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Capillary gel electrophoresis of oligonucleotides: prediction of migration times using base-specific migration coefficients

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Abstract

Chemically synthesized oligodeoxyribonucleotides were subjected to capillary gel electrophoresis on three different polyacrylamide-based matrices. Analysis of about 1000 samples over a 1-year period showed that the gel matrix evolved with time resulting in shifting migration times, making it essential to use an internal standard. Cross-linked polyacrylamide matrices had the highest stability, allowing an average of 100 injections on the same capillary. Computer-aided prediction of migration times was subsequently evaluated to confirm the size and base composition of oligonucleotides more accurately. A number of problems were noted when using this approach on a routine basis, such as insufficient stability of the gel matrices, effects of secondary structure on migration and insufficient differences in migration times for oligonucleotides containing >50 bases. Capillary gel electrophoresis at pH 3.5 in replaceable gels showed that migration was mainly dependent on the charge per base ratio resulting in separations of significantly altered selectivity which complemented analyses under the commonly used basic pH conditions.

1. Introduction

Capillary electrophoresis (CE) has found widespread applications as an analytical technique in the field of biomedical research since its introduction by Jorgenson and Lukacs [1]. In particular, CE in gel-filled capillaries (CGE) has provided separations of high efficiency for both proteins [2] and nucleic acids [3]. Application of CGE to the separation of DNA fragments has resulted in some of the most efficient separations reported to date with over 10^7 theoretical plates per metre [4].

Since the introduction of automated solid-phase chemical synthesis, oligodeoxyribonucleotides (referred to as oligonucleotides) [5,6] have become indispensable tools in molecular biology and biotechnology. Further, ongoing developments of modified oligonucleotides as antisense therapeutic agents [7–9] and synthetic ribozymes for anti-viral therapy [10] have put increasing demands on the reliability of analytical techniques used for their quality control.

CGE as an instrumental approach to gel electrophoresis has allowed efficient separations of synthetic oligonucleotides in surface-coated fused-silica capillaries filled with either cross-linked or linear polyacrylamide gels [11]. Further, CGE may be automated for quality control purposes and quantitative results can be obtained, as oligonucleotides are detected on-line by UV absorption measurement. Reproducible

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routine analyses are limited, however, by the gel matrix (generally polyacrylamide), which shows rapid signs of fatigue allowing about 100 injections on the same capillary in favourable cases. Although alternative gels that can be replaced after each run have been described for the separation of oligonucleotides [12,13], the resolution is generally not as good as with permanent gels.

In this work, three different types of gel-filled capillaries were evaluated with respect to stability using a 28-mer oligonucleotide. In addition, the feasibility of obtaining more detailed information on oligonucleotide length, base composition and secondary structure based on the prediction of electrophoretic migration times as described previously [14] was assessed. As previous investigations had established that electrophoretic migration of oligonucleotides depended not only on size but also on base composition and the capacity to form secondary structures of sufficient stability [14–17], the effect of duplex formation on CGE will be described. Finally, initial results on CGE at pH 3.5 in replaceable gels showed that complementary migration data can be obtained under these conditions where differences in the ionization of the four nucleobases are most pronounced [18].

2. Experimental

2.1. Apparatus

All separations were performed on a P/ACE 2100 system (Beckman Instruments, Palo Alto, CA, USA) working in reversed polarity (cathode at the capillary inlet). Separations were monitored at 254 nm. Capillaries were thermostated using the liquid cooling system provided with the instrument. Data acquisition was done on a 386/33 microcomputer using System Gold software (Beckman Instruments).

2.2. Gel-filled capillaries

Commercialized gel-filled capillaries (100 μm I.D.) were obtained from Beckman Instruments (U100P capillary, composition not specified) or J&W Scientific (Folsom, CA, USA) (5% T, 5% C polyacrylamide)[1] and pre-run for 45 min by increasing the voltage in a stepwise fashion up to the separation voltage. Capillaries filled with 10% T linear polyacrylamide were prepared by coating the interior wall of a 100-μm I.D. fused-silica capillary with a layer of polyacrylamide according to Hjertén [19]. Linear polyacrylamide was prepared by mixing 3.3 ml of a 30% (w/v) acrylamide solution with 6.7 ml of 100 m*M* tris(hydroxymethyl)aminomethane–borate–ethylenediaminetetraacetate (TBE) (μPAGE, J&W Scientific) buffer (pH 8.5) containing 7 *M* urea and degassing at 100 mbar for at least 3 h with magnetic stirring. The solution was subsequently cooled on ice while still degassing and 50 μl of 10% (v/v) aqueous N,N,N′,N′,-tetramethylethylenediamine and 50 μl of 10% (w/v) aqueous ammonium peroxodisulfate were added followed by further degassing for a few minutes. About 1 m of capillary was filled with a syringe and polymerization was continued overnight at 4°C. Gel-filled capillaries were inspected under a light microscope for bubble formation and cut to the appropriate length to fit the capillary cartridge. Finally, the external polyimide coating was removed from the gel-filled capillary with a scalpel under a light microscope to form the detection window.

