary by dipping the injection end into the sample solution for 15–30 s (diffusion injection [9]). Because the response time of the software or the CE system requires 14 s to start a run (from the time of starting the method to the beginning of voltage application), the injection time should be least 14 s. Generally, the run should be started at the injection time minus 14 s after the capillary is dipped into the sample solution.

3. Results and discussion

3.1. Detection sensitivity

When using highly condensed gel-filled capillaries for the separation of polyamino acids, the optimum UV detection sensitivity is reached at

220 nm [1]. If the wavelength is shorter than 214 nm, no peaks can be detected. However, even at 220 nm, the sensitivity is very poor, as shown in Figs. 2A and 3A. In contrast, with a buffer-filled capillary as a detection cell, the coupled capillaries (types A and B) allow the use of a short-wavelength UV radiation for detection and a high detection sensitivity is obtained at 200 nm, which is the shortest wavelength available using our CE system. Both Figs. 2B and 3D show that, at 200 nm, the coupled capillaries (with the same bore as the detection cell) increase the peak height of poly(Asp) at least fivefold compared with the gel-filled capillaries at 220 nm (Figs. 2A and 3A). The sensitivity is further improved by coupling a wider capillary to the gel-filled capillaries (Fig. 2B–D), but at the cost of lower resolution. For maintaining the resolution, it is better to use a detection capillary slightly thinner than the separation capillary (Fig. 3C). If the detection capillary is too narrow, both the resolution and detection sensitivity will be reduced, as shown in Fig. 3B.

The detection sensitivity of the coupled capillaries also depends on running buffer, similarly

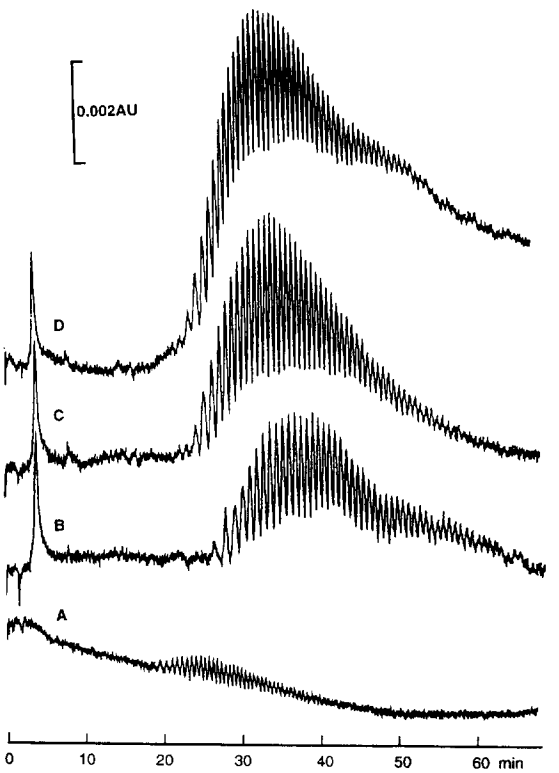

Fig. 2. Comparison of the detection sensitivity of a 15%T + 5%C gel-filled capillary (A) without detection cell and with (B–D) untreated buffer-filled detection cell (type A). A sample of poly(Asp) in water (50 mg/ml) was injected by diffusion into the capillary. Electrophoreses was performed at constant current (4.4 μA) and monitored at (A) 220 nm or (B–D) 200 nm. Detection cells were filled with 0.1 *M* tricine–0.05 *M* Tris. The following capillaries were used: (A) 30/37 cm (separation/total length) × 50 μm I.D.; (B) 32 cm × 50 μm I.D. (gel) + 8.5 cm × 50 μm I.D. (buffer); (C) 32 cm × 50 μm I.D. (gel) + 8.5 cm × 75 μm I.D. (buffer); (D) 32 cm × 50 μm I.D. (gel) + 8.5 cm × 100 μm I.D. (buffer).

