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Capillary electrophoresis of oligonucleotides using a replaceable sieving buffer with low viscosity-grade hydroxyethyl cellulose

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Abstract

Short model homo-oligomeric deoxynucleotides ranging in length from 12- to 24-mer were separated using a 20 m*M* Tris(hydroxymethyl)aminomethane–N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid buffer containing 4% hydroxyethy cellulose of low viscosity as the polymer additive (pH 7.0). The separation was performed using a DB-17-coated capillary. The influence of instrumental parameters such as field strength and temperature was evaluated. It required a separation voltage of 12 kV at reversed polarity and a temperature of 25°C. Efficiencies of up to 2.5×10^6/m were obtained. The most important parameters influencing the separation were the concentration and the viscosity of the polymer used. Different viscosity grades of hydroxyethy cellulose were evaluated for their loading time on the capillary and their ability to separate $p(dA)_{12-18}$ and $p(dA)_{19-24}$. The use of low viscosity-grade hydroxyethy cellulose at a relatively high concentration (4%) made it possible to replace the buffer after every run and permitted the use of hydrodynamic injection of oligonucleotide samples. The entangled polymer solution system was found to be applicable on automatic capillary electrophoresis (CE) equipment. For quantitation, the use of an internal standard has been shown to improve both migration time and peak area repeatability. This method using low viscosity-grade hydroxyethy cellulose has been demonstrated to have the repeatability, linearity and selectivity required for stability studies of oligonucleotides.

Keywords: Buffer composition; Oligonucleotides; Hydroxyethyl cellulose; Nucleotides

1. Introduction

Antisense oligonucleotides (generally 12 to 30 bases in length), are currently being evaluated as potential drugs for the treatment of cancer and viral infections [1]. Analysis and purification of synthetic oligonucleotides by reversed phase [2] and ion-exchange high performance liquid chromatography (LC) [3] using gradient elution have been reported previously. This requires large samples and is well suited for preparative purposes. Traditionally, polyacrylamide slab gel electrophoresis has been employed [4]. Although high resolution separation of multiple samples can be achieved with this methodology, this technique has the limitation of post-electrophoresis visualization of DNA by radiolabeling or by staining that is only semi-quantitative.

The capillary format allows the use of a higher field strength and is thus more efficient. The advantages of a column-operated technique are on-line sample detection, easy quantitation and automation. Capillary zone electrophoresis (CZE) can not be

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applied to resolve short oligonucleotides ranging in length from 12–24mers because these have a similar charge-to-mass ratio. Capillary gel electrophoresis (CGE) employing crosslinked polyacrylamide gels under denaturing conditions has been used for analyzing oligonucleotides [5,6]. The resolving power and speed of CGE for the separation of oligonucleotides have been shown to be much better than that of slab gel electrophoresis [7,8] and LC [9]. However, a problem is encountered in the performance of these gel-filled capillaries as they degrade over time, and in practice their lifetime is severely limited. The use of concentrated solutions of uncrosslinked linear polyacrylamide (LPA) containing 10% T (0% C) was reported for the separation of oligonucleotides, with either a manual pre-filling of the capillary [10,11] or a polymerization reaction inside the capillary [12]. Automatic filling of capillaries with LPA solutions of this concentration is excluded because of their high viscosity.

Recently, capillary electrophoresis (CE) using replaceable polymer solutions other than LPA, has become a popular technique for the separation of double stranded DNA molecules [13–17]. DNA fragments are separated due to entanglement with the entangled polymer network inside the capillary [18–20]. In this technique, double stranded DNA molecules can be separated using a dilute polymer solution that can be loaded into the capillary freely after each run, thus refreshing the separation matrix before every analysis, and avoiding any sample material remaining on the column.

The intention of this study was to explore a replaceable polymer solution (in this case hydroxyethyl cellulose, which has not been reported before for the separation of oligonucleotides) that could be loaded into the capillary automatically with a minimum loading time and which could resolve the short oligonucleotides in question.

2. Experimental

2.1. Chemicals

Oligonucleotide samples $p(dA)_{12-18}$, $p(dA)_{19-24}$, $p(dT)_{12-18}$ and $p(dA)_{21}$ were purchased from Pharmacia Biotech (Roosendaal, Netherlands). Hydroxyethyl cellulose (HEC) of single viscosity (0.08–0.125 Pa·s at 2% m/v) was purchased from Aldrich (Bornem, Belgium), while HEC samples of different viscosity grades (HEC EP09, QP-300, QP-4400H and HEC QP-100MH, >85.5% purity containing less than 7.5% sodium acetate) were kindly donated by Union Carbide (Antwerpen, Belgium).

