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Review

Capillary gel electrophoresis and antisense therapeutics Analysis of DNA analogs

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Abstract

Capillary gel electrophoresis (CGE) has become an effective tool for the analysis of antisense oligonucleotides. As these compounds begin to show promise in the pharmaceutical field, CGE is often used to determine the quality of chemically synthesized DNA analogs, which are presently being studied as potential antisense therapeutics. The demand for gel capillaries to possess high resolving power and provide statistically meaningful data has indirectly provided a better understanding of what is required to denature single-stranded oligonucleotides. For CGE to be useful for the analysis of oligonucleotides in general, an internal standard is often employed; however, apart from being a strictly quantitative tool, CGE has the capability to be useful in a wide range of applications within the field of antisense therapeutics. CGE can be used in conjunction with HPLC to determine an effective method for the purification of crude oligonucleotide solutions. It has also proven useful in determining whether or not a DNA analog can promote the ribonuclease H-mediated hydrolysis of RNA. An understanding of the interactions between antisense oligonucleotides and nucleases in general is critical for determining how antisense oligonucleotides function within a biological system.

Keywords: Reviews; DNA; RNA; Oligonucleotides; Antisense oligonucleotides; Homooligodeoxyribonucleotides

Contents

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1. Introduction

CGE has been demonstrated to be effective in the separation of phosphodiester deoxyoligonucleotides (ODNs) [1,2]. A polyacrylamide solution polymerized in buffer containing 7–8 *M* urea is the gel matrix most commonly used for these separations. Entangled polymer matrices such as Micro-Gel™ 100 have also been employed in the separation of ODNs [3]. However, applying the above techniques to the separation of DNA analogs (ODNs that have been synthetically modified) can be challenging [4]. Various DNA analogs are presently being studied for pharmaceutical uses, and CGE has been envisaged as an analytical tool for purity determination after their chemical synthesis. Initial experiments into the separation of DNA analogs by CGE led to the evaluation of certain electrophoretic parameters such as pH, buffer concentration and organic additives [4,5].

Varying these parameters in a capillary gel gave significant insight into what effects the electrophoretic separation of oligonucleotides in general. It has been demonstrated that varying the pH has a substantial effect on the mobilities of homooligodeoxyribonucleotides [6,7]. It is also known that with polyacrylamide gel electrophoresis (PAGE), homooligodeoxyribonucleotides of the same length migrate with different mobilities, which do not correspond to their size, i.e., molecular mass [8]. This phenomenon, observed with CGE as well, calls into question whether or not a particular electrophoretic method can be described as "denaturing". Consequently, certain single-stranded ODNs can form secondary structures that 7–8 *M* urea in a 20% gel cannot disrupt [9,10]. Standard polyacrylamide-gel electrophoresis (PAGE) is also ineffective at denaturing secondary structures formed by certain single-stranded RNA oligonucleotides [11].

Discovering ideal conditions for the separation of oligonucleotides has recently accelerated due to the inherent advantages of CGE. A capillary gel can be reliably re-used for at least 40 runs [4]. It is unnecessary to prepare a new gel, as must be done with PAGE, for each set of samples. Due to the durability of CGE, a capillary gel can therefore handle a larger number of samples than can a typical slab gel (PAGE) which contains about 12 wells per gel. The consequence of this feature has led to a better understanding of the conditions necessary to denature single-stranded oligonucleotides.

1.1. Capillary gel electrophoresis and the analysis of DNA analogs

DNA analogs can be defined as ODNs that have been chemically modified in a specific manner. This modification can be placed anywhere within the nucleotide sub-unit and is often repeated throughout the length of the oligonucleotide. DNA analogs have shown promise as possible antisense therapeutics in the treatment of viral infections and certain cancers [12–14]. Antisense DNA can be defined as a strand of DNA that hybridizes in a complimentary manner to a target strand of RNA or DNA. The therapeutic utility of antisense compounds were first investigated with unmodified DNA [15,16]. However, DNA nucleases within cells degrade unmodified DNA; thus, in order to insure nuclease resistance, it is necessary to chemically modify an antisense oligonucleotide thereby producing a DNA analog.

At present, most antisense studies involving DNA analogs have utilized phosphorothioate DNA (SODN) (Fig. 1). As of yet this class of DNA analog has not shown efficacy in a clinical setting. CGE is used to determine the purity of chemically synthesized SODNs, and there are instances where CGE is used routinely within quality control departments [17]. As in the analysis of ODNs, polyacrylamide-based gels are most commonly used for the analysis of SODNs. However, we have found that utilizing an entangled polymer gel matrix such as Micro-Gel is sufficient for the analysis of synthetic SODNs [4,5]. We have also utilized these capillary gels for DNA/enzyme digestion assays and for monitoring HPLC purification processes.

1.2. Capillary gel electrophoresis and the quantitative analysis of oligonucleotides

As a quantitative tool for oligonucleotide analysis, CGE should be used with an internal standard [2,6]. This is necessary for two reasons. First, the electrokinetic injection, which is the method of sample loading for CGE, inherently causes variations in sample size. Second, migration times generally increase with the age of the capillary gel. Choosing an

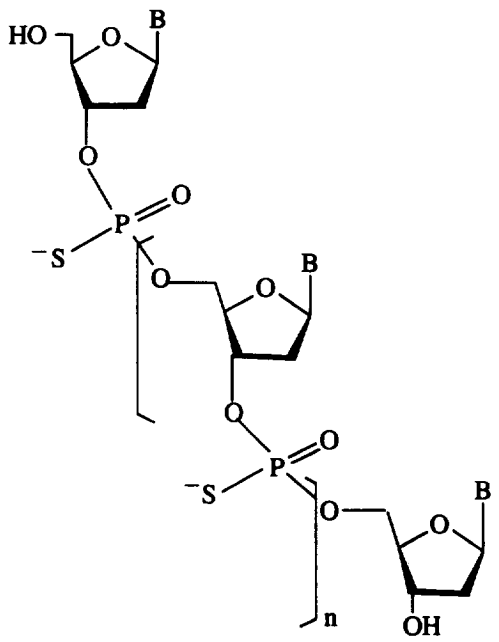

Fig. 1. Molecular structure of phosphorothioate DNA (SODN). Phosphodiester deoxyoligonucleotides (ODNs) possess an O^- in place of the S^- atom found in SODNs. The length of the oligonucleotide = $n+2$. B represents one of the four nucleotide bases, adenine (A), guanine (G), cytidine(C) and thymine (T), found in naturally occurring DNA.

appropriate internal standard allows for the correction of sample load variation and inconsistent migration time.