Capillaries containing a commercially available replaceable gel (DNA 1000, Beckman Instruments) were surface coated as described above and filled while installed using the "high-pressure" capability of the P/ACE 2100 system. The pH was adjusted to 3.5 with citric acid. Surface-coated capillaries containing 2% (w/v) hydroxyethylcellulose (HEC; medium viscosity) (Fluka, Buchs, Switzerland) were filled as described above. HEC was dissolved in water at 50°C with subsequent addition of 2 *M* citric acid (final concentration 36 m*M*) and 0.5 *M* Na_2HPO_4 (final concentration 14 m*M*) to reach pH 3.5. Electrode buffers (5 ml) were changed after every tenth analysis to avoid pH shifts.

[1] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = (g acrylamide + g Bis)/100 ml solution.

2.3. Calculation of base-specific migration coefficients

Base-specific migration coefficients were calculated according to Ref. [14] using software written in language C. A copy of this program can be obtained on request from the authors for research purposes only.

2.4. Synthetic oligonucleotides

Synthetic oligonucleotides were prepared on a 0.2-μmol scale using solid-phase phosphoramidite chemistry [5,6] on either a Model 380B (Applied Biosystems, Foster City, CA, USA) or a Model 7500 (Milligen, Bedford, MA, USA) automated synthesizer. Oligonucleotides were purified by reversed-phase HPLC before removing their 5'-dimethoxytrityl protecting groups (μBondapak C_{18}, particle size 10 μm, pore size 12.5 nm) using a gradient of 20–30% (<50 nucleotides) or 5–30% acetonitrile (>50 nucleotides) in 100 m*M* triethylammonium acetate (pH 6.9).

2.5. Secondary structure predictions

Predictions of the thermodynamic stabilities of hairpin loops and duplexes were performed using OLIGO software version 4.0 (National Biosciences, Plymouth, MN, USA) on a 486/33 microcomputer. Duplex stability was calculated according to Breslauer et al. [20] based on nearest-neighbour thermodynamic parameters. Hairpin stabilities were calculated according to Refs. [21] and [22].

3. Results and discussion

3.1. Stability of gel-filled capillaries

Polyacrylamide gels in slab gel electrophoresis are generally prepared for single use. It is therefore not surprising that narrow-diameter fused-silica capillaries filled with either cross-linked or linear polyacrylamide gels have a limited lifetime. Analysis of about 1000 synthetic oligonucleotides over a time period of 1 year by CGE on three different types of gels (two types of commercialized capillaries and one type prepared in-house) showed that migration times tended to increase with time while the resolution diminished to a point where single-base resolution was lost (Fig. 1). In a few cases the migration velocity increased again at the very end of a capillary's lifetime accompanied by a severe decrease in resolution and a drop in electric current. These analyses indicated that cross-linked polyacrylamide has a higher structural stability than linear polyacrylamide, allowing approximately 100 injections per capillary. On

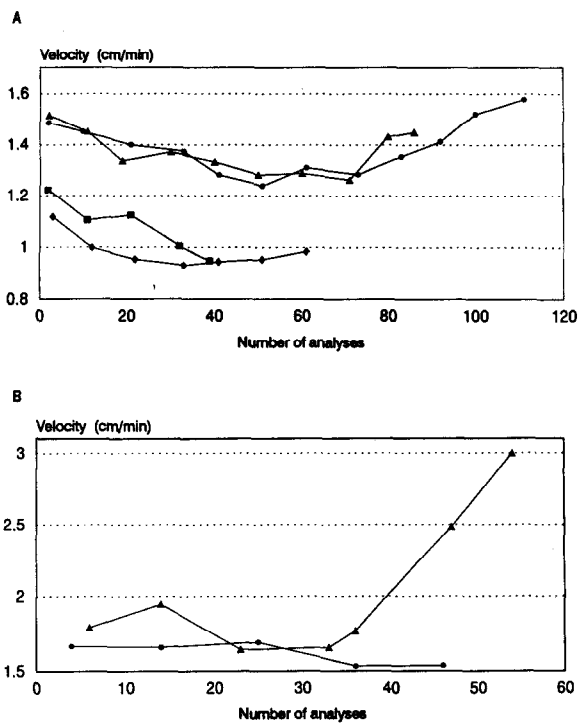

Fig. 1. Determination of the stability of gel-filled capillaries based on the migration velocity of the 28-mer (5'-ATCGTTACGGCATTAGCAGCTTGAGCAC-3'). Capillaries were rejected after the indicated number of injections owing to severe decrease in resolution or current and/or baseline fluctuations (the second 10% T capillary is still in use at analysis No. 60). (A) Comparison of two 5% T, 5% C polyacrylamide capillaries (▲, ●; J&W Scientific) with two 10% T linear polyacrylamide capillaries (■, ◆; prepared in-house) at 250 V/cm and 20°C (capillary length 37 cm, 30 cm to the detector). (B) Comparison of two U100P capillaries (▲, ●; Beckman Instruments) at 300 V/cm and 30°C (conditions as recommended by the manufacturer).