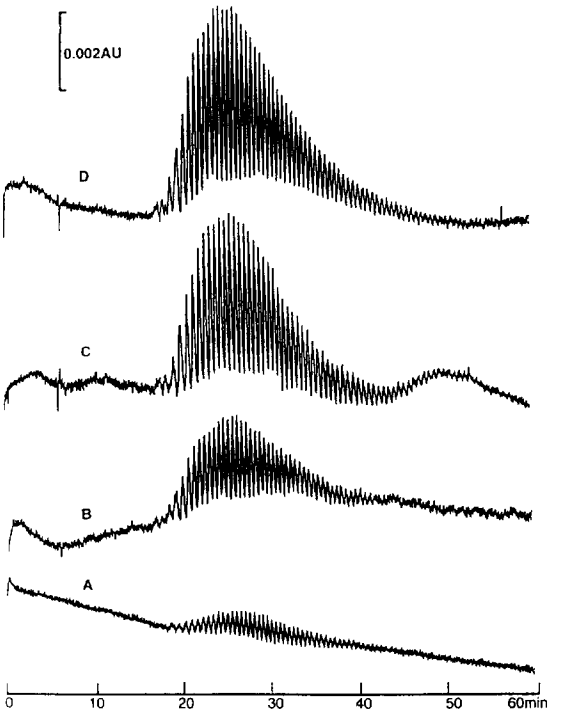

Fig. 3. Comparison of the detection sensitivity and performance of a 15%T + 5%C gel-filled capillary (A) without detection capillary and (B–D) with coated detection capillaries (type B). Electrophoresis was performed at 16 μA with the following capillaries: (A) 30/37 cm (separation/total length) × 100 μm I.D.; (B) 28.5 cm × 100 μm I.D. (gel) + 5.5 cm × 50 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel); (C) 28.5 cm × 100 μm I.D. (gel) + 5.5 cm × 75 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel); (D) 28.5 cm × 100 μm I.D. (gel) + 5.5 cm × 100 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel). Other conditions as in Fig. 2.

to free-solution capillary electrophoresis. Fig. 4A, B, D and G show that the peak height of poly(Lys), which is positively charged, may be increased another fivefold when a low-background running buffer is used such as Bis-Tris–HCl or Bis-Tris–H_3PO_4. The overall improvement of the detection sensitivity is greater than one order of magnitude in this case.

3.2. Resolution

The resolution will be reduced after coupling since an interface and/or a dead volume exist inside the connected part. To improve the resolution, we first used the tube type A with an untreated (bare) capillary as the detection cell. For the separation of anionic samples, the bare detection capillaries will induce an electroosmotic sheath flow, that is, the buffer flows towards the gel end in the layer near the capillary wall and returns to the electrode reservoir from the centre of the tube. However, although the detection sensitivity is dramatically improved, the resolution losses are greater than 20% (Fig. 2B–D). The reasons are no clear. It seems that the interaction of the solutes with the capillary wall may account for this phenomenon because the baseline generally rises when or after the peaks emerge (see Fig. 2D). It is also possible that the electroosmotic sheath flow causes mixing of the separated peaks. We then turned to the tube type B with coated detection capillaries or non-flow detection cells. To decrease the influence of the interface and the dead volume, wider capillaries of 75–100 μm I.D. were used. Fig. 3B–D show that, after the detection capillary has been coated, the system peaks (before 10 min in both Figs. 2 and 3, which can be observed without the injection of samples) are greatly reduced, the separation time is similar to that with the gel-filled capillary and the resolution losses are less than 10%, depending on the bore of the detection capillaries. Some part of the resolution losses in Fig. 3B–D is caused by the shorter separation gels used.

Interestingly, the buffer distance between the gel tip and the detection window, ranging from 0.6 to 5 cm, only slightly influences the resolution (but influences the separation time). In the separation of poly(Lys) with the linearly coupled capillaries, we found that the running buffer greatly influenced the resolution. The use of tricine–Tris as a running buffer, which is an excellent buffer for the separation of anionic polyamino acids, yielded no or an extremely poor separation of the poly (Lys) (Fig. 4A and D), often accompanied by spikes, which implies that this buffer might induce aggregation of the sample. Some separation was observed when the Tris as the buffer was replaced with Bis-Tris (Fig. 4E) and the separation was improved by using Bis-Tris–MES buffer at pH 5.5–6.5 (compare Fig. 4E and F). Interestingly, the counter ions of the buffer (having opposite charge to the solutes) greatly affect the resolution of poly-(Lys). Fig. 4B and G show that chloride reduces the resolution whereas phosphate increases the resolution compared with MES (Fig. 4C and F). These phenomena mean that, when using the coupled capillaries for the separation of cationic polyamino acids, it is possible to improve the resolution by optimizing the running buffer and pH, but further investigations should be made.