The detection system used most often with CGE is on-line UV (260 nm) detection. In most automated CE instruments, the detector is positioned at some point along the length of the capillary. Analytes move past the detector window at different rates based on their charge to mass ratios (q/m) [2]. In general, a compound with a relatively low q/m resides in the detector window for a longer period of time than a compound with a relatively high q/m. The peak resulting from the slower moving compound will be broad, and thus will integrate inaccurately. Table 1 lists the molecular weights (M_r), q/m, and the change in q/m ($\Delta q/m$) for 20 homooligonucleotides [poly$(dA)_{2-20}$]. These relationships are graphically represented in Fig. 2. Fig. 2A indicates that the $d(A)_4$ mer will move at a slightly slower rate than the $d(A)_{15}$ mer, and thus the $d(A)_4$ mer resides at the detector for a longer period of time. In general,

Table 1
M_r, q/m and $\Delta q/m$ values for poly$(dA)_{2-20}$

Oligonucleotide	M_r	q/m	$\Delta q/m$
$(dA)_2$	565	0.001770	0.000511
$(dA)_3$	877	0.002281	0.000242
$(dA)_4$	1189	0.002523	0.000142
$(dA)_5$	1501	0.002665	0.000093
$(dA)_6$	1813	0.002758	0.000066
$(dA)_7$	2125	0.002824	0.000048
$(dA)_8$	2437	0.002872	0.000038
$(dA)_9$	2749	0.002910	0.000030
$(dA)_{10}$	3061	0.002940	0.000025
$(dA)_{11}$	3373	0.002965	0.000020
$(dA)_{12}$	3685	0.002985	0.000017
$(dA)_{13}$	3997	0.003002	0.000015
$(dA)_{14}$	4309	0.003017	0.000013
$(dA)_{15}$	4621	0.003030	0.000011
$(dA)_{16}$	4933	0.003041	0.000010
$(dA)_{17}$	5245	0.003051	0.000008
$(dA)_{18}$	5557	0.003059	0.000008
$(dA)_{19}$	5869	0.003067	0.000007
$(dA)_{20}$	6181	0.003074	

longer oligonucleotides have larger q/m values, i.e., they migrate faster (Fig. 2A). However, the longer the oligonucleotide, the smaller the $\Delta q/m$ becomes between two oligonucleotides that differ in length by one nucleotide. For example, the $\Delta q/m$ between a 4-mer and a 5-mer is greater than the $\Delta q/m$ between an 18-mer and a 19-mer (Fig. 2B). Dividing the peak area by migration time is often done in an attempt to "correct" for relative differences in the residence time at the detector caused by variations in the q/m of a sample's constituents [17]. Studies into the impact of this type of data manipulation have shown, that in some cases, these corrections provide more accurate peak integration [18].

2. Capillary gel electrophoresis and HPLC

Prior to CGE, HPLC was used as an analytical tool to determine the purity of synthetic ODNs and DNA analogs (specifically SODNs). Ion- exchange HPLC was one of the first methods utilized in an attempt to separate SODNs that differ in length by one nucleotide base. Fig. 3 illustrates an ion-exchange chromatogram of a series of SODNs that differ in length by two nucleotides. While ion-exchange HPLC has shown itself useful in the sepa-

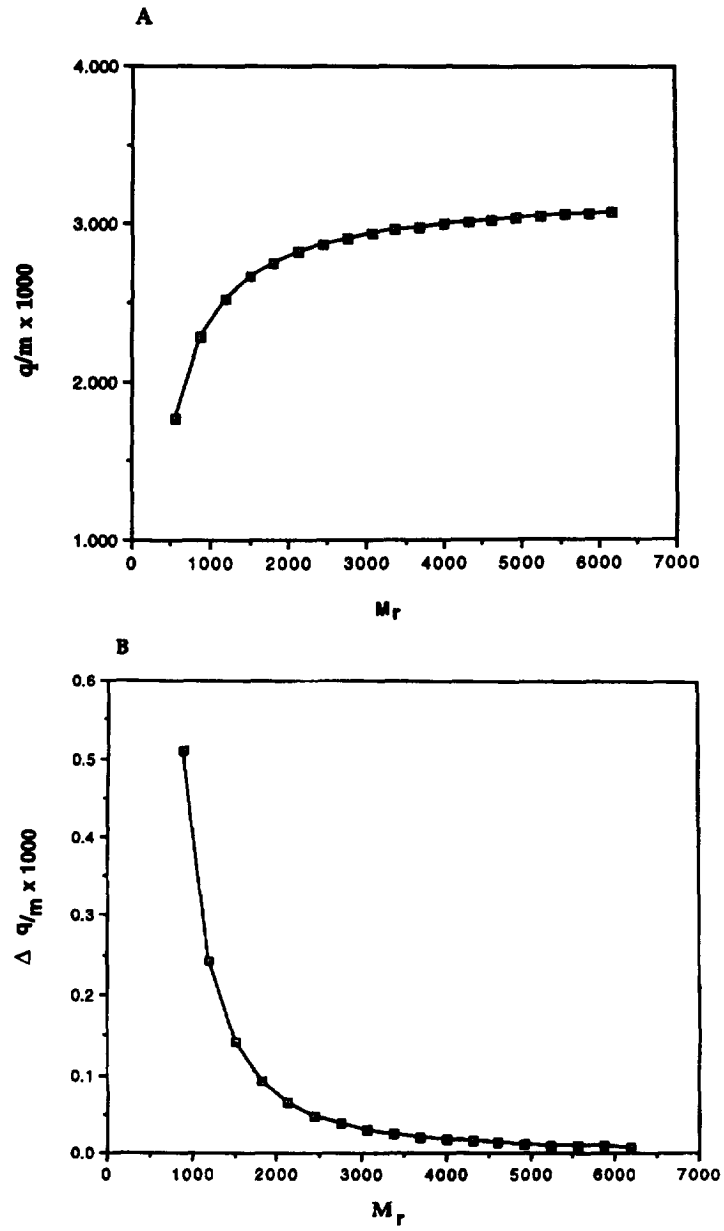

Fig. 2. The relationship between the charge to mass ratio (q/m) of an oligonucleotide and its M_r. M_r values for homooligodeoxyribonucleotides of adenylic acid $(dA)_{2-20}$ (see Table 1) were plotted against their (A) q/ms and (B) $\Delta q/ms$.

ration of ODNs and some DNA analogs, its utility for analyzing SODNs, as demonstrated in Fig. 3, is inadequate. HPLC is presently incapable of baseline resolving two SODNs that differ in length by one nucleotide [19].

On the other hand, CGE has proven itself effective in the separation of both ODNs and SODNs [5]. Fig. 4 is an electropherogram of six ODNs that differ in length by one nucleotide. The system utilized for the separation illustrated in Fig. 4 was modified to achieve a similar separation of SODNs in Fig. 5. As in Fig. 4, the SODNs separated in Fig. 5 differ in

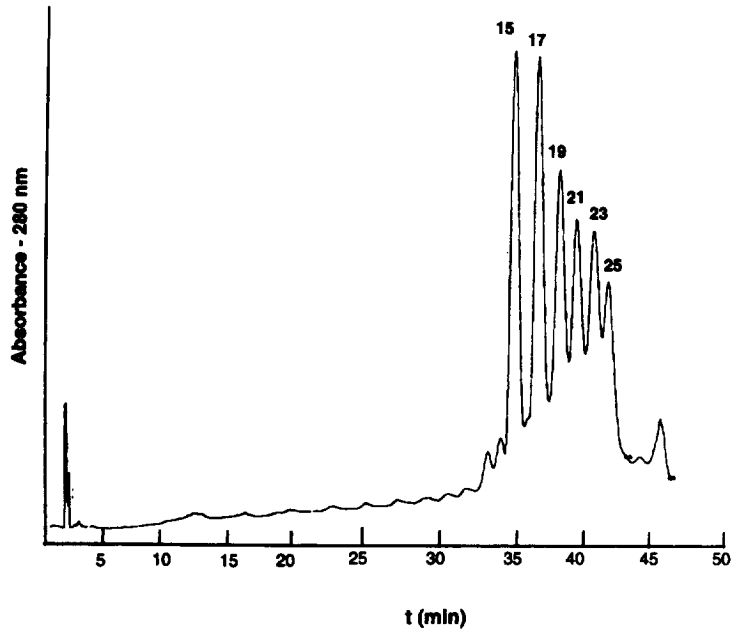

Fig. 3. Ion-exchange HPLC of a mixture containing six SODNs (15–25 mers) differing in length by 2 nucleotides. Chromatographic conditions were as follows: Buffer A was 50 m*M* Na_3PO_4 (pH 8.1), buffer B was 1.0 *M* NaSCN, buffer C was CH_3CN; the flow-rate was 1 ml/min, and a linear gradient of 0.8%/min buffer B was applied over 50 min. Buffer C was kept constant at 30%. A Dionex (Sunnyvale, CA, USA) Nucleopac PA-100 (250×4 mm) column was used, and the detector was set at 280 nm.

