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Short communication

Separation of $pd(GC)_{12}$ from $pd(AT)_{12}$ by free solution capillary electrophoresis

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Abstract

The two synthetic self complementary oligonucleotides $pd(AT)_{12}$ and $pd(GC)_{12}$ were separated by free solution capillary electrophoresis (CZE) using simple borate buffers. The effects of pH (7.5–9) and the concentration of the buffer (0.03–0.35 *M*) were investigated. Higher pH values and buffer concentrations led to better resolution and longer migration times, the pH having a more pronounced effect on the separation than the concentration of the buffer. It is proposed that the conformation and effective length of the oligonucleotides may have a role in their separation in free solution capillary electrophoresis.
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1. Introduction

Synthetic oligonucleotides are used extensively in biochemistry and molecular biology as probes for gene isolation, primers for DNA sequencing and for template amplification. The emergence of the anti-sense nucleotides as potential therapeutic agents [1] has stimulated further research into the synthesis and modification of oligonucleotides [2,3]. Thus fast and reliable methods for the quality control of oligo-nucleotides and modified analogues are necessary. HPLC (for short oligonucleotides) [4,5] and capillary gel electrophoresis [6–9] have been used.

With the use of a sieving medium, e.g., cross-linked polyacrylamide [7,10], single base resolution (separation of oligonucleotides differing by one nucleotide in length) is readily achievable for oligo-mers in the range 30–60 nucleotides. If capillary zone electrophoresis (CZE) is used without a sieving medium (free solution) the primary separation mech-anism is primarily a function of the charge to mass ratio of the analytes. Since oligonucleotides above ca. 15 bases have almost the same charge to mass ratio, they are expected to have similar electro-phoretic mobilities in free solution (CZE) almost irrespective of length [7,11,12] and the presence of a sieving medium would be expected to be necessary to achieve separation. Even more challenging is the separation of oligonucleotides with identical lengths but with different base composition (base specific recognition). With capillary affinity gel electropho-resis (cAGE), in which an affinity ligand is incorpo-rated into the gel matrix, e.g., poly(9-vinyladenine) [13,14], oligomers of different base composition have been separated by their differential interactions.

In this Short communication we describe the separation of two 24-mer oligonucleotides [$pd(AT)_{12}$ and $pd(GC)_{12}$] in free solution CZE with an un-

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treated silica capillary and simple buffer system. To the best of our knowledge this type of separation has not been previously reported. The effect of pH and the concentration of the buffer on the separation are also discussed.

2. Experimental

2.1. Chemicals

Boric acid (reagent grade) and sodium tetraborate decahydrate (electrophoretic grade) were purchased from Sigma (St. Louis, MO, USA). The 24-mers [pd$(AT)_{12}$ and pd$(GC)_{12}$] were chemically synthesised using a DNA/RNA synthesiser (Applied Biosystems, model No. 392) and purified using commercial cartridges (Applied Biosystems, OPC, API P/N 400771). Samples of the oligomers were diluted in distilled water (0.5–1 ml) and stored at −20°C until use. Before injection, samples were freshly thawed and diluted with distilled water to yield a concentration of 17 pmol/μl of each oligomer.

2.2. Apparatus

CZE separations were carried out using a TSP-CE 1000 capillary electrophoretic separation system (Thermo Separation Products, USA). Data were acquired and processed using OS/2 warp software version 3. Untreated fused silica capillaries (375 μm O.D. and 50 μm I.D., Composite Metal, Worcester, UK) were used with an effective length of 32 cm and a total length of 40 cm. New capillaries were conditioned by sequential washing with 1 *M* phosphoric acid, 1 *M* NaOH, distilled water, followed by borate buffer solution (each at 60°C for 10 min). Four concentrations (0.02, 0.04, 0.06, 0.08 *M*) of sodium tetraborate solutions were prepared and the pH of each solution was adjusted using 0.5 *M* boric acid solutions (Table 1).

All buffers were made with water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) and filtered through 0.2 μm pore size filters (Whatman, Maidstone, UK) and carefully vacuum degassed. Samples of the oligomers were hydrodynamically injected into the capillary for 5 s. The applied voltage was 25 kV, and the capillary temperature was maintained at 20°C. The capillary

Table 1
Composition of buffers used to separate the 24-mer oligonucleotides with the resulting migration times and resolution values

Initial Na borate conc. (*M*)	Final borate conc. (*M*)	Final Na^+ conc. (*M*)	Migration time (min)		Resolution value[a]
			pd$(AT)_{12}$	pd$(GC)_{12}$	
pH 7.5					
0.02	0.22	0.012	4.35	4.40	0.59
0.04	0.25	0.015	4.37	4.45	0.65
0.06	0.31	0.017	4.90	4.99	0.72
0.08	0.35	0.020	5.60	5.73	0.90
pH 8.0					
0.02	0.15	0.014	4.55	4.60	0.70
0.08	0.29	0.04	6.77	6.95	2.75
pH 8.5					
0.02	0.09	0.018	5.72	5.90	0.81
0.08	0.22	0.053	8.07	8.46	5.25
pH 9.0					
0.02	0.03	0.019	7.10	7.51	1.40
0.08	0.14	0.07	15.05	16.10	3.31

[a] Baseline separations were obtained when resolution values were 1.3 or above. The equation used to calculate the resolution values was: $R_s=2(t_2-t_1)/(w_1+w_2)$ where t_1 and t_2 are the migration times, w_1 and w_2 are the peak widths at the base. Migration times and resolution values were obtained at the final borate concentration, which have the stated pH.

was washed after each run for 1 min with distilled water followed by 1 *M* NaOH for 1 min. Oligonucleotides were detected and identified using high speed scanning in the UV region (200–350 nm) and the electropherograms were recorded at 260 nm.