the other hand, capillaries filled with linear polyacrylamide were easier to prepare without the introduction of bubbles.

3.2. Prediction of migration times

In order to confirm the size and base composition of synthetic oligonucleotides based on their migration in CGE, the approach described by Satow et al. [14] was evaluated to predict electrophoretic migration times. A computer program in language C served to solve the system of five equations given below and to obtain the respective base-specific migration coefficients (a, g, t, c and constant k).

$$[AA]a + [AG]g + [AT]t + [AC]c + [A]k = [AM]$$
$$[AG]a + [GG]g + [GT]t + [CG]c + [G]k = [GM]$$
$$[AT]a + [GT]g + [TT]t + [CT]c + [T]k = [TM]$$
$$[AC]a + [CG]g + [CT]t + [CC]c + [C]k = [CM]$$
$$[A]a + [G]g + [T]t + [C]c + [K]k = [M]$$

where [AA] corresponds to $\Sigma_i A_i A_i$ with i being the number of oligonucleotide standards used for calculation and [A] corresponds to $\Sigma_i A_i$ etc. The constant k reflects the time required for an oligonucleotide of zero length to reach the detector while M is the experimentally determined migration time of the respective standard oligonucleotide (see Ref. [14] for more details).

Nine oligonucleotides ranging from 20 to 48 nucleotides in length were selected as standards to calculate migration coefficients with each oligonucleotide being identified in the mixture by spiking (Fig. 2 and Table 1). Extending this approach to longer oligonucleotides (up to 100 bases) on a 5% T, 5% C polyacrylamide gel was unsuccessful owing to the small differences in migration times. Polyacrylamide gels of 3% T, 3% C have subsequently proved to be more suitable for the analysis of longer oligonucleotides. This will necessitate the use of a second set of standards to cover the higher molecular mass range, however.

All of the standard oligonucleotides were analysed by electrospray mass spectrometry under negative ionization conditions to confirm their structure [23], showing that the measured mass values were in agreement with the expected molecular mass. The following order of relative contributions of each base to the overall migration time of the oligonucleotide was observed (in min/base): t (0.210) > a (0.166) > g (0.150) > c (0.135) as derived from analysis No. 13 (Table 2). These coefficients are significantly smaller than those reported previously [14] using a U100P gel-filled capillary [analysis No. 11 in Ref. [14]; t (0.362) > g (0.345) > a (0.240) > c (0.168)], indicating not only that the U100P gel matrix was less restrictive but also that different gels may exercise base-specific effects on the migration of oligonucleotides (note the inversion of coefficients a and g). Subsequent analyses using U100P capillaries did not, in our hands, result in migration coefficients corresponding to those previously described, indicating batch-to-batch variations between capillaries to account for these discrepancies. Batch-to-batch variations were also observed for the 5% T, 5% C capillaries (Table 2). The method of determining base-specific migration coefficients may thus be used as a sensitive test of gel reproducibility and to help to standardize the production of gel-filled capillaries.

The results in Table 1 confirmed earlier observations that oligonucleotides of the same length can have significantly different migration times as a result of base-specific influences on migration (e.g., OTG4598 and OTG2040). Further, inversions of migration times have been observed on gel ageing (compare analyses Nos. 13 and 60 for OTG3156 and OTG4598 in Table 1). At present it cannot be excluded that OTG4598 migrated faster owing to its capacity to form a stable duplex (Table 1 and results described below).

The observed increase in base-specific migration coefficients of 19.8% (a), 34.2% (g), 35.4% (c), 39.7% (t) and 18.2% (k) over a period of 47 injections on a 5% T, 5% C gel-filled capillary (see Table 1) showed that the gel became more restrictive to the migration of oligonucleotides with increased use. Concomitant with increasing migration times was an increase in peak width, leading to an overall decrease in resolution