length by one nucleotide; thus, CGE is capable of providing baseline resolution for the analysis of SODNs. The average resolution between peaks in the electropherogram represented in Fig. 5 is 1.95. Resolution (R_s) is defined here as $2(t_y - t_x)/(W_y + W_x)$, where y and x refer to the peaks of interest, t is

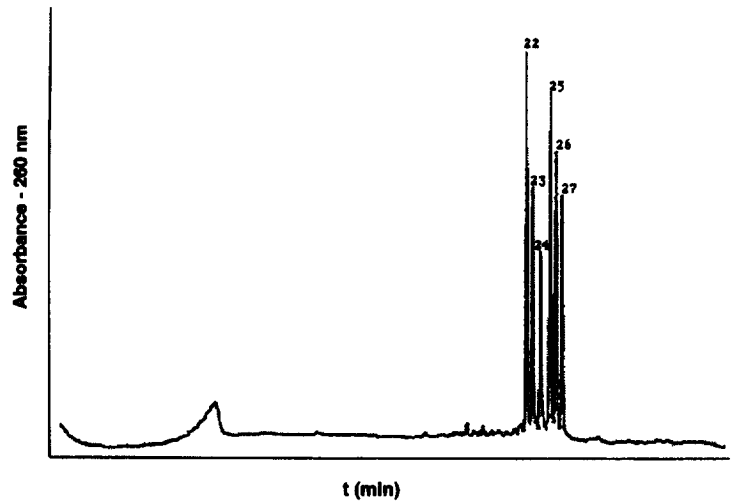

Fig. 4. Electropherogram of a mixture of six ODNs (22–27 mers) differing in length by 1 nucleotide. Sample concentration was 0.2 absorbance units (AU)/ml. Electrophoresis was conducted with an electrokinetic injection at −5 kV for 5 s, and a constant running voltage of −10 kV was used. Gel matrix and running buffer consisted of 10% Micro-Gel in 75 m*M* Tris-phosphate–10% methanol (pH 7.5) with 50 cm × 100 μm I.D. capillaries.

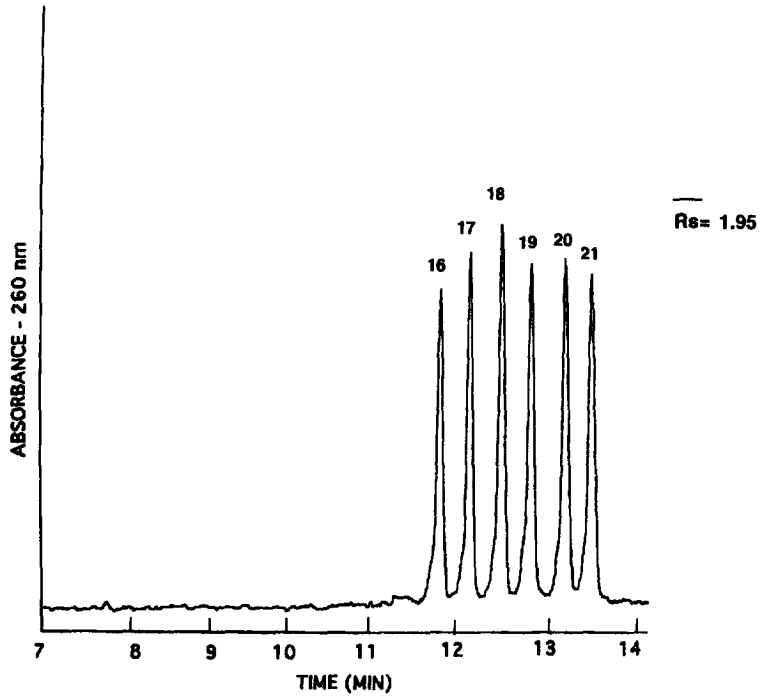

Fig. 5. Electropherogram of a mixture of six SODNs (16–21 mers) differing in length by 1 nucleotide. Sample concentration was 0.5 AU/ml. Electrophoresis was conducted with an electrokinetic injection at −8 kV for 5 s, and a constant running voltage of −22 kV was used. Gel matrix and running buffer consisted of 10% Micro-Gel in 35 m*M* Tris–5.6 m*M* H_3BO_3-15% ethylene glycol (pH 9.0) with 50 cm × 100 μm I.D. capillaries. The average resolution (R_s) between the six major peaks is 1.95.

retention time in decimal min and *W* is the peak width in decimal min at the base.

2.1. Capillary gel electrophoresis used in conjunction with preparative HPLC

Preparative HPLC is often used to purify both ODNs and DNA analogs. In fact, HPLC is critical for the purification of SODNs [20]. CGE can be used to determine the effectiveness of a particular HPLC method chosen for the purification of synthetic oligonucleotides. Fig. 6 is a chromatogram that illustrates an HPLC purification of a DNA analog. At 31.5 min, fractions (six total) were collected, and the purity of each fraction was determined by CGE. Fig. 7 illustrates six different electropherograms corresponding to the fractions collected from the HPLC purification represented by the chromatogram in Fig. 6. The CGE analysis of this purification revealed that fractions 2 and 3 were the most pure of the those collected. This evaluation process has become routine in our laboratory, for it has provided a means to determine the most efficient HPLC parameters necessary for the purification of a given oligonucleotide.

3. The preparation and testing of gel-filled capillaries

The gel matrix used in the preparation of our gel-filled capillaries consists of 10% Micro-Gel hydrated in 35 m*M* Tris–5.6 m*M* H_3BO_3–15% ethylene glycol (EG), pH 9.0. Micro-Gel is an entangled polymer that was originally developed by Applied Biosystems (ABI) [3]. Presently, Perkin-Elmer (PE), AB division (Foster City, CA, USA), sells Micro-Gel 100. Micro-Gel 100 consists of a 50 μm I.D. capillary filled with 8.5% Micro-Gel hydrated in 75 m*M* Tris-phosphate (TP)–10% methanol, pH 7.5. The buffer system used for Fig. 4 is the same as that used for Micro-Gel 100, except that the