In contrast, the pH (>7) and the components of running buffer have only a limited influence on the separation of anionic polyamino acids when using the coupled capillaries. Various organic buffers such as tricine, bicine, HEPES and MES with Tris, TEA or Bis-Tris as the counter ions yield similar resolutions of poly(Asp) and poly(Glu) (data not shown). However, some inorganic buffers such as borate yield no or very broad peaks of the anionic polyamino acids.

The simplest way to improve the separation is to increase the gel concentration (compare Fig. 4F and C), but the separation time will also be increased dramatically. For the separation of the above-mentioned polyamino acids, a gel of 20%T + 5%C is adequate.

3.3. Baseline drift

A problem in using linearly coupled capillaries is that the baseline drifts after the capillaries have been run for several hours. The drift may be greater than 0.01 absorbance per hour at 200

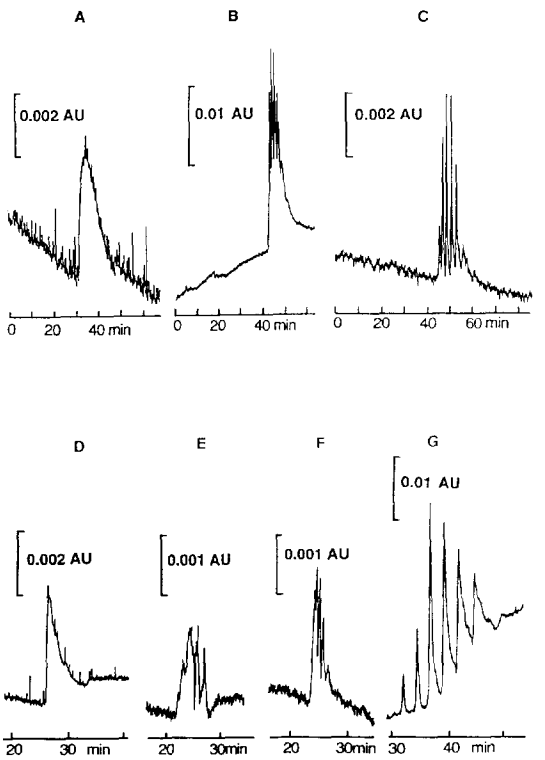

Fig. 4. Effects of pH and buffer composition on the detection sensitivity and resolution of poly(Lys) with a type B capillary. A sample of poly(Lys) in water (50 mg/ml) was injected by diffusion (15 s) into the capillaries and detection was effected at 200 nm. Different capillaries and running conditions were compared. (A) Capillary, 24 cm × 75 μm I.D. (20%T + 5%C gel) + 5.5 cm × 100 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel); buffer, 0.1 *M* Tricine–Tris (pH 7.7); conditions, 5 kV/6.8 μA. (B) Capillary, 24 cm × 75 μm I.D. (20%T + 5%C gel) + 5.5 cm × 100 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel); buffer, 0.1 *M* Bis-Tris–HCl (pH 6.0); conditions, 4.5 kV/12 μA. (C) Capillary, 24 cm × 75 μm I.D. (20%T + 5%C gel) + 5.5 cm × 100 μm I.D. (buffer) + 3 cm × 100 μm I.D. (5%T gel); buffer, 0.05 *M* Bis-Tris–0.1 *M* MES; conditions, 7 kV/8 μA. (D) Capillary, 30 cm × 75 μm I.D. (15%T + 5%C gel) + 5.5 cm × 75 μm I.D. (buffer) + 3 cm × 75 μm I.D. (5%T gel); buffer, 0.1 *M* tricine–Tris (pH 7.7); conditions, 6.5 kV/8.6 μA. (E) Capillary, 30 cm × 75 μm I.D. (15%T + 5%C gel) + 5.5 cm × 75 μm I.D. (buffer) + 3 cm × 75 μm I.D. (5%T gel); buffer, 0.1 *M* Bis-Tris–tricine (pH 7.7); conditions, 6.5 kV/ca. 3 μA. (F) Capillary, 30 cm × 75 μm I.D. (15%T + 5%C gel) + 5.5 cm × 75 μm I.D. (buffer) + 3 cm × 75 μm I.D. (5%T gel); buffer, 0.05 *M* Bis-Tris–0.1 *M* MES; conditions, 6 kV/7.7 μA. (G) Capillary, 30 cm × 75 μm I.D. (15%T + 5%C gel) + 5.5 cm × 75 μm I.D. (buffer) + 3 cm × 75 μm I.D. (5%T gel); buffer, 0.05 *M* Bis-Tris–H_3PO_4 (pH 6.0); conditions, 7 kV/12 μA.