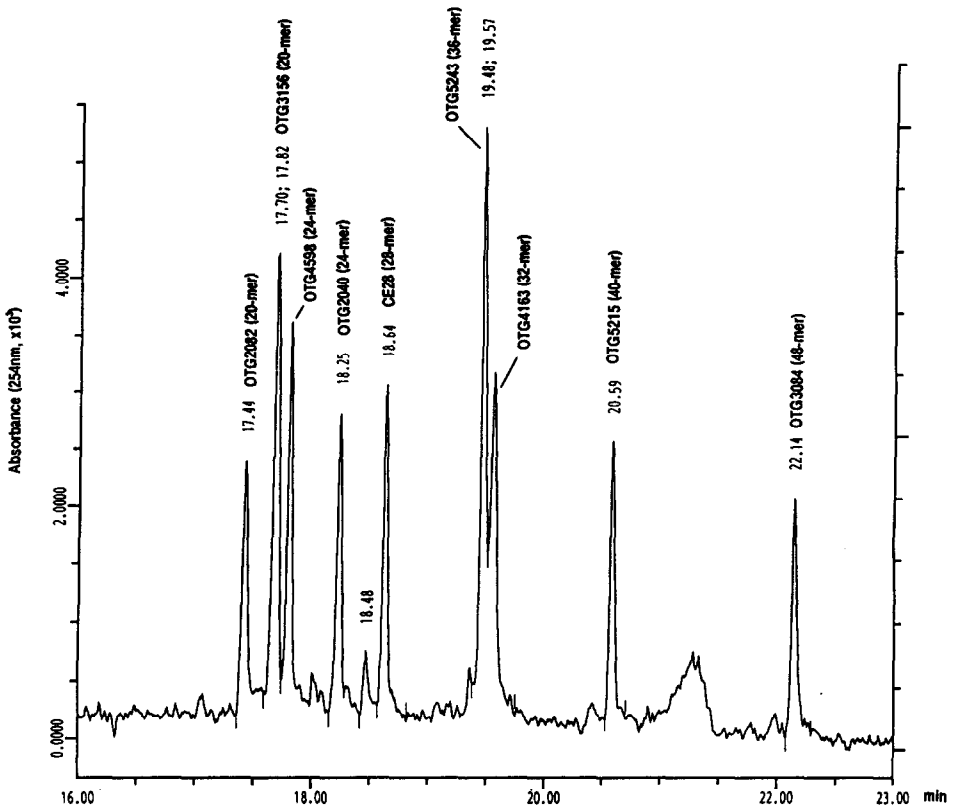

Fig. 2. Analysis of nine standard oligonucleotides (see Table 1) on a 5% T, 5% C polyacrylamide gel-filled capillary (100 μm I.D., 37 cm total length, 30 cm to detector, 250 V/cm, 8.8 μA, 20°C, TBE buffer, pH 8.5). Individual oligonucleotides were identified by spiking and are labelled with their length and identification number (see Table 1 for sequences).

accompanied by an increase in electric resistance. It therefore does not seem to be possible to extrapolate migration coefficients from one batch of capillaries to another, nor is it possible to use the same set of migration coefficients on a single capillary over an extended time period owing to age-related changes in migration time, efficiency and selectivity.

3.3. *Influence of secondary structure on migration*

The formation of intra- or intermolecular secondary structures such as hairpin loops or duplexes of sufficient stability may result in shorter electrophoretic migration times in CGE, as indicated by previous work [14,17]. In order to investigate whether deviations from predicted migration times might be due to more compact secondary structures, nine standard oligonucleotides (Table 1) and two pairs of oligonucleotides of identical composition (Table 3) were analysed for thermodynamic stability of potential hairpin structures [21,22] and of duplexes [20]. These calculations provided estimates of the relative thermodynamic stabilities of internal hairpins and duplexes ranging from $\Delta G < 0$ (no stable structure) to a maximum hairpin stability of 7.2 kcal/mol (OTG656; Table 3) and duplex stabilities reaching 37.6 kcal/mol (OTG5899; Table 3). While formation of hairpin loops did not correlate with shortened migration times, probably owing to insufficient stabilities, duplex formation correlated well with decreased migration times, as shown for two pairs of oligonucleotides having identical base compositions but

Table 1
Comparison of predicted and measured migration times for nine standard oligonucleotides on a 5% T, 5% C polyacrylamide capillary (see Fig. 2) using base-specific migration coefficients (Table 2)