All viscosity measurements were performed on a Contraves Rheomat 115. All other reagents used were of analytical grade. Tris [Tris(hydroxymethyl)aminomethane] and formamide were obtained from Acros Chimica (Geel, Belgium). TAPS [N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid], urea and disodium hydrogen phosphate were purchased from Sigma (St. Louis, MO, USA), UCB (Brussels, Belgium) and Merck (Darmstadt, Germany), respectively. Milli-Q water (Millipore, Milford, MA, USA) was used throughout. Stock solutions of oligonucleotide samples were prepared in Milli-Q water as follows: $p(dA)_{12-18}$ (0.5 mg/ml), $p(dA)_{19-24}$ (0.1 mg/ml), $p(dT)_{12-18}$ (0.25 mg/ml) and $p(dA)_{21}$ (0.93 mg/ml).

2.2. Preparation of running buffer

The running buffer, at a pH of 7.0, used in this study contained 20 m*M* TAPS, 7 *M* urea (as a denaturing agent) and an appropriate amount of HEC. It was prepared as follows. A 20-ml volume of water at 50°C was added to HEC while stirring. After obtaining a clear solution, 121.5 mg of TAPS were added (20 m*M*) and mixed. Then, 10.5 g of urea were added (7 *M*) and the mixture was stirred for a further 5–10 min. Finally, the pH was adjusted to 7.0 with solid Tris and the final volume was made up to 25 ml with water.

2.3. HPCE apparatus

CE separation was performed on Spectra Phoresis 1000 (Thermo Separation Products, Fremont, CA, USA), which was driven by CE software (version 1.04) operating under IBM OS/2TM (version 1.2). The vacuum system of the instrument applies a constant negative pressure of 0.75 p.s.i. for loading the capillary with running buffer. Two different types of coated capillaries were used in this study. An evaluation sample of BioCAP^{TM} linear poly-

acrylamide (LPA)-coated capillary (75 μm I.D.×44 cm total length and 36 cm effective length) was kindly provided by Bio-Rad (Nazareth, Belgium). The capillary was washed with fresh running buffer containing 4% HEC (0.08–0.125 Pa.s at 2% m/v) for 30 min, every four to six runs. The J&W DB-17 capillary (50% methyl silicone, 50% phenyl silicone coating) (100 μm I.D.×44 cm total length and 36 cm effective length) was purchased from Alltech (Laarne, Belgium). In this case, the prewash time was 5 min in the case of 4% HEC EP09 and therefore the capillary was filled with fresh buffer before every analysis. The capillary was cleaned at the beginning of the day with HPLC-grade methanol for 5 min followed by a water wash for the same period of time. The oligonucleotide samples were either introduced electrophoretically at negative polarity (5 kV for 1 s) or hydrodynamically (30 s) into the capillary and were separated in the above-mentioned capillaries filled with running buffer, at a negative voltage of 12 kV (273 V/cm) at room temperature (25°C). Oligonucleotides were detected at 260 nm.

3. Results and discussion

3.1. Qualitative development work

3.1.1. Choice of capillary

In this study, the first aim was to resolve oligonucleotides with a buffer containing HEC on a fused-silica capillary. Although a better separation of DNA restriction fragments was achieved by Barron et al. [21] with an uncoated capillary, in comparison to a coated capillary using ultra-dilute polymer solution, in our case, no separation of sample components was achieved due to their similar mobilities. In addition, baseline disturbances were observed, due to the generation of electroosmotic flow (EOF) and extrusion of viscous buffer solution from the capillary [10]. For this reason, two different types of coated capillaries with reduced EOF were evaluated. The LPA-coated capillary was characterized by carrying out an EOF measurement test, as prescribed by the manufacturer [22]. The value obtained for the electroosmotic mobility ($\mu_{eo}=1.8\times10^{-5}$ cm^2/V·s), was close to the value demonstrated for a well-coated capillary. The separation of $p(dA)_{12-18}$ and $p(dA)_{19-24}$ using an LPA-coated capillary with a buffer adjusted to pH 8.7 [23] is presented in Fig. 1A–B, respectively.