nm. This phenomenon is possibly due to the unbalanced migration of the buffer ions from the gel to the buffer or vice versa, as the applied electric field across the capillaries is discontinuous. This can be demonstrated by using T-shaped capillaries. The T-shaped capillaries can suppress the unbalanced migration because of the buffer flow from the upper capillary (Fig 1B, 6) to the detection capillary (Fig. 1B, 2). Fig. 5 shows a comparison between T-shaped and linearly coupled capillaries. We found that the baseline stability of the T-shaped capillaries depended on the hydrodynamic height or the difference in the buffer levels between vial 7 and the electrode reservoir 9 (*H* in Fig. 1B). When *H* was between 0 and 2 cm, baseline drift occurred, but when *H* was greater than 10 cm, the baseline became stable. Unfortunately, although the coupled T-shaped capillaries can stabilize the baseline by increasing the value of *H*, the detection sensitivity is dramatically reduced because the separated bands will be diluted when they pass through the detection capillary. To maintain a moderate detection sensitivity, the gel-filled capillary should be at least as wide as 75 μm I.D. and *H* should be less than 15 cm. If *H* is between 10 and 15 cm, the detection sensitivity of the T-shaped capillary is reduced to about 50% compared with the linearly coupled capillary. The peak height shown in Fig. 5B is higher than that in Fig. 5A. The reasons are that, first, the

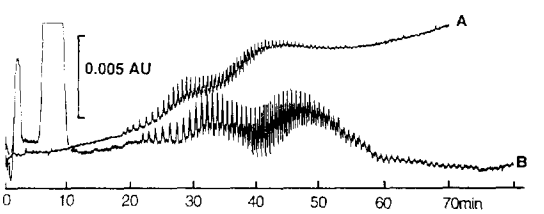

Fig. 5. Comparison of the baselines with differently coupled capillaries. A sample of (A) 50 or (B) 100 mg/ml of poly(Glu) in water was injected by diffusion (30 s) into the capillary coupled to a 0.1 *M* tricine–0.05 *M* Tris buffer-filled detection cell. Detection was effected at 200 nm. (A) Linearly coupled capillary (type A), 30 cm × 50 μm I.D. (15%T + 5%C gel), with 8.5 cm × 50 μm I.D. detection capillary; conditions, 6 kV/ca. 4 μA. (B) T-shaped capillary, 30 cm × 100 μm I.D. (15%T + 5%C gel), with 9.5 cm × 100 μm I.D. detection capillary; conditions, 6 kV/ca. 15.5 μA.

capillary used to obtain Fig. 5B is wider than that for Fig. 5A, and second, the sample concentration in Fig. 5B is higher than that in Fig. 5A. Another problem in using the T-shaped capillary is that bubbles accumulate very easily inside the connector, disrupting the separation. The capillaries should be re-coupled in this case.

The baseline drift of the linearly coupled capillaries also depends on the running buffers, samples and detection wavelengths. The baseline drift at 220 nm is about 60% less than that at 200 nm. When the detection wavelength is set at 280 or 254 nm, the baseline becomes fairly stable. For the separation of poly(Lys), Bis-Tris–MES buffer yields a fairly stable baseline, but if the MES is replaced with phosphate or chloride, the baseline drifts positively (Fig. 4C–B and F–D). In the analysis of anionic polyamino acids with tricine–Tris as the running buffer, the baseline drifts positively but can be suppressed by replacing Tris with TEA. The baseline can further be stabilized if the detection capillary is inserted between gels (tube type B). Hence, the general approaches for overcoming the problem of baseline drift include (1) using a type B capillary and replacing the detection capillary when the baseline begins to drift substantially; the replacement or re-coupling takes about 10–15 min if the coated detection capillaries are at hand and a gel-filled capillary allows more than ten re-couplings; (2) carefully selecting the components of the running buffer; (3) using a longer detection wavelength whenever possible. In addition, an electronic approach is to subtract a baseline obtained just before or after the separation from the electropherograms. This is possible by using the System Gold software.

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