Oligo-nucleotide	Sequence (5′ to 3′)	Migration time (min)						Duplex stability[a]: ΔG (kcal/mol)	Hairpin stability[b]: ΔG (kcal/mol)
		Analysis No. 13, batch No. 1			Analysis No. 60, batch No. 1				
		Measured	Calculated[c]	Deviation[d]	Measured	Calculated[c]	Deviation[d]		
OTG2082 (20-mer)	GGCACACAATTCCTAGTGTG	17.44	17.37	0.51	21.66	22.15	−2.34	5.8	2.3
OTG3156 (20-mer)	GGGCTCGTTTCTTCTCGTTT	17.70	17.60	0.78	23.13	22.85	1.34	none	−0.5
OTG4598 (24-mer)	TGAGAGGCTGCATGCCCCCCCCCC	17.82	17.72	0.75	22.90	22.73	0.81	14.1	1.8
OTG2040 (24-mer)	TTACCTTCATTTTTCCTCTTCTGA	18.25	18.39	−1.05	23.95	24.02	−0.33	1.8	1.4
CE28 (28-mer)	CTAGTTACGGCATTAGCAGCTTGAGCAC	18.64	18.69	−0.40	24.12	24.13	−0.05	1.7	1.0
OTG4163 (32-mer)	ATAAGCTTGGTGAATCAATAAAGTTTGGGTGG	19.57	19.59	−0.18	25.56	25.43	0.62	6.0	−0.5
OTG5243 (36-mer)	GATCCCAGCGCACGCGTCGGATCGCTCGGCGCG TAA	19.48	19.67	−1.41	n.d.[e]	25.39	n.d.[e]	10.0	6.4
OTG5215 (40-mer)	CCCAAAGCATGCTGATCAGAAAATTATCGCCATA AAAGAC	20.59	20.61	−0.12	26.80	26.60	0.96	none	−0.1
OTG3084 (48-mer)	GAGCCAGTATCATGACCCTGGCCCTTCTACAGG AGGATTAACCAGT	22.14	21.99	1.13	28.86	29.07	−1.00	none	5.2

[1] cal = 4.184 J.
[a] Calculated according to Ref. [20].
[b] Calculated according to Refs. [21] and [22].
[c] Calculated using the respective base-specific migration coefficients (see Table 2).
[d] In multiples of coefficient c.
[e] Not determined.

Table 2
Base-specific migration coefficients calculated from the data given in Table 1 and from a similar analysis on a second batch of 5% T, 5% C polyacrylamide gel-filled capillaries in comparison with those obtained with a capillary containing a replacable gel at pH 3.5 (Fig. 5) (calculated according to Ref. [14]

Migration coefficient (min/base)	Analysis No. 13, batch No. 1	Analysis No. 60, batch No. 1	Analysis No. 13, batch No. 2	Analysis at pH 3.5
Coefficient *a*	0.166	0.207	0.216	0.100
Coefficient *g*	0.150	0.228	0.128	−0.224
Coefficient *c*	0.135	0.209	0.149	0.202
Coefficient *t*	0.210	0.348	0.310	0.000
Constant *k*	14.065	17.185	18.254	13.130

Table 3
Influence of secondary structure on electrophoretic migration times of two pairs of oligonucleotides with identical base composition in a 10% T linear polyacrylamide gel (see Fig. 3)

Oligonucleotide	Sequence (5 to 3)	Migration time (min)[a]	Duplex stability[b]: ΔG (kcal/mol)	Hairpin stability[c]: ΔG (kcal/mol)
OTG3598 (18-mer)	TGACCCGGAAGGCGGCCT	24.71 (−4.53)	5.2	−0.5
OTG5731 (18-mer)	GCAAGCGGTCCACGCTGG	23.23 (−6.17)	14.5	−0.2
OTG656 (36-mer)	CCTTTTATAGCCCCCTTAGTCGACCATGGGGCGGAG	31.21 (1.52)	5.7	7.2
OTG5899 (36-mer)	CAAGAGAGTCCCCCTGGATCCGGGGTACTCTCTTTG	29.43 (−0.43)	37.9	5.2

[a] The difference in migration time relative to the co-injected 28-mer (CE28, Table 1) is given in parentheses.
[b] Calculated according to Ref. [20].
[c] Calculated according to Refs. [21] and [22].

clearly different migration times (Fig. 3 and Table 3).

Electrospray mass spectrometry confirmed that the four oligonucleotides had the expected molecular masses, thus ruling out other modifications as possible reasons for this abnormal migration behaviour. The difference in migration times for the two 18-mers (OTG3598 and OTG5731, 1.48 min; Fig. 3A) and the two 36-mers (OTG656 and OTG5899, 1.78 min; Fig. 3B) indicated that the structures of OTG5731 and OTG5899 were more compact, in agreement with predicted duplex stabilities of 14.5 and 37.9 kcal/mol, respectively. Predictions of the migration of oligonucleotides in CGE will thus have to take secondary structure formation into account. As such predictions are difficult at present owing to the lack of data relating thermodynamic stabilities to electrophoretic migration, it would be preferable to suppress secondary structure formation by using more powerful denaturants such as formamide and/or by operating at elevated temperatures [17]. Investigations are in progress to evaluate this possibility.