Because of the commercial unavailability of LPA-coated capillaries at the time of submission of this paper, we chose a J&W surface-modified fused-silica DB-17 capillary for this study.

3.1.2. HEC: different viscosity grades

The performance of several HEC viscosity grades as a sieving agent for CE separation of $p(dA)_{12-18}$ was investigated. The different viscosity grades of HEC along with their molecular masses, viscosities at different concentrations, loading time on the capillary and the current observed are outlined in Table 1. The separation of $p(dA)_{12-18}$ is shown in Fig. 2. It was observed that the current values increased with an increasing concentration of HEC. Considering the fact that Tris, TAPS, urea and HEC do not contribute very much to the current, this increase in current value is related to the salts present in HEC (see experimental for purity of HEC). As the concentration of HEC increases, the salt concentration also increases, which is responsible for the rise in current observed.

In this study, it was not possible to compare the

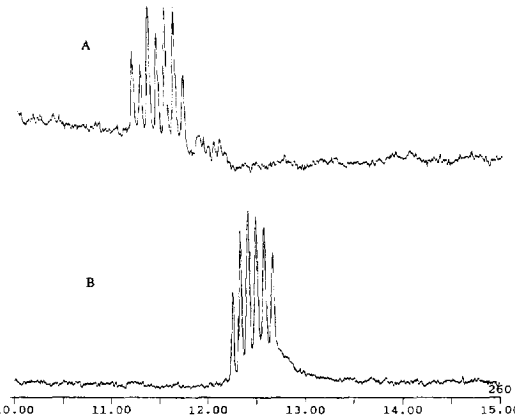

Fig. 1. Electropherograms obtained using 4% m/v HEC (Aldrich) as a polymer additive in the buffer for the separation of samples; $p(dA)_{12-18}$ (A) and $p(dA)_{19-24}$ (B). Electrolyte, 20 m*M* Tris–TAPS, 4% m/v HEC (Aldrich) and 7 *M* urea at pH 8.7; capillary, BioCAP™ LPA-coated; temperature, 25°C; voltage, 12 kV (at reversed polarity); injection, electrokinetic (1 s at 5 kV); detection, UV at 260 nm. *x*-Axis gives time in min.

Table 1
Different viscosity grades of HEC evaluated (for experimental conditions, see Fig. 3).

HEC Name and source	Molecular mass[a] (MW)	Viscosity[a,b] (mPa·s)	Viscosities at different concentrations (mPa·s)[c]	Loading time on J&W capillary (min)	Observed current (μA)
HEC EP09 (UC)[d]	$1–50\times10^4$	9	84.7 (4% m/v)	2–2.5	16.5
HEC (Aldrich)	$1–1.2\times10^5$	80–125	73.6 (2% m/v)	2–2.5	11.1
HEC QP-300 (UC)	$1–100\times10^4$	300	40.3 (1% m/v)	1	5.2
HEC QP-4400H (UC)	$1–100\times10^4$	4400	36.5 (0.6% m/v)	2	3.0
HEC QP-100MH (UC)	$1–100\times10^4$	100 000	22.9 (0.25% m/v)	0.75	1.7

[a] Data supplied by manufacturer.
[b] Average viscosity of 2% m/v aqueous solution measured at 25°C.
[c] Viscosity measured at 27°C.
UC=Union Carbide.

different grades of HEC at the same concentration (e.g. 1–4%), because of the difficulty of dissolving the highly viscous grades of HEC at these concentrations and it would have been impossible to load them on the capillary due to the very high viscosity. When a buffer containing 4% HEC (80–125 mPa·s at 2% m/v) was tried on the LPA-coated capillary, a loading of about 25 min was required. Also the lifetime of such a capillary was decreased as it became clogged after a few days. On the other hand, the same concentration (4% HEC EP09) could be successfully used with a short loading time.

These results indicate that HEC EP09, having a shorter polymer chain length, is easier to replace at higher concentration in comparison to high viscosity-grade HECs (HEC QP-300 to HEC QP-100MH).