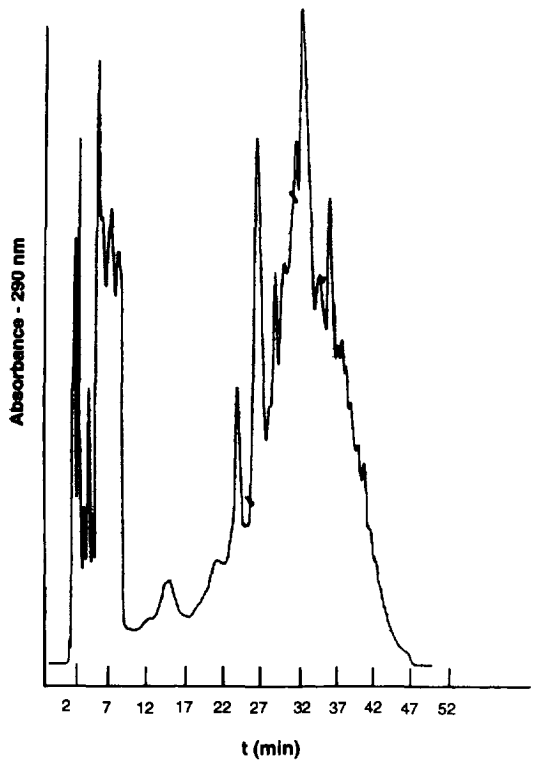

Fig. 6. Ion-exchange preparative HPLC of a crude DNA analog solution. Chromatographic conditions were as follows: buffer A was 10 m*M* NaOH, buffer B was 1.5 *M* NaCl–10 m*M* NaOH (pH 12.0); the flow-rate was 2 ml/min, and a linear gradient from 15 to 45% buffer B was applied over 40 min (0.75%/min). A Pharmacia (Piscataway, NJ, USA) Mono-Q HR 10/10 (100×10 mm) column was used, and the detector was set at 290 nm.

capillary gel used for Fig. 4 had a polymer concentration of 10%, and a 100 μm I.D. capillary was used instead of a 50 μm. We have found that 100 μm I.D. capillaries result in larger sample injections which enhances detection. However, there are limitations on sample injections due to zone broadening effects [21,22]. In other words, as the sample size increases, resolution can decrease due to a general increase in the peak width.

3.1. Native buffers versus denaturing buffers

There is another important difference between Micro-Gel 100 capillaries and those that we prepare besides the difference in the capillary I.D. Each system utilizes different hydrating and running buffers. The buffer system (75 m*M* TP–10% methanol, pH 7.5) chosen by PE-AB division could be described as a native buffer, for these buffer conditions will promote the formation of secondary structure [5]. The ability for oligonucleotides to form secondary structures or compression artifacts is a function of the oligonucleotide's nucleotide sequence [10,23,24]. Compression artifacts can be specifically defined as hairpin loops or generally defined as any occurrences of hydrogen bonding between adjacent nucleotides (Fig. 8A and Fig. 8B). Cations and a buffer pH between 7.0 and 7.5 gives rise to compression artifacts.

To counteract the tendency for single-stranded oligonucleotides to form compression artifacts, we have raised the pH of our buffer to 9.0, and we routinely perform electrophoresis at 55°C. These conditions disrupt most hydrogen bonding; however, we have discovered that oligonucleotides high in guanine (G) content require pre-heating at 95°C for 5 min. G-rich oligonucleotides are capable of forming both intramolecular hydrogen bonds as well as intermolecular hydrogen bonds in which up to four strands can orient themselves either parallel or antiparallel to one another [25–27]. The hydrogen bonds for these higher-order inter-molecular secondary structures occur between G nucleotides that are adjacent to one another, and the presence of particular cations, i.e., K^+, Na^+, NH_4^+, Ca^{2+} and Mg^{2+} stabilizes them. We have observed intermolecular hydrogen bonding between G-rich oligonucleotides that have as few as 4 G nucleotides in sequence. For example, the oligonucleotide with the following nucleotide sequence:

5′-AAC GTT GA G GGG CAT-3′

gave two distinct peaks on electropherograms and two bands on denaturing PAGE (data not shown). The faster migrating peak/band corresponded to a single-stranded 15-mer while the slower moving peak/band corresponded to a 30-mer. After preheating the sample containing the above 15-mer to 95°C, the peak/band corresponding to the 30-mer disappeared indicating that this peak/band was actually a duplex stabilized by hydrogen bonds between G nucleotides. Thus, neither a pH 9.0 buffer utilized with CGE, nor 20% PAGE in 7 *M* urea were able to disrupt hydrogen bonds that occurred in the above

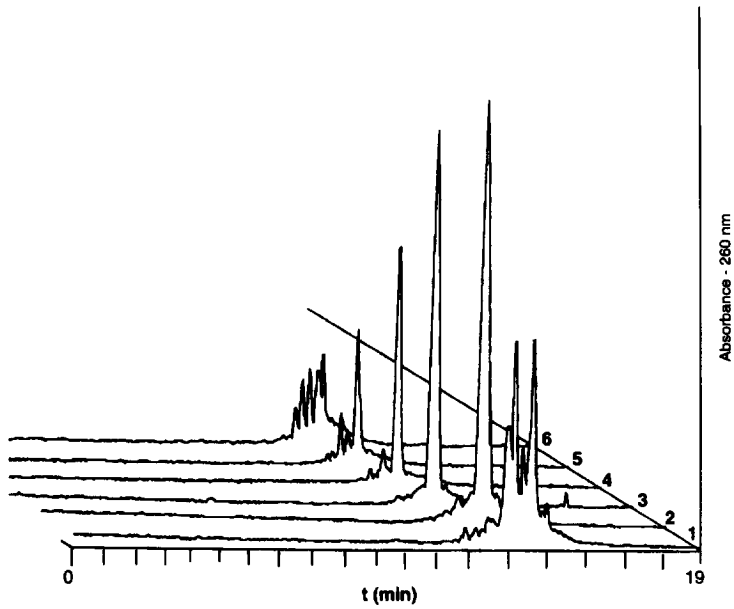

Fig. 7. Electropherograms of six fractions collected at approximately 31 min from the ion-exchange preparative HPLC illustrated in Fig. 6. Electrophoretic conditions were the same as those used for Fig. 5.

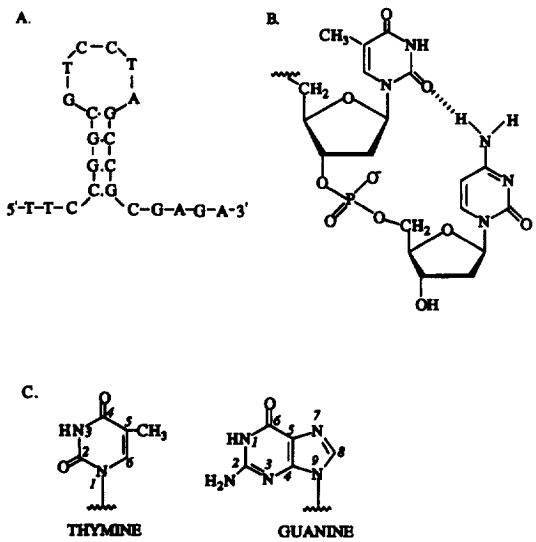

Fig. 8. Possible secondary structures: (A) hairpin loop in a single-stranded oligonucleotide and (B) hydrogen bonding between two adjacent nucleotides (C and T) on the 3′ end and (C) the molecular structure of thymine and guanine. At pH 10.0 the NH at position 3 on thymine and position 1 on guanine looses a proton which confers a negative charge on each of these molecules.

G-rich oligonucleotide when preheating the sample solution was omitted.

As of yet, an electrophoretic system does not exist that will universally denature all oligonucleotides. A buffer that is stable at pH 10.0 or greater would disrupt all secondary structures caused by hydrogen bonds; however, the pK_a values of T and G are approximately 10.0 (Fig. 8C). These nucleotides become ionized at pH 10.0 or greater, and consequently, the overall charge and q/m of oligonucleotides rich in T or G would increase. Any effects on the migration properties of oligonucleotides caused by this potential increase in charge remains to be seen, for we have not found a suitable buffer of pH 10.0 or higher in conjunction with CGE and the analysis of oligonucleotides.