3.4. Capillary gel electrophoresis at pH 3.5 in replaceable gels

The above results underscore the difficulties in predicting the migration times of synthetic oligonucleotides with sufficient precision to confirm their size and base composition on a routine basis by CGE in polyacrylamide gels at the basic pH values generally employed. It was therefore attempted to obtain complementary migration data under modified run conditions in order to augment the reliability of purity control by CGE. Earlier work showed that a shift in pH under which electrophoretic separations are done may influence the migration of oligonucleotides in CGE [24] indicating that base-specific migration

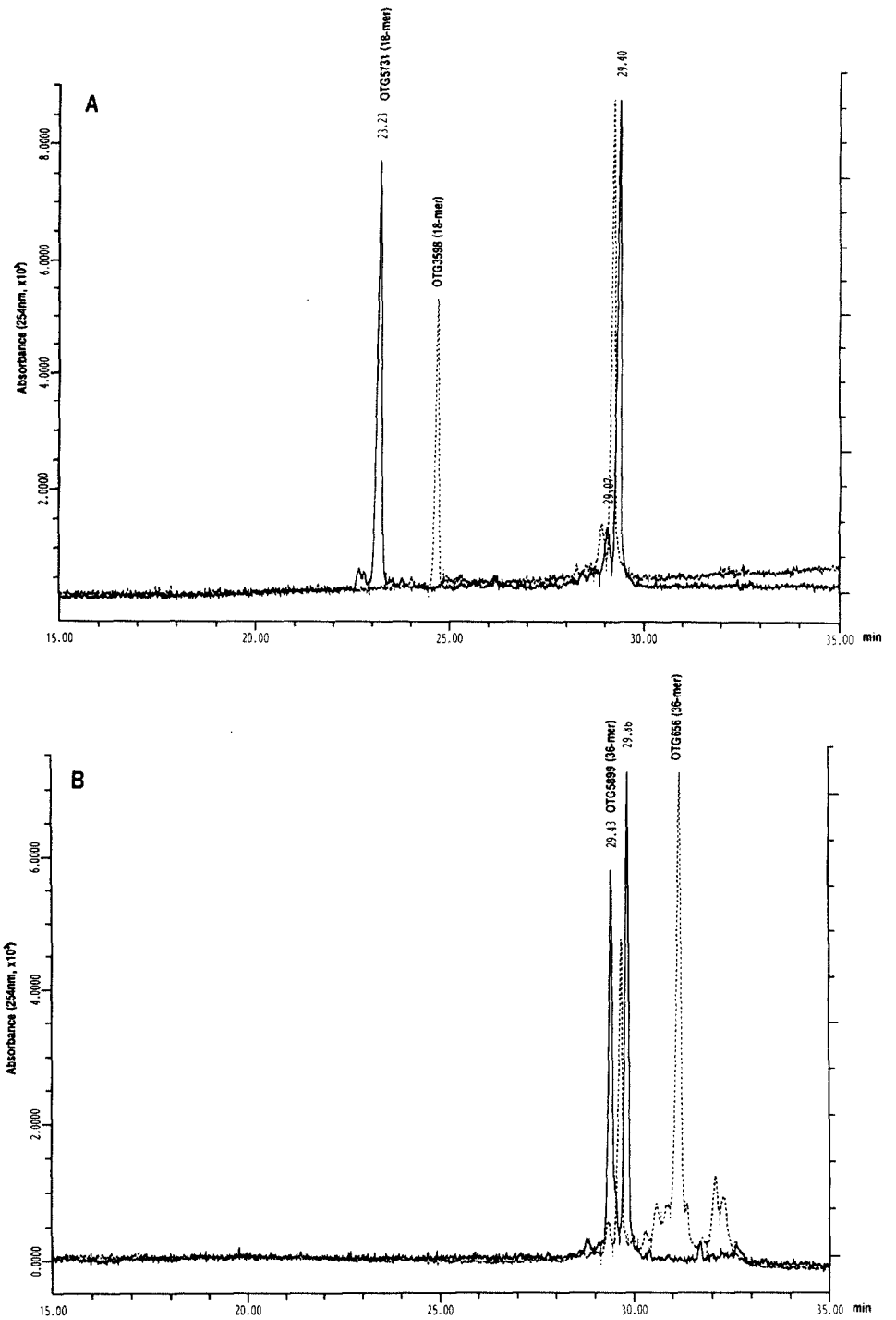

Fig. 3. Influence of duplex formation on electrophoretic migration of oligonucleotides having identical base compositions (see Table 3) in a 10% T linear polyacrylamide gel (run conditions as given in the legend to Fig. 1, 7.8–8.0 μA). (A) 18-mer (OTG3598), duplex stability 5.2 kcal/mol; 18-mer (OTG5731), duplex stability 14.5 kcal/mol. (B) 36-mer (OTG656), duplex stability 5.7 kcal/mol; 36-mer (OTG5899), duplex stability 37.9 kcal/mol. The 28-mer was co-injected to account for slight variations in migration times.