3.1.3. Effective concentration of HEC

In the literature, dilute to ultra-dilute solutions of HEC were reported for the separation of DNA restriction fragments [15,16,18,24]. We tried to use a higher concentration (1–4%) of HEC of low viscosity grade for the separation of oligonucleotides. Because the concentration of polymer in entangled polymer solutions is the most important parameter influencing the separation of $p(dA)_{12-18}$, we therefore evaluated the influence of the concentration of HEC EP09 on the separation of $p(dA)_{12-18}$ as shown in Fig. 3. Separation of $p(dA)_{12-18}$ and $P(dA)_{19-24}$ could not be obtained using more dilute polymer solutions (0.1–1%). The effective pore diameter decreases with increasing concentration of HEC, providing improved separation of oligonucleotides. These findings are in agreement with the statement of Singhal and Xian [24] and Baba et al. [17] that higher concentrations (0.5–0.9%) of cellulose derivative are most effective for separating small fragments of DNA (50–100 bp). Fig. 4A represents typical electropherograms obtained with a buffer containing 4% HEC EP09 for samples $p(dA)_{12-18}$ and $p(dA)_{19-24}$. The prewash run time was only 5 min. From these results, it can be concluded that the size (viscosity) and the concentrations of HEC are the most influential parameters for manipulating migration time and resolution. The same conditions could also be applied to separate $p(dT)_{12-18}$ (see Fig. 4B). The migration time of $p(dA)_{12-18}$ was shorter in comparison to $p(dT)_{12-18}$, which is consistent with a report [25] where a polyacrylamide slab gel was used. Furthermore, CE with crosslinked polyacrylamide under denaturing conditions and with LPA under non-denaturing conditions gave the same migration order for A- and T-containing homo-oligonucleotides [23].

A Ferguson plot was constructed to determine the size selectivity of the prepared solution. The log of the measured electrophoretic mobility versus the percentage concentration of HEC EP09 for $p(dA)_{16}$ showed a linear relationship ($r=0.985$). Fig. 5 shows the plot of ln [mobility (μ)] vs. N (base number) obtained at 12 kV. A linear relationship was obtained ($r=0.9999$) and this demonstrates an Ogston sieving mechanism for migration. Therefore, it can be concluded that the separation of short oligonucleotides follows Ogston theory, where the separation is modeled as simple molecular sieving. A plot of relative migration time [migration time of $p(dA)_n$ (where $n=12–18$)/migration time of I.S.] vs. base

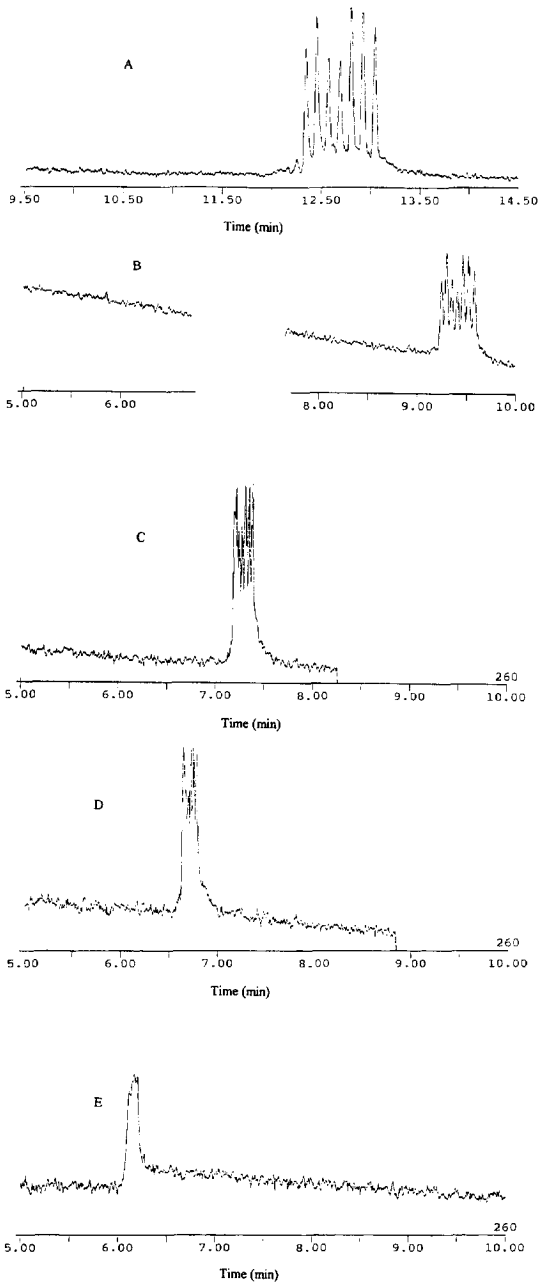

Fig. 2. The separation of $p(dA)_{12-18}$ using different viscosity grades of HEC. Electrolyte, 20 m*M* Tris–TAPS and 7 *M* urea at pH 7.0, containing 4% HEC EP09 (A), 2% HEC (Aldrich) (B), 1% HEC QP-300 (C), 0.6% HEC QP-4400H (D) and 0.25% HEC QP-100MH (E). Capillary, J&W DB-17; temperature, 25°C; voltage, 12 kV (at reversed polarity); injection, electrokinetic (1 s at 5 kV); detection, UV at 260 nm.

