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Influence of pH on the migration properties of oligonucleotides in capillary gel electrophoresis

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ABSTRACT

The effect of pH on the electrophoretic migration properties of single-stranded oligodeoxyribonucleotides in capillaray gel electrophoresis was investigated. Different homooligodeoxyribonucleotides of equal chain length showed significant differences in relative migration when the pH of the gel buffer was varied from pH 6 to 8, parallel with the running buffer. A similar variation in migration order was observed during the electrophoretic equilibration of a pH 8 gel-filled capillary column with a pH 6 running buffer. In the latter instance, the current reached the new level after 20 min of electrophoretic equilibration with the pH 6 running buffer. However, it was observed that the migration order characteristic of the pH 6 gel was achieved only after 4 h of electrophoretic equilibration. To avoid this time-consuming equilibration process, these results suggest that gel-filled capillary columns should be prepared with the same buffer (composition and pH) that will be used as the running buffer during the separations.

INTRODUCTION

An instrumental approach to electrophoresis, capillary electrophoresis, is currently being developed with the potential for automation and increasing importance as a separation tool in analytical biochemistry [1–4]. Using narrow-bore fused-silica capillaries filled with polyacrylamide gels, separations of biologically important molecules are possible with high performance [5–7]. At present, there is a great deal of interest in the separation and characterization of DNA molecules. We have previously demonstrated the high resolving power of gel-filled capillaries for the separation of oligonucleotides [8]. In continuation of previous work [9], a mixture of homodecamers was electrophoresed using running buffer systems with the same and different pH as the buffer used to prepare the gel, in order to determine its effect on the migration properties and the electrophoretic equilibration time.

EXPERIMENTAL

Apparatus

High-performance capillary gel electrophoresis was performed in fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) of 75 μm I.D. and 375 μm O.D. with column lenghts of 650 mm. The polyimide coating was carefully peeled off by means of a razor blade under a micro-

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scope along a 2 mm length at 150 mm from one end of the capillary. A 60-kV high-voltage d.c. power supply (Model PS/MK 60P00.5 × 66, Glassman, Whitehouse Station, NJ, USA) was used to produce the potential across the gel-filled capillary column. A UV detector (V-4, ISCO, Lincoln NE, USA) modified as decribed previously [7] was used at a wavelength of 260 nm. The detector slit was 0.1 × 0.1 mm. The tubing and the detector were cooled with ambient air using a fan. Each end of the capillary was connected to a separate 3-ml vial filled with the appropriate running buffer. Platinium wire electrodes were inserted in the two vials for connection to the electrical circuit. The electrophoretic data were acquired and stored on an IBM PC/AT computer.

Materials

The homodecamers polydeoxyadenylic [$p(dA)_{10}$], polydeoxycytidilic [$p(dC)_{10}$] and polydeoxythymidilic [$p(dT)_{10}$] acids were purchased from Pharmacia (Piscataway, NJ, USA). The samples were diluted to 20 μg/ml with water before injection and were stored at −20°C when not in use. Ultrapure, electrophoresis-grade acrylamide, N,N′-methylenebisacrylamide, Tris, boric acid, urea, ammonium peroxodisulfate and tetramethylethylenediamine (TEMED) were employed (Schwartz Mann Biotech, Cambridge, MA, USA). Methacryloxypropyltrimethoxysilane reagent was purchased from Petrarch Systems (Bristol, PA, USA). The buffer solutions were filtered through a Nylon GC filter unit of 0.2-μm pore size (Schleicher and Schuell, Keene, NH, USA) and carefully vacuum degassed.

Preparation of capillary gel columns

The fused-silica capillary column was filled with polyacrylamide gel of 3% monomer (acrylamide + N,N′-methylenebisacrylamide) and 0.15% cross-linker (N,N′-methylenebisacrylamide) in 0.1 *M* Tris–boric acid–7 *M* urea buffer adjusted to the appropriate pH between 6.0 and 8.0 by varying the concentration of boric acid. The polymerization reaction was driven to completion in 25–30 min in all instances in order to achieve a similar polymerization rate, *i.e.*, gel structure. This was accomplished by using increasing amounts of ammonium peroxodisulfate initiator and TEMED catalyst as the pH of the gel buffer was decreased. For stabilization, the polyacrylamide gel was covalently bound to the wall of the fused-silica capillary column by using a bifunctional reagent (methacryloxypropyltrimethoxysilane). An electric field of 300 V/cm was applied to the gel-filled capillary columns in all the experiments. In order to remove impurities from the polyacrylamide gel, the capillary column was pre-electrophoresed with the appropriate running buffer at 100, 200 and 300 V/cm for ten min each. The samples were injected electrokinetically on to the column by applying *ca.* 0.1 W s of power.