coefficients are affected by pH. Further, electrophoresis on cellulose acetate at pH 3.5 is part of an established method to sequence short oligonucleotides based on the differential contribution of each base to the overall electrophoretic mobility [18], as protonation of the four nucleobases is significantly different at pH 3.5 (net charge of 5-deoxyribonucleotides-5′-monophosphates at pH 3.5:dTMP = −1.0, dGMP = −0.83, dAMP = −0.40, dCMP = −0.15, according to ref. [25]). It was therefore investigated whether the selectivity of CGE could be changed by operating at pH 3.5. Initial results with capillaries containing 10% T linear polyacrylamide showed that the migration times were significantly longer at pH 3.5, in agreement with a decreased net negative charge of the oligonucleotides owing to partial protonation of the nucleobases. In an attempt to shorten migration times and to overcome the problem of gel stability, replaceable gels were employed, resulting in efficient separations of even fairly small oligonucleotides (Fig. 4). Further, the selectivity was drastically changed, as a 20-mer oligonucleotide (OTG2082) which migrated significantly faster than the 28-mer internal standard (CE28) in a cross-linked gel at pH 8.5 (see Fig. 2) had almost the same mobility when analysed in a replaceable gel at pH 3.5 (see Fig. 4). This result was in agreement with OTG2082 having a predicted net charge per base of −0.552 (−11.05 total net charge), which was identical with that of CE28 (−0.552 charge per base; −15.47 total net charge), as both oligonucleotides contained an equal number of each base (see Table 1; net charge calculated with data from Ref. [25]). In contrast, OTG3156, a 20-mer having −0.720 charge per base (−14.35 total net charge), migrated significantly faster than both the 28-mer (CE28) and the 20-mer (OTG2082), indicating that this gel had little sizing capacity in this range, as expected, resulting in separations governed by the charge per base ratio of each oligonucleotide.

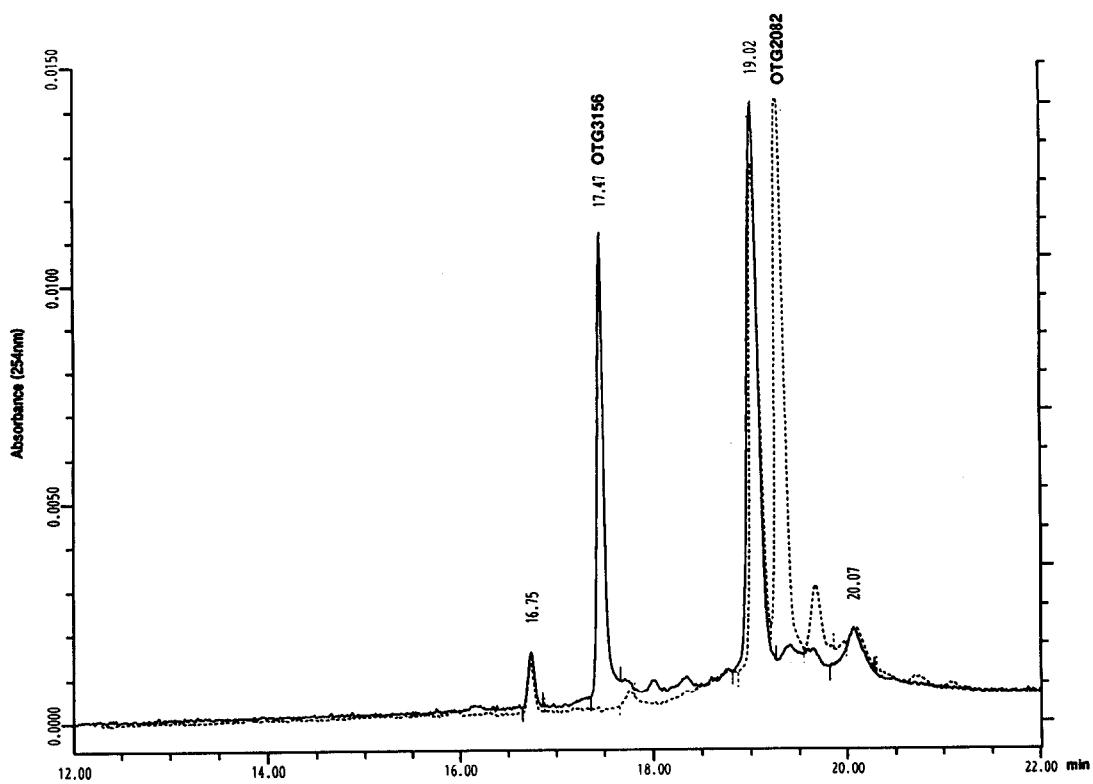

Fig. 4. Capillary gel electrophoresis at pH 3.5 of two 20-mer oligonucleotides in a replaceable gel (DNA1000, Beckman Instruments, 250 V/cm, 85 μA, 13°C) relative to a 28-mer standard (CE28).