3.2. Testing the precision of gel-filled capillaries

Our CGE system (10% Micro-Gel; 35 m*M* Tris–5.6 m*M* H_3BO_3–15% EG, pH 9.0 in 100 μm I.D. capillaries) is routinely tested for its consistency to resolve oligonucleotides like the separation illustrated in Fig. 5. Fig. 9 represents an electropherogram of a standard solution that we utilize to test

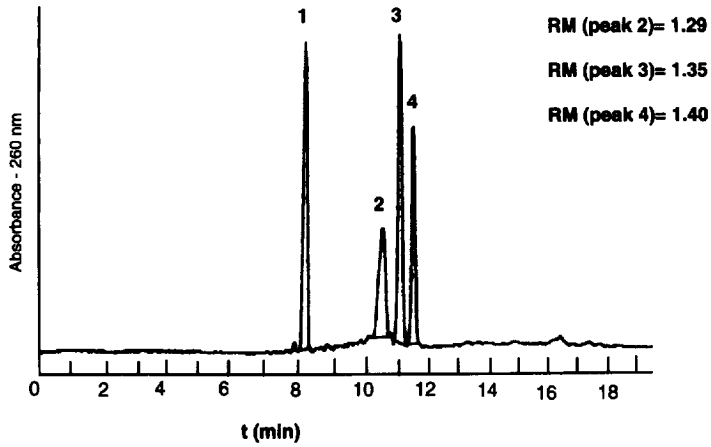

Fig. 9. Electropherogram of a standard used to check batch to batch consistency. Peak 1 is the internal standard $(dT)_{10}$, peak 2 is a $(dC)_{16}$ SODN, peak 3 is $(dT)_{18}$ SODN and peak 4 is a $(dA)_{18}$ SODN. Relative mobility (RM) for peaks 2, 3 and 4 was calculated by dividing their migration times by the migration time of the internal standard. Electrophoretic conditions were the same as those used for Fig. 5.

batch to batch consistency. The four main peaks in Fig. 9 represent four different homooligodeoxyribonucleotides. Peak 1 is a $(dT)_{10}$ ODN and is the reference peak. Relative mobilities (*RM*) of the three additional peaks are calculated by dividing the migration time for the peak of interest by the migration time of the reference peak, $(dT)_{10}$. For example, peak 2 in Fig. 9 is a $(dC)_{16}$ SODN and its *RM* is 10.58 min/8.22 min=1.29. Similarly, peak 3, a $(dT)_{18}$, SODN, had a migration time of 11.13 min. Its *RM* is 11.13 min/8.22 min=1.35, and the *RM* of peak 4, a $(dA)_{18}$ SODN is 11.52 min/8.22 min= 1.40. Table 2 represents *RM* data accumulated over a 6-month period from electropherograms of this standard mixture. These data were collected from several different batches of gel-filled capillaries. The statistical data from this table (R.S.D.<2.5%) indicate that with a reference compound, serving as an internal standard, this system exhibits the precision necessary for quantitative analysis.

4. Generating a standard curve for the analysis of antisense oligonucleotides

The fact that homooligodeoxyribonucleotides of the same length migrate with different mobilities [6–8] gives rise to difficulties in determining the length or size of a mixed-nucleotide oligonucleotide. In the electropherogram represented in Fig. 9, peaks 3 and 4 are two different SODNs, $[(dA)_{18}$ and $(dT)_{18}]$ of the same length. Even though they are the same length, they clearly migrate with different mobilities and in fact, are baseline resolved. It is generally recognized that if electrophoresis is utilized as an analytical tool for the analysis of oligonucleotides, a series of homooligodeoxyribonucleotides of appropriate lengths must be synthesized and their electrophoretic mobilities established [6,8,28]. After the mobilities of the homooligodeoxyribonucleotides are determined, a series of equations can be applied to predict the *RM*s of mixed-nucleotide oligonucleotides. Fig. 10 illustrates a linear relationship between the *RM*s and the lengths of each of the homooligodeoxyribonucleotides. The following line equation describes this relationship: $t'(Yn)=sn+a$, where n is the number of nucleotides present in the oligonucleotide, Y represents one of the four nucleotide bases (A, G, C or T), $t'(Y)=RM$, s is the slope of the line and a is the y intercept. The values for s and a can be obtained from the graph in Fig. 10. Thus, the *RM* for a specific homoligodeoxyribonucleotide of length n can be described as follows. For:

$$Y = A, t'(An) = 0.035370n + 0.65216; \quad (1a)$$

$$Y = G, t'(Gn) = 0.035600n + 0.66507; \quad (1b)$$

Table 2
Accumulated *RM* data for CGE standard gathered over six MOS on several different gel-filled capillaries

	RM		
	$(dC)_{16}$	$(dT)_{18}$	$(dA)_{18}$
	1.3072	1.3824	1.4394
	1.3176	1.3889	1.4468
	1.3036	1.3738	1.4296
	1.2986	1.3616	1.4177
	1.3059	1.3796	1.4335
	1.2972	1.3616	1.4177
	1.2793	1.3400	1.3924
	1.2729	1.3301	1.3823
	1.2805	1.3374	1.3912
	1.3029	1.3717	1.4266
	1.2876	1.3514	1.4047
	1.2574	1.3122	1.3609
	1.2642	1.3189	1.3656
	1.2598	1.3128	1.3628
	1.3053	1.3768	1.4343
	1.2805	1.3433	1.3962
	1.2610	1.3166	1.3667
	1.2546	1.3084	1.3576
	1.2954	1.3625	1.4165
	1.2929	1.3599	1.4152
	1.2749	1.3355	1.3870
	1.2889	1.3490	1.4071
	1.3392	1.4153	1.4783
	1.2871	1.3534	1.4015
	1.2758	1.3416	1.3883
	1.3216	1.3955	1.4572
$\bar{X}$	1.2889	1.3531	1.4068
S.D.	0.02118	0.02788	0.03149
%R.S.D.	1.64	2.06	2.24

RM for each homooligodeoxyribonucleotide [$(dC)_{16}$, $(dT)_{18}$ and $(dA)_{18}$] is calculated by dividing its migration time by the migration time of the internal standard [$(dT)_{10}$] (see Fig. 9).

$$Y = C, t'(Cn) = 0.033185n + 0.65653; \quad (1c)$$

$$Y = T, t'(Tn) = 0.034865n + 0.64602; \quad (1d)$$

The graph in Fig. 10 is reliable for the determination of *RM* values of homooligodeoxyribonucleotides in the size range 10–25-mers.