RESULTS AND DISCUSSION

Varying the pH of the gel buffer parallel to the running buffer

The effect of the pH of the gel buffer on the sep-

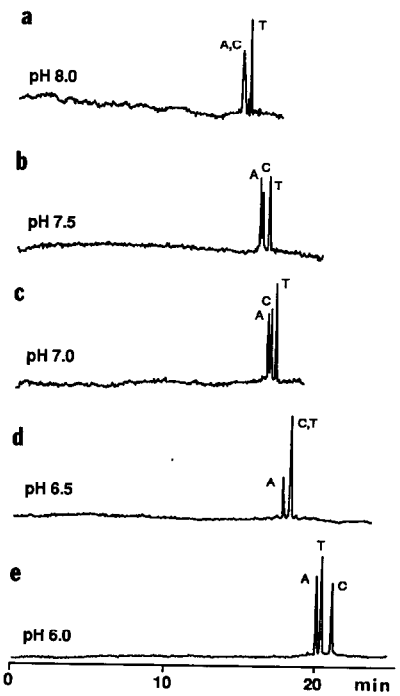

Fig. 1. Separations of the homodecamer test mixture by capillary gel electrophoresis at different pH values. All the gels were prepared at the same pH as the running buffer: (a) pH 8.0; (b) pH 7.5; (c) pH 7.0; (d) pH 6.5; (e) pH 6.0. Peaks: A = $p(dA)_{10}$; C = $p(dC)_{10}$; T = $p(dT)_{10}$. Conditions: isoelectrostatic, 300 V/cm; polyacrylamide gel, 3% monomer, 0.15% cross-linker, total column length, 650 mm (effective length from the injection point to the detector point, 500 mm); buffer, 0.1 *M* Tris–7 *M* urea, adjusted to the final pH with boric acid.

aration of the homodecamer mixture of $p(da)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$ was examined first. The omission of the $p(dG)_{10}$ from the test mixture was done on purpose in order to avoid possible self-association problems [10,11]. Fig. 1a–e compare the separations obtained when the pH of both the gel buffer and the running buffer were varied over the range 8.0–6.0. The peak-height differences observed at different pH values are caused by the pH-dependent UV absorbance of the nucleotide bases [12]. The identification of the homooligomers was accomplished by spiking with the individual compounds.

Fig. 1a shows the separation of the homodecamer test mixture [$p(dA)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$] when both the gel preparation buffer and the running buffer were pH 8.0. At this pH, the $p(dA)_{10}$ and $p(dC)_{10}$ migrated together, preceding the $p(dT)_{10}$, which migrate more slowly. When the pH of both buffers was reduced to 7.5 by increasing the concentration of boric acid in the buffer, almost complete separation of the three components was achieved, as shown in Fig. 1b [migration order $p(dA)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$]. The separation was even better when the pH of both buffers was reduced to 7.0, as shown in Fig. 1c [migration order $p(dA)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$]. A further decrease in the pH of both buffers to 6.5 led to incomplete separation of the components. Fig. 1d shows that the $p(dC)_{10}$ and $p(dT)_{10}$ migrated together, preceded by the $p(dA)_{10}$. At pH 6.0, all three homodecamers were baseline separated again, with a different migration order of $p(dA)_{10}$, $p(dT)_{10}$ and $p(dC)_{10}$, as shown in Fig. 1e. As mentioned under Experimental, the polymerization rate of the gels prepared at different pH values was maintained at the same level; hence the porosity of the gel (sieving matrix) may be considered to be similar in the pH range examined [13]. Therefore, the migration inversion effect is assumed to be due mainly to the pH changes.