3. Results and discussion

The 24-mer oligodeoxynucleotides $pd(AT)_{12}$ and $pd(GC)_{12}$ were chosen as model intermediate-length self-complementary oligonucleotides that are long enough to complete one hairpin turn to form the B-form DNA helix. Starting with a total borate concentration (TBC) of 0.22 *M* at pH 7.5, little separation was obtained. The order of migration of the oligonucleotides was determined from the UV spectra of the peaks in the region 200–350 nm. As the two oligonucleotides have distinctive UV spectra it was confirmed that $pd(AT)_{12}$ passes the detector before $pd(GC)_{12}$. When the TBC was increased from 0.22 to 0.35 *M* with the pH constant at 7.5 a significant improvement in resolution was obtained with only a small increase in migration time (Table 1). However complete separation of the two oligonucleotides was not achieved even at a TBC of 0.35 *M*.

As sodium tetraborate solutions (0.02 *M*) were adjusted to pH 7.5, 8.0, 8.5 and 9.0 with 0.5 *M* boric acid the resulting TBC changed to 0.22, 0.15, 0.09 and 0.03 *M*, respectively (Table 1). From the results at pH 7.5, it would be expected that these buffer solutions would show an improvement of resolution at higher ionic strengths if the pH had no effect on the separation. However, the separation improved greatly with increasing pH, in spite of decreasing ionic strengths (Table 1), showing that pH changes had a more significant effect on resolution than the ionic strength of the buffer. The selective effect of pH on the separation of oligonucleotides has previously been reported [15] with crosslinked-polyacrylamide-gel filled capillaries, an optimum separation of three different oligonucleotide decamers being achieved at pH 6 [15].

In order to test if the combined effect of higher ionic strengths and higher pH values could lead to better resolution, 0.08 *M* sodium tetraborate solutions were adjusted to pH 7.5, 8.0, 8.5 and 9.0 with

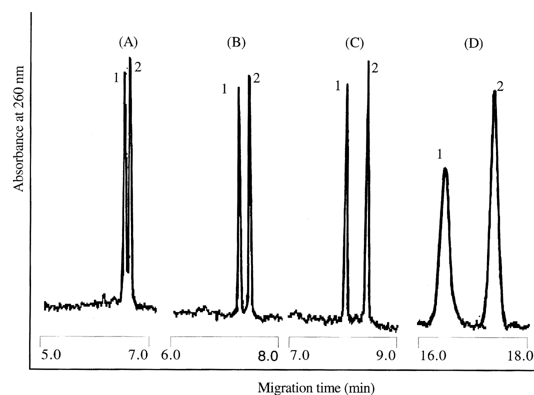

Fig. 1. Sample electropherograms of an equimolar mixture of $pd(GC)_{12}$ and $pd(AT)_{12}$ obtained with 0.08 *M* sodium tetraborate solution adjusted to different pH values (Table 1). (A) pH 7.5, (B) pH 8.0, (C) pH 8.5, (D) pH 9.0. Peaks: $1=pd(AT)_{12}$; $2=pd(GC)_{12}$. Conditions: Free solution CZE, applied voltage=25 kV, untreated silica capillary of total length 40 cm and effective length 32 cm.

0.5 *M* boric acid giving solutions of 0.35, 0.29, 0.22 and 0.14 (*M*) TBC. The results (Fig. 1) show that the effects of ionic strength and pH can be combined to produce yet better resolution with baseline separation being obtained at pH 8.0, 8.5 and 9.0. Migration times were found to change considerably with changes in pH (Fig. 1). It should be noted that at pH 9 the current was higher than the maximum limit (100 μA) for the applied voltage (25 kV) which resulted in an automatic adjustment of the applied voltage to 20 kV thereby causing a doubling of the migration times and significant band spreading. The differential peak broadening observed in Fig. 1D together with the pH dependence of the UV absorbance of the nucleotide bases [16] are responsible for the observed changes in relative peak height.

In all cases increasing the ionic strength of the buffer and/or the pH produced an increase in the average current during the run. Deleterious Joule heating effects on the separation are unlikely except at pH 9 and 0.14 *M* TBC since the capillary temperature was controlled at 20°C and the recorded currents were less than 100 μA.

Baseline separation was obtained without any sieving medium at 0.22 *M* TBC and pH 8.5 in less than 10 min (Fig. 1C). Using several capillaries from different batches, the results were reproducible with only slight differences in resolution from one capil-

lary to another, irrespective of the order of injection (i.e. high to low pH or vice versa). Good migration time reproducibility was obtained at 0.22 *M* borate and pH 8.5 with a relative standard deviation of less than 2.5% for the same capillary.

These results were not expected according to the charge-to-mass ratio concept which is the current basis for explaining separation by CZE, because the two 24-mers would be expected to have the same charge-to-mass ratio in the pH range studied [17]. Moreover, the separation was improved by increasing the ionic strength of the buffer at constant pH (Table 1) which is consistent with the expectation that the separation is not induced by differential ionisation of the oligonucleotides.

It would seem likely that the conformation of the oligonucleotides may have a role in their separation. In a detailed study [18] it has been established that: (a) the minor groove is wide in G:C and mixed sequence B-DNA but narrow in hetero- or homopolymer A:T sequences and (b) propeller twist is low for G:C base pairs and may (but need not) be high for A:T base pairs. More specifically it has been reported that poly d(GC)·poly d(GC) can undergo salt-induced conformational changes [19] which have been shown to be a transition from the A or B right handed conformation to the left handed Z conformation [20]. It has also been found that this transition occurs with poly d(GC)·poly d(GC) but not with poly d(AT)·poly d(AT) [21,22]. Thus differences in the helix conformation and hence overall shape might be responsible for the separation.

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