To determine the *RM* of a hetero oligonucleotide, the following equation [8] is applied:

$$t'(TtAaCcGg) = t/n \cdot t'(Tn) + a/n \cdot t'(An) + c/n \cdot t'(Cn) + g/n \cdot t'(Gn) \quad (2)$$

where $t'(T_tA_aC_cG_g) = RM, t + a + c + g = n$ is the total number of nucleotides in the full-length oligonucleotide. Fig. 11 shows two analyses of hetero oligonucleotides. The nucleotide composition for the sequence analyzed in Fig. 11A is $A_{10}T_{10}C_4$. Applying Eq. 2, the *RM* for this sequence can be calculated as follows. First, after applying 1a and 1c and 1d, respectively, with $n = 24$, the *RM* values of the corresponding homooligodeoxyribonucleotides are: $t'(A_{24}) = 1.5010, t'(C_{24}) = 1.4530, t'(T_{24}) = 1.4828$, and $t'(T_{10}A_{10}C_4G_0) = 10/24(1.4828) + 10/24(1.5010) + 4/24(1.4530) = 1.4893$. Since this calculation is based on the *RM* of the three corresponding homooligodeoxyribonucleotides, $(dA)_{24}$, $(dT)_{24}$ and $(dC)_{24}$, 1.4893 is a theoretical value for the *RM* of the mixed-nucleotide 24-mer analyzed in Fig. 11A. The experimental *RM* for this analysis was 1.5047 (Fig. 11A), a 1.03% difference from the theoretical. Fig. 11B is an analysis of a mixed-nucleotide 11-mer. The nucelotide composition for this oligonucleotide is AT_6C_4 and the calculated *RM* is 1.0277. As indicated in Fig. 11B, the experimental value for the *RM* of this oligonucleotide is 1.0418, 1.4% higher than the theoretical *RM*.

There is a draw-back in the above approach to predicting experimental *RM* values for hetero oligonucleotides. The mobilities of the dG homooligodeoxyribonucleotides must be indirectly obtained. Oligonucleotides consisting solely of dG nucleotides form highly ordered aggregates [29], and these aggregates produce two obstacles. First, the aggregates consist of higher ordered secondary structures that, due to their higher M_r, will migrate at a slower rate than a single-stranded oligonucleotide. Secondly, the optical density at 260 nm decreases significantly when aggregate formation occurs. Since most automated CE units utilize UV detection for oligonucleotide analysis, dG homooligodeoxyribonucleotides become difficult to detect. To prevent these aggregates from forming, electrophoresis must be performed at temperatures in excess of 60°C. We are unable to conduct CGE at these temperatures, and so three oligonucleotides, $(dAdG)_8$, $(dAdG)_9$ and $(dAdG)_{10}$, were synthesized and used to indirectly determine the *RM* values of the homooligodeoxyribonucleotides $(dG)_{16}$, $(dG)_{18}$ and $(dG)_{20}$, and these theoretical *RM* values were used to generate the standard curve for G in Fig. 10.

Fig. 10 illustrates a unique aspect of these gel-

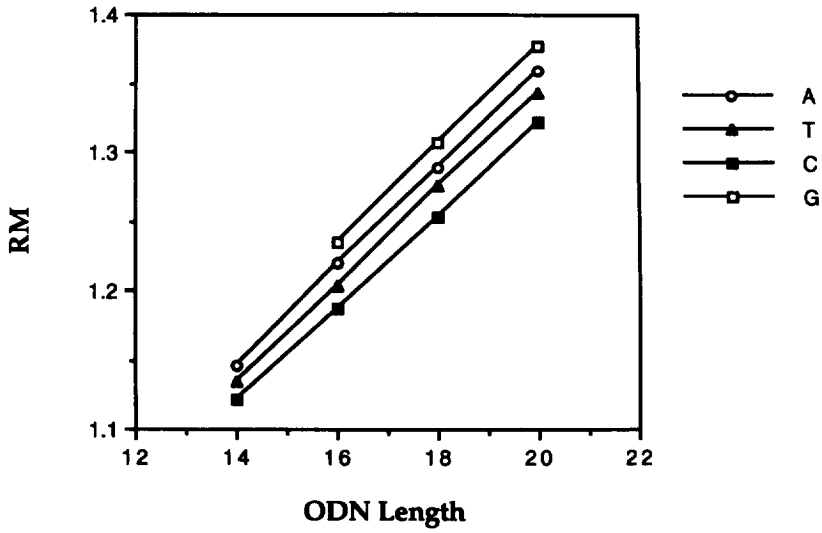

Fig. 10. *RM* data for a series of homooligodeoxyribonucleotide ODNs. A linear correlation exists between the length of the homooligodeoxyribonucleotide and its *RM*. *RM*s were calculated by dividing the migration time of each ODN by the migration time of the internal standard, $(dT)_{10}$.

filled capillaries that distinguishes them from acrylamide-based capillary gels. Each set of homooligodeoxyribonucleotides is separated from one another in a manner that is directly proportional to their M_r or size. For the size range of homooligodeoxyribonucleotides utilized here (14–20-mers) the order of migration was poly: dG>dA>dT>dC, which equals the order of their corresponding M_r values. Table 3 lists the 15 homooligodeoxyribonucleotides represented graphically in Fig. 10 and in Fig. 12. Fig. 12 plots the RM values against the M_r values of each of the homooligodeoxyribonucleotides. The linear correlation coefficient (r) for this plot is 0.993, which suggests that with further studies and improvements to our running condition, i.e., conducting the electrophoresis at temperatures of 60°C or higher, our CGE system may be able to predict the M_r of any given oligodeoxyribonucleotide. It must be noted that the *RM*s which were indirectly determined for the dG homooligodeoxyribonucleotides are probably erroneously low. The M_r of $(dG)_{16}$ is higher than the M_r of $(dC)_{18}$ (Table 3); however, the calculated *RM* for $(dG)_{16}$ is smaller than the experimentally obtained *RM* for $(dC)_{18}$. This discrepancy likewise occurs between $(dG)_{18}$ and $(dC)_{20}$ as indicated in Table 3 and Fig. 12.

Others have conducted similar studies with acrylamide-based electrophoresis systems. For polyacrylamide-gel-filled capillaries the order of migration for the homooligodeoxyribonucleotides was dT>dG>dC>dA with poly(dT) having the longest migration or slowest mobility, and poly(dA) having the fastest mobility and shortest migration time [6]. For PAGE, the order of migration is dG>dT>dA>dC. Poly(dG) moves the slowest on slab gel, while poly(dC) moves the fastest [8].

4.1. Micro-gel and 7 M urea

We have also filled capillaries with 10% Micro-Gel hydrated in a 7 *M* urea–Tris-borate buffer (pH 9.0). The result of adding urea to the buffer is illustrated in Fig. 13. The SODN mixture analyzed in this figure is the same as the mixture analyzed in Fig. 5. Comparing the average resolution in the electropherogram of Fig. 13 to that of Fig. 5, there was an increase in resolution from 1.95 for the capillary gels without urea to 2.61 for gels containing urea. This increase in resolution resulted in shoulders on the primary peaks resolving into separate peaks (Fig. 13). Although the addition of urea resulted in higher resolving gel capillaries that allowed for the de-