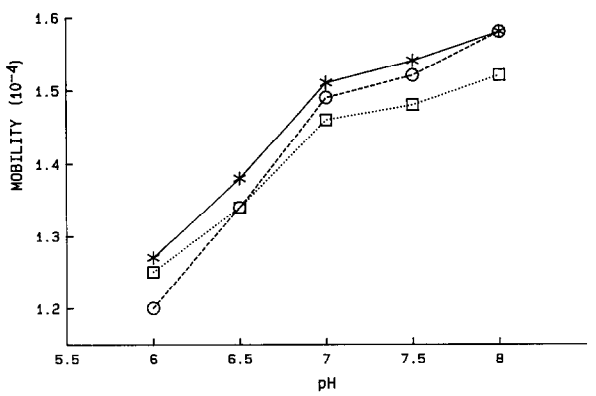

Fig. 2. Relationship between the pH of the gel–running buffer system and the electrophoretic mobility of the homodecamers. Conditions as in Fig. 1. * = $p(dA)_{10}$; ○ = $p(dC)_{10}$; □ = $p(dT)_{10}$.

The effect of pH on the mobilities of the components is plotted in Fig. 2; the change in relative migration order of the three homodecamers can be clearly followed. The plots for $p(dA)_{10}$ and $p(dT)_{10}$ are almost parallel to each other, whereas the mobility of the $p(dC)_{10}$ changes differently with pH.

As the pH of both the gel and running buffers was reduced, the migration times of the components became higher. This phenomenon can be explained by the fact that with a decrease in pH the overall charge of a deoxyribonucleotide polyion decreases so the electrophoretic mobility also decreases. However, this migration time shift is different for the three homodecamers because each homooligomer is protonated differently at different pH values [12]. A major pH shift effect can be observed in the migration behavior of $p(dC)_{10}$, which migrates together with the $p(dA)_{10}$ at pH 8.0, preceding the $p(dT)_{10}$, but moves behind both of them at pH 6.0.

Electrophoretic equilibration of a gel-filled capillary column by varying the pH of the running buffer

It is possible to manipulate the pH of a given gel by varying the composition of the running buffer as described by Chrambach [14] in the use of multiphasic buffer systems in slab or rod gel electrophoresis. A major difference is that in capillary electrophoresis, the gel-filled tubing can be used for up to hundreds of runs, not only once as for the slab or rod gels. Hence it was important to examine the possibility of changing the buffer system in a gel-filled capillary column after preparation or even after use.

A capillary gel column, prepared with pH 8.0 buffer, was connected to buffer reservoirs filled with running buffer of pH 6.0, then the electrophoretic equilibration was started by applying a 300 V/cm electric field to the capillary. After 20 min, the current stabilized at a new, higher level that was typical of a pH 6.0 buffer system. Based on this observation, the entire buffer system appears to have

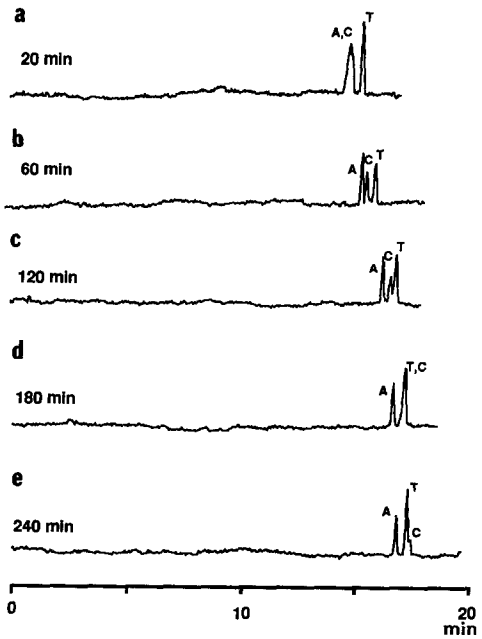

Fig. 3. Capillary electrophoretic separations of the homodecamer test mixture using a pH 8.0 capillary gel column after different equilibration times with the pH 6.0 running buffer: (a) 20; (b) 60; (c) 120; (d) 180; (e) 240 min. Peaks: A = $p(dA)_{10}$; C = $p(dC)_{10}$; T = $p(dT)_{10}$. Conditions: gel buffer and running buffer, 0.1 *M* Tris–boric acid–7 *M* urea at pH 8.0 and 6.0, respectively, adjusted with boric acid in both instances. Other conditions as in Fig. 1.