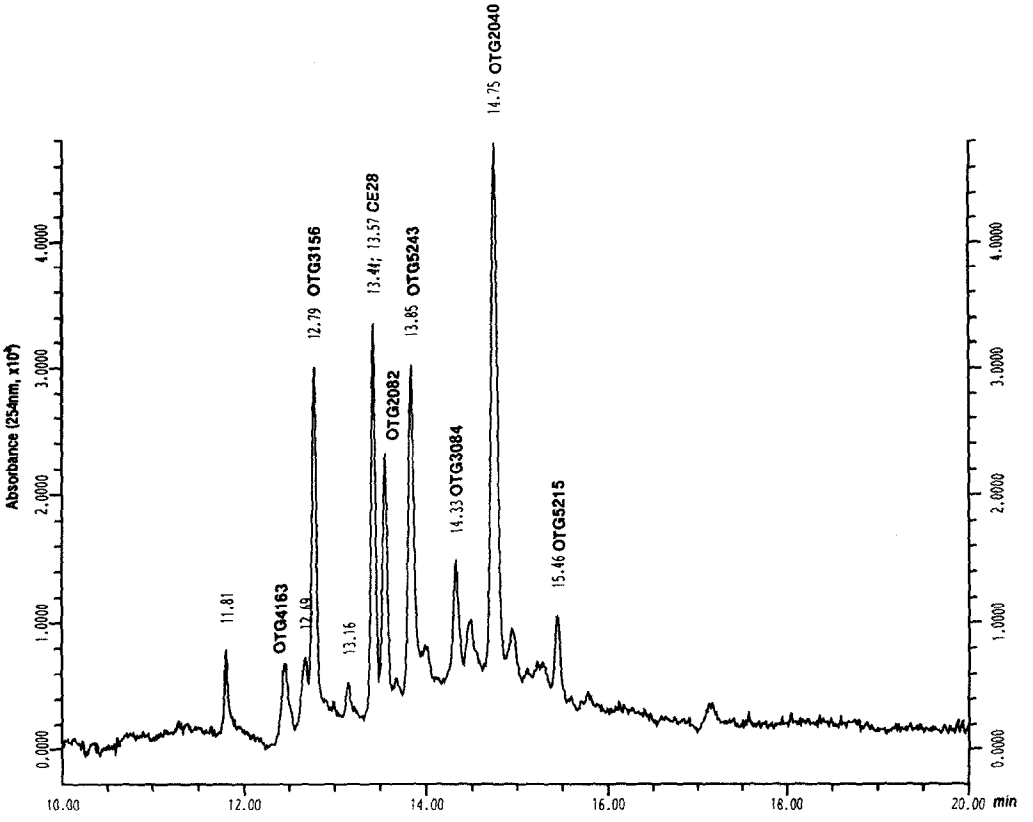

Fig. 5. Capillary gel electrophoresis of standard oligonucleotides at pH 3.5 in HEC (2%, w/v) (see Table 1 for sequences and Table 2 for migration coefficients) (100 μm I.D., 37 cm total length, 30 cm to detector, 250 V/cm, 50 μA, 15°C). Individual oligonucleotides were identified by spiking.

In order to evaluate the base-specific effects on migration under acidic conditions, HEC (2%, w/v) was used as a replaceable gel matrix to separate eight standard oligonucleotides (Fig. 5). Initial results indicated that the migration coefficients had changed dramatically, confirming that results obtained in this separation system should be different from those obtained at basic pH values, thus providing an additional criterion of purity (see Table 2). While the migration coefficients c (0.202), a (0.100) and t (0.00) followed the order as expected from the individual basicities, g (−0.224) showed a fairly abnormal behaviour, actually increasing the electrophoretic mobility. This effect may be related to G inducing a reduced hydrodynamic volume of the oligonucleotide under these conditions potentially as a result of secondary structure formation. The predicted migration times of the eight standards employing this set of base-specific migration coefficients differed from measured values by 0.03–0.22 min. These initial results showed that the migration coefficients were different from each other and that separations under these conditions should allow discrimination between oligonucleotides that are difficult to separate at pH 8.5 (e.g., exchange of G for C).

The possibility of resolving oligonucleotides in replaceable gel matrices at pH 3.5 with the possibility of renewing the gel after each analysis should result in separations of higher reproducibility and ruggedness, being more adapted to routine use. Future investigations will concentrate on developing this approach to complement analyses at pH 8.5 in permanent gels.

Acknowledgements

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