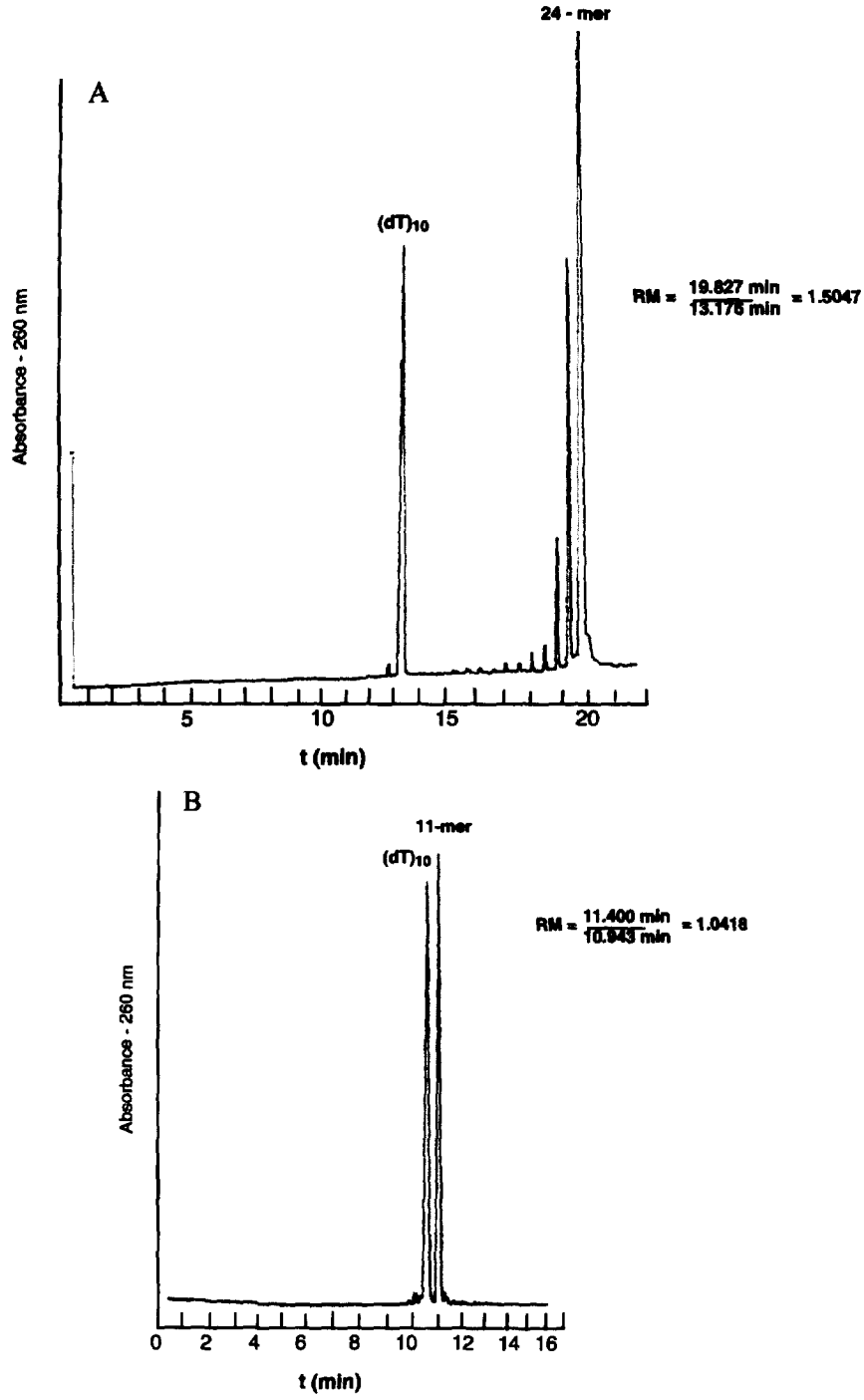

Fig. 11. Electropherogram illustrating the use of an internal standard, $(dT)_{10}$. The *RM* for (A) a mixed-nucleotide 24-mer was calculated (using Eq. 2) to be 1.4893, a 1.03% difference from the *RM* (1.5047) taken from the electropherogram. The *RM* for (B) a mixed-nucleotide 11-mer was calculated (using Eq. 2) to be 1.0277, a 1.37% difference from the *RM* (1.0418) taken from the electropherogram. Electrophoretic conditions were the same as those used for Fig. 5. Experimental *RM* values were determined like those in Fig. 9.

Table 3
M_r and *RM* of 15 ODN homooligodeoxyribonucleotides used to generate a standard curve

Homooligodeoxyribonucleotide	M_r	*RM*
$(dA)_{14}$	3959	1.1215
$(dT)_{14}$	4169	1.1342
$(dA)_{14}$	4295	1.1466
$(dC)_{16}$	4533	1.1874
$(dT)_{16}$	4773	1.2031
$(dA)_{16}$	4917	1.2192
$(dC)_{18}$	5107	1.2529
$(dG)_{16}$	5173	1.2346
$(dT)_{18}$	5377	1.2749
$(dA)_{18}$	5539	1.2888
$(dC)_{20}$	5681	1.3209
$(dG)_{18}$	5827	1.3060
$(dT)_{20}$	5981	1.3427
$(dA)_{20}$	6161	1.3592
$(dG)_{20}$	6481	1.3770

See also Fig. 10 and Fig. 12. The *RM* for each homooligodeoxyribonucleotide was calculated as described in Table 2.

tection of small impurities between the major peaks in the SODN mixture, the durability of these gel-filled capillaries was unsuitable for practical use. They last for approximately 20 runs.

The addition of urea to the gel matrix and the running buffer causes the buffer conductivity to increase which leads to higher running currents. Higher current produces higher temperatures from Joule heating [22] which leads to the formation of air bubbles or gaps and eventual gel failure. To counter this, the running voltage must be decreased in order to keep the current below 10 μA. For gel-filled capillaries without urea, the running voltage is kept constant at -22 kV which results in a current of approximately 5 μA. Decreasing the running voltage consequently leads to longer run times.

The longest SODN in the mixture analyzed in Fig. 5 and Fig. 13 was a 21-mer. The migration time for this SODN doubled from approximately 15 min in Fig. 5 to 30 min in Fig. 13. Thus, the gel-filled capillaries that contained urea lasted half as long as the capillaries filled with gel matrix without urea which are dependable for at least 40 runs. Consequently, we feel that the resilience of gel-filled capillaries without urea outweigh the benefits gained in resolution from gel capillaries that contain 7 *M* urea. However, if a particular analysis requires a higher degree of resolution, then gel-filled capillaries with urea are used.

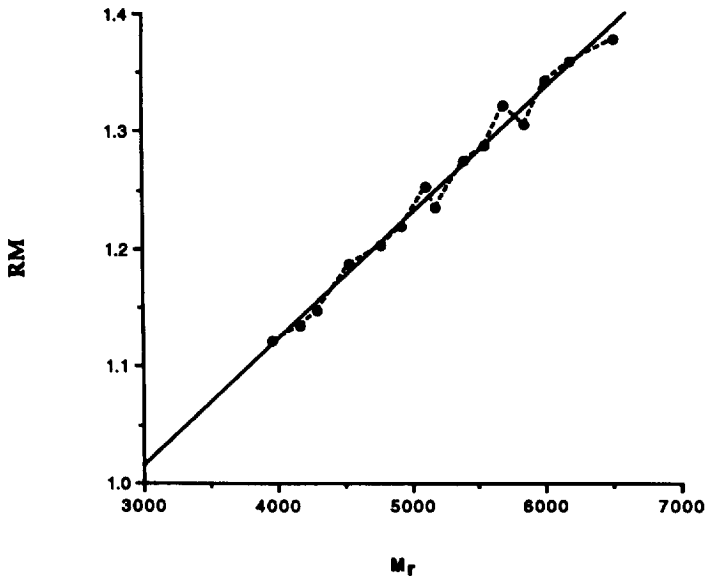

Fig. 12. The linear relationship between the M_r of 15 different homooligodeoxyribonucleotides and their *RM* values ($y = 0.69174 + 1.0764 \cdot 10^{-4}x$). See Table 3 for the identity of each oligonucleotide. $r^2 = 0.987$ for the regression line generated by the data.