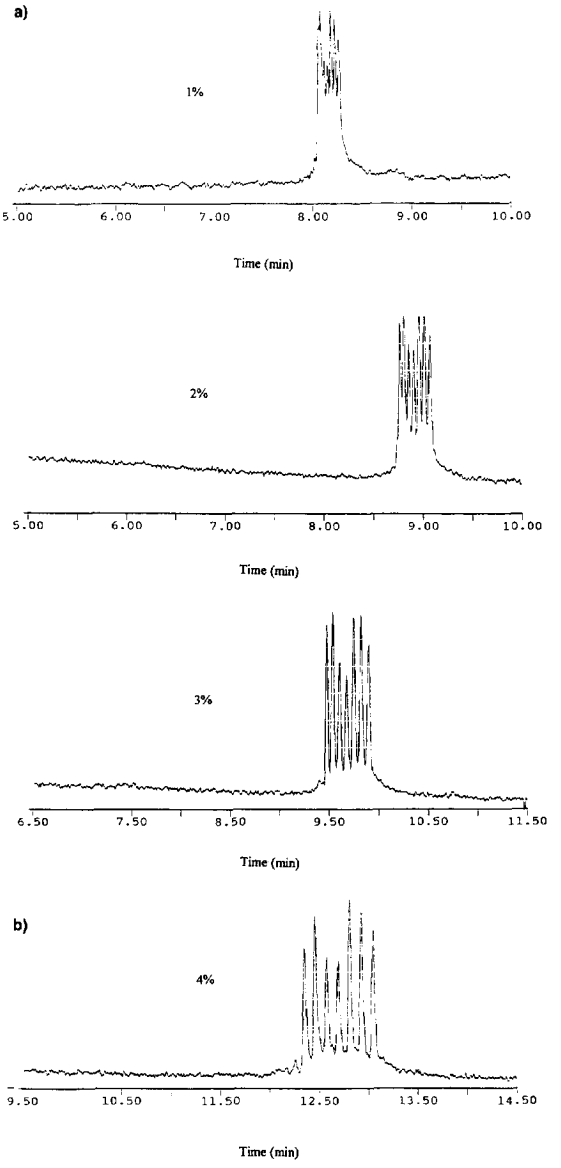

Fig. 3. The effect of the concentration of HEC EP09 on the separation of $p(dA)_{12-18}$. Electrolyte, 20 m*M* Tris–TAPS, HEC EP09 (1–4%), 7 *M* urea at pH 7.0; capillary, J&W DB-17; temperature, 25°C; voltage, 12 kV (at reversed polarity); injection, electrokinetic (1 s at 5 kV); detection, UV at 260 nm.

number gave a straight regression curve with $y = 0.009x + 0.807$ (where y=relative migration time and x=number of bases) and a correlation coefficient of 0.9999. Therefore, the chain length and molecular mass of oligonucleotides can be determined from this

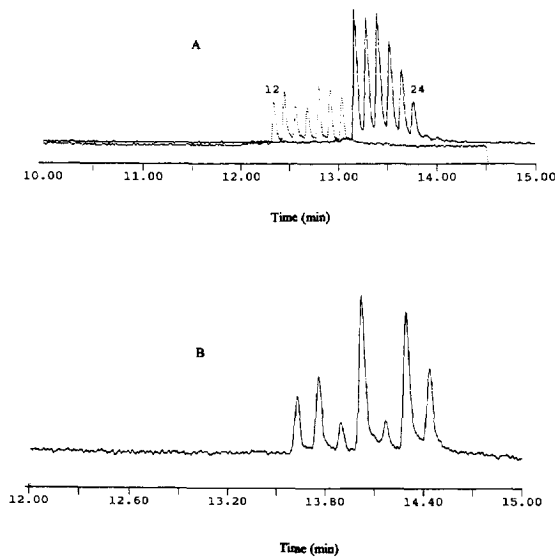

Fig. 4. Separation of $p(dA)_{12-18}$, $P(dA)_{19-24}$ (A) and $p(dT)_{12-18}$ (B) using 4% HEC EP09. Other experimental conditions were identical to those in Fig. 3.