changed throughout the gel within 20 min. However, when the separation performance of the gel was checked, almost the same electrophoretic separation and migration order were obtained as with pH 8.0 gel with pH 8.0 running buffer (Fig. 3a). Only after 4 h of electrophoretic equilibration did a retention pattern appear that was characteristic of a gel prepared and run at pH 6.0. After 60 min of electrophoretic equilibration, the migration pattern of the test mixture was similar to that of a column prepared with a pH 7.5 buffer [Fig. 3b, migration order $p(dA)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$]. After 120 min of equilibration the gel had a performance similar to a pH 7.0 gel [Fig. 3c, migration order $p(dA)_{10}$, $p(dC)_{10}$ and $p(dT)_{10}$]. When the homooligomer test mixture was injected after 180 min of equilibration (Fig. 3d), the $p(dC)_{10}$ and $p(dT)_{10}$ migrated together, preceded by the $p(dA)_{10}$, as was observed on a pH 6.5 gel-filled capillary. The separation

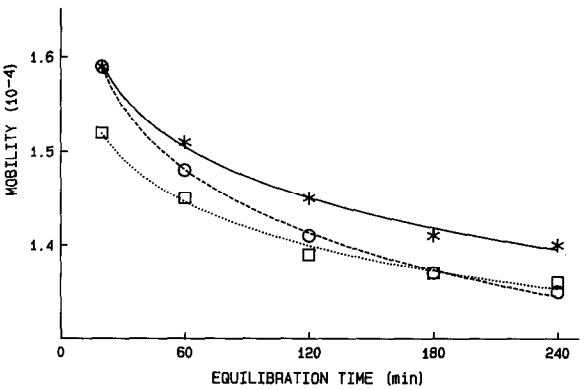

Fig. 4. Relationship between the equilibration time and the electrophoretic mobility of the homodecamers in the pH 8.0 gel-filled capillary column running with a pH 6.0 buffer. Conditions as in Fig. 3. * = $p(dA)_{10}$; ○ = $p(dC)_{10}$; □ = $p(dT)_{10}$.

shown in Fig. 3e was the result of a 300 V/cm electric field applied to a pH 8.0 gel-filled capillary column using a pH 6.0 running buffer for 4 h. The $p(dA)_{10}$ and $p(dT)_{10}$ preceded the $p(dC)_{10}$, as is characteristic of the pH 6.0 gel column.

In conclusion, it is important to note that the current does not seem to be a sufficient indication for determining buffer equilibration in capillary gel electrophoresis. However, the rapid change in current indicates that the small buffer ions in the running buffer have replaced the buffer ions in the gel column, so some other phenomena must occur during the electrophoretic equilibration of the polyacrylamide gel-filled capillaries. However, the reason for this behavior is not clear, and requires more investigation into the physical properties of the gel structure during the electrophoretic equilibration.

Fig. 4 shows plots of the mobilities of the three homodecamers as a function of the equilibration time of the pH 8.0 gel-filled capillary column using a pH 6.0 running buffer. In this instance, as with the data shown in Fig. 2, the mobility of $p(dC)_{10}$ again shifts faster than that of the other two components. The shape of the curves suggest that equilibration is still not complete after 4 h.

CONCLUSIONS

The pH of the buffer system used in capillary gel electrophoresis has a marked effect on the migration properties of the different homooligomers. A

mixture of homooligodecamers was co-electrophoresed using buffer systems of different pH values between 6.0 and 8.0 in order to determine its effect on the separation. Gel-filled capillaries, prepared at pH 8.0, were equilibrated with a running buffer of pH 6.0 for various times to determine when full equilibration had taken place, as judged by separation performance. Although it was found to be possible to re-equilibrate the buffer system in a gel-filled capillary, it was found to be extremely time consuming. For example, the electrophoretic equilibration time required for changing the buffer system from pH 8.0 to 6.0 takes more than 4 h for a 650 mm × 0.075 mm I.D. capillary column filled with 3% polyacrylamide gel. Unfortunately, the time required for completion of this equilibration appears to be much longer than would be appropriate for the capillary gel electrophoretic analysis of oligonucleotides (the average separation time is up to 20–30 min). However, in some instances it is difficult to prepare polyacrylamide gels at the desired pH, *e.g.*, highly acidic gels, special additives. In these instances, after the gel has completely polymerized, equilibration with a suitable running buffer is necessary. Based on these results, it is suggested that for capillary gel electrophoresis of oligonucleotides, the gel should be prepared using buffer of the same pH as the running buffer in order to avoid time-consuming equilibration procedures.

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