plot. The efficiency of separation for $p(dA)_{12-18}$ was calculated under these conditions. Using a polymer solution containing 4% HEC EP09, the plate number was estimated to be 8.0×10^5 (2.2×10^6/m) for $p(dA)_{12}$ and 9.0×10^5 (2.5×10^6/m) for $p(dA)_{18}$, which is slightly less than with CGE using cross-linked gels containing 7% T and 1.5% C (for 30mer, 7×10^6/m) [26], but is comparable to LPA containing 10% T (for 20mer, 8.3×10^5/m) [27] and polyvinylpyrrolidone [28].

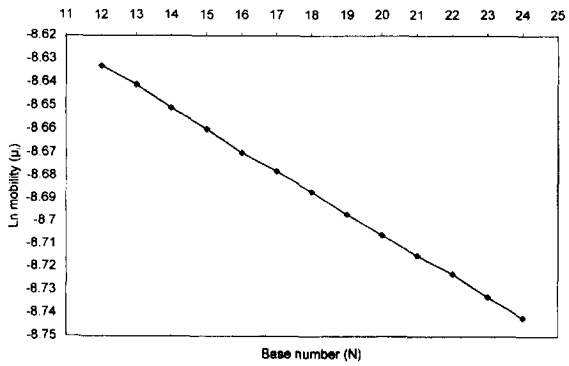

Fig. 5. Plot of [ln mobility (μ_i)] vs. base no. for $p(dA)_{12-24}$. For experimental conditions, see Fig. 3.

3.1.4. Instrumental parameters: effect of applied voltage and temperature

The effect of varying the electric field strength (205 to 409 V/cm) on the separation for $p(dA)_{12-18}$ was studied. The analysis time increases at lower voltage due to the lower mobility of oligonucleotides, as the velocity of charged analytes in CE is directly proportional to the field strength. It was found that the resolution values for 12–18mers remained unaffected (figure not shown). A separation voltage of 12 kV (273 V/cm, 16–17 μA) was chosen, because it gave an acceptable analysis time of 15 min.

The temperature range 20–28°C was evaluated for capillary temperature. Like applied voltage, the change in capillary temperature did not show any important influence on the resolution for $p(dA)_{14-15}$ (results not shown). The overall migration time of $p(dA)_{16}$ decreased by about 1.6%/°C, which is somewhat higher than the 1.1%/°C mentioned in the literature [29]. A separation temperature of 25°C was chosen as the optimum value.

3.1.5. Denaturing agent

The use of urea (7 *M*) as a denaturing agent is recommended in the literature [30]. The use of a combination of 3.5 *M* urea with 30% (v/v) formamide is an alternative way to denature the oligonucleotide samples. Therefore, an experiment was performed to evaluate these two possibilities. The resolution value for $p(dA)_{14-15}$ was decreased in the second case (1.8 vs 1.4). Therefore, 7 *M* urea was preferred as a denaturing agent. A combination of 3.5 *M* urea and 30% formamide would be beneficial in cases where higher viscosity-grade HEC is needed in the buffer, in order to keep the viscosity down, which in turn helps when loading the capillary.

3.1.6. Investigation of buffer and buffer pH

Different types of buffers containing HEC and denaturing agent at pH 8.7 were studied for the separation of oligonucleotides. Tris–acetate buffer produced higher current which was responsible for poorer separation (52.5 μA). Tris–borate and Tris–TAPS gave comparable results in terms of separation and current (16–17 vs. 24–25 μA), but a difficulty was encountered in the preparation of the former when removing the dissolved air and the prolonged

time needed for its preparation ultimately led to the selection of Tris–TAPS buffer.

The Tris–TAPS buffer was investigated at pH 8.7. It was seen that the alkaline pH caused a deterioration of the capillary coating after some time. In an attempt to study the effect of acidic pH, a different kind of buffer (phosphate–citrate) [31] was tried out, because it was not possible to use Tris–TAPS buffer at acidic pH. The concentration of HEC EP09 used was 4% and the pH was 3.6. At lower pH, the mobility of oligonucleotides decreases due to a decrease in the overall charge of the deoxyribonucleotide polyion [32]. Separation deteriorated for $p(dA)_{12-18}$ and was completely lost in the case of $p(dA)_{19-24}$.

In view of the stability of the capillary coating, it was advisable to use a neutral pH. Although this pH is at the extreme of the acceptable range for buffers made with Tris, the pK_a of which is 8.0, the selection was made for a buffer adjusted to pH 7.0.

3.2. Quantitation in entangled polymer solution buffer

3.2.1. Injection mode

CGE, by necessity, employs an electrokinetic mode of introduction of the sample into the capillary. With replaceable gels however, sample introduction can be done either by hydrodynamic (pressure, vacuum) or electrokinetic injection. Hydrodynamic injection is generally preferred for quantitative work. Therefore, we performed an experiment to compare the results for electrokinetic and hydrodynamic injection, because the buffer containing 4% HEC EP09 still permits the use of hydrodynamic injection. The results are compared in Fig. 6. No deterioration in resolution, peak shape and efficiency were observed when oligonucleotide samples were injected hydrodynamically, which contradicts previous results [33].

3.2.2. Migration time and corrected area repeatability: effect of an internal standard

Within-day repeatability was calculated for both migration time and observed (corrected) peak areas for $p(dA)_{16}$, using a sample solution of $p(dA)_{12-18}$ [240 μg/ml containing $p(dA)_{21}$ (58 μg/ml) as an internal standard], which was hydrodynamically

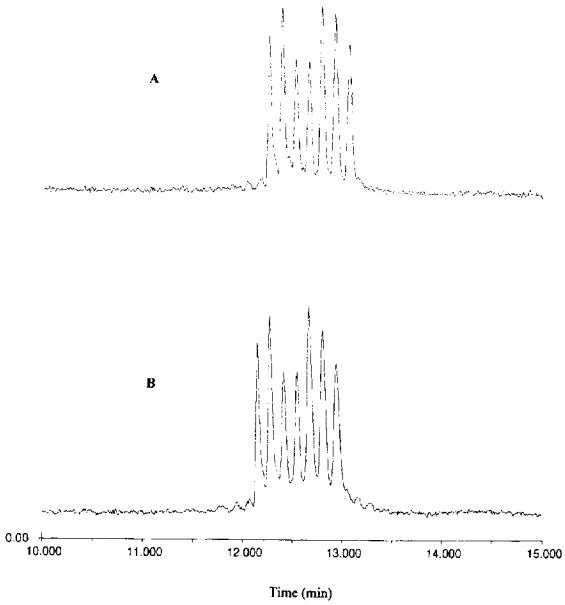

Fig. 6. Comparison of electrokinetic (5 kV, 1 s) (A) and hydrodynamic (30 s) (B) injection for the separation of $p(dA)_{12-18}$. For experimental conditions, see Fig. 4.

injected six consecutive times. An R.S.D. value of 0.16% was found for migration time and an improvement of the percentage R.S.D. was seen for the observed area (8.6 to 1.4), when $p(dA)_{21}$ was used as an internal standard. The results for day-to-day repeatability ($n=4$) showed an R.S.D. of 0.12% for migration time and 2.7% for peak area. It can be concluded that the use of an internal standard is necessary for quantitative purposes [28].

3.2.3. Linearity and sensitivity

The linearity of the detector response for $p(dA)_{16}$ was studied in the range of 15–240 μg/ml of $p(dA)_{12-18}$, containing $p(dA)_{21}$ (58 μg/ml) as the internal standard, and using hydrodynamic injection. The calibration data for $p(dA)_{16}$ is shown below. The regression equation was $y=0.0023x+0.0186$, where y is the ratio [peak area $p(dA)_{16}/p(dA)_{21}$] and x is the concentration of $p(dA)_{12-18}$ (μg/ml). Five different concentrations were injected, the total number of analyses was fifteen, the correlation coefficient (r) was 0.9991 and the standard error of estimate $s_{y,x}$ was 0.0085 and standard error of slope was 0.000026.

The limit of detection at $S/N=3$, was 31.2 pg for a

solution containing a total concentration of 3.25 μg/ml of $p(dA)_{12-18}$, using a 30 s hydrodynamic injection (if the apparatus injects 0.3 nl/s for a 100-μm capillary). To determine the limit of quantitation, six injections of a solution containing 7.5 μg/ml showed an R.S.D. of 14.6% at a S/N ratio of 6.

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