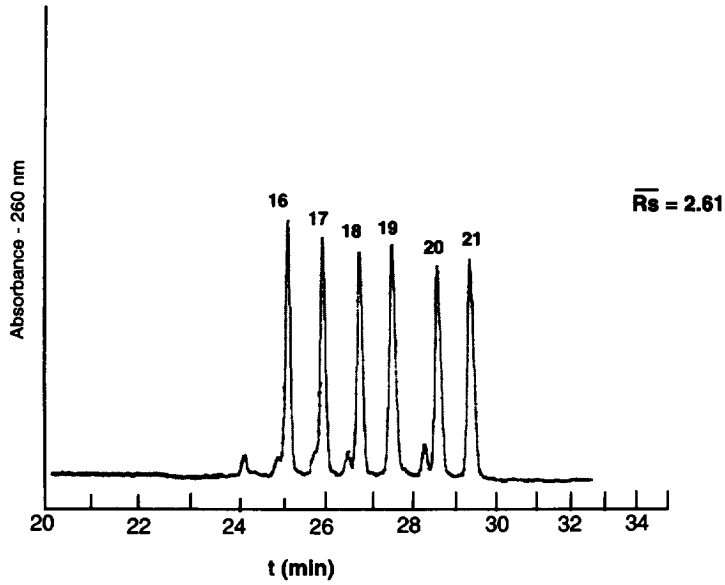

Fig. 13. Electropherogram illustrating increased resolution after the addition of 7 *M* urea to the gel matrix. Sample mixture is the same as that used in Fig. 5. Electrophoresis was conducted with an electrokinetic injection at −8 kV for 5 s, and a constant running voltage of −16 kV was used. Gel matrix and running buffer consisted of 10% Micro-Gel in 35 m*M* Tris–5.6 m*M* H_3BO_3–15% ethylene glycol–7 *M* urea. The R_s between the six major peaks was 2.61. For capillary gels that do not contain urea, the R_s = 1.95 for this sample (see Fig. 5).

4.2. Estabilishing the purity of an antisense oligonucleotide with capillary gel electrophoresis

Presently, we do not use CGE to quantitatively determine the purity of our synthetic antisense oligos. However, others who are involved in developing antisense therapeutics routinely use CGE as a quantitative tool for establishing an oligonucleotide's purity [17]. If used in conjunction with a suitable internal standard, CGE can provide the precision and accuracy necessary for determining the amount of full-length compound present.

The most commonly occurring impurity present in a typical oligonucleotide sample is termed the "$n-1$"-mer. Synthetic antisense oligonucleotides are normally manufactured with an automated synthesizer that builds the DNA chain one nucleotide at a time [20]. This process yields small quantities of "failure sequences" or shorter pieces of DNA. The full-length oligonucleotide is referred to as the n-mer and failure sequences are catagorized as $n-1$, $n-2$, etc., with the $n-1$-mer present as the largest impurity. While most quantitative CGE has been performed with acrylamide-based gels, other gel matrices such as the one described here (with and without urea) should prove equally effective for determining the purity of synthetic antisense oligonucleotides.

5. Capillary gel electrophoresis and the analysis of enzyme digestion assays involving antisense oligonucleotides

Synthetic DNA analogs are designed to be resistant to certain enzymes, called nucleases, which are present in cells for the purpose of hydrolyzing DNA and RNA. Resistance of an antisense oligonucleotide to nuclease activity is critical for any therapeutic activity to occur. One measure of antisense activity occurs when the antisense compound hybridizes to RNA in a complimentary orientation. This short double-stranded nucleic acid can potentially provide a substrate for ribonuclease H (RNase H), an enzyme that hydrolyzes RNA only if the RNA strand exists in a duplex form with DNA or a DNA analog. CGE

has proven useful in determining if an antisense oligonucleotide can elicit the RNase-H-mediated hydrolysis of RNA.

Several studies involving RNase H, RNA and various types of antisense oligonucleotides have been conducted [30–32], but these investigations utilized either PAGE or agarose-gel electrophoresis as methods of analysis. Conducting a CGE analysis of an enzyme digest is viewed as problematic due to restrictions on the sample buffer. Customarily, samples are dissolved in pure H_2O to minimize zone broadening that can occur with UV detection [21]. However, if the sample is dissolved in a solvent that has a lower ionic strength than the running buffer, and if the injection parameters are optimized, zone broadening is readily controlled [22].

Fig. 14 illustrates the analysis of an RNase H digestion assay executed with an ODN and its complimentary strand of RNA. The digestion buffer consisted of 10 m*M* Tris–HCl (pH 7.2) and 10 m*M* $MgCl_2$ in the presence of 1.1 unit of RNase H purchased from Pharmacia (Piscataway, NJ, USA). The diminishing RNA peak over time indicated that the RNA strand was enzymatically cleaved due to its hybridization to the complimentary ODN. This was the expected result. A similar ODN/RNA reaction without the presence of RNase H was also incubated in parallel for 3 h. There was no indication of RNA hydrolysis for this control (data not shown). The injection voltage and injection time for this experiment were increased from 8 V to 15 kV (negative polarity) and from 5.0 to 60.0 s, respectively. This served to counter any zone broadening that may have resulted from salts present in the sample buffer.

Fig. 15 represents the results of a similar experiment conducted with a DNA analog, that we are presently studying, in place of the ODN used in Fig. 14. The RNA peak for this experiment was identified

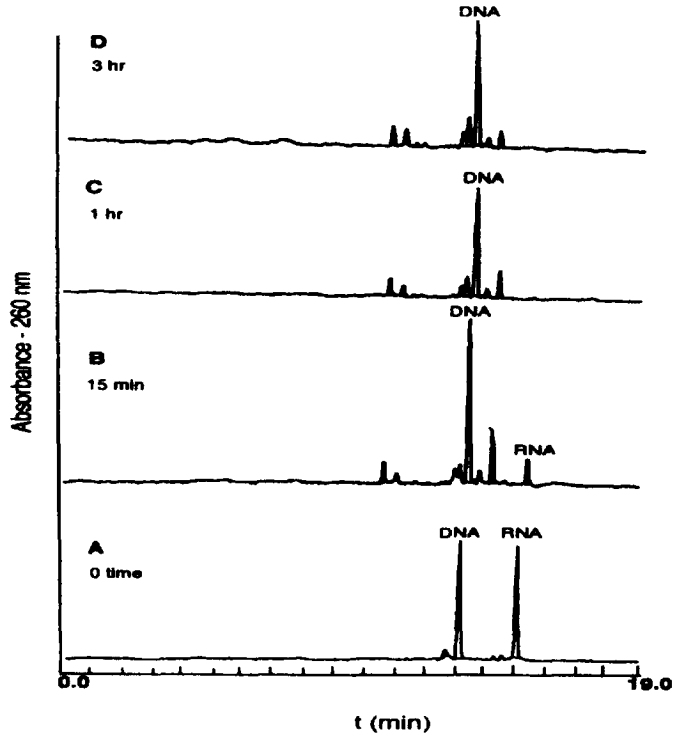

Fig. 14. CGE analysis for an RNase H digestion of an ODN/RNA duplex. Sample (1.0 AU/ml) was buffered in 10 m*M* Tris–HCl (pH 7.2)–10 m*M* $MgCl_2$. Electrophoresis was conducted with an electrokinetic injection of 15 kV for 60 s, and a constant running voltage of −22 kV was used. The gel matrix and running buffer were the same as that used for Fig. 5. Sample was incubated at room temperature (RT) with 1.1 units of RNase H. Electropherograms were obtained at (A) 0 min, (B) 15 min, (C) 1 h and (D) 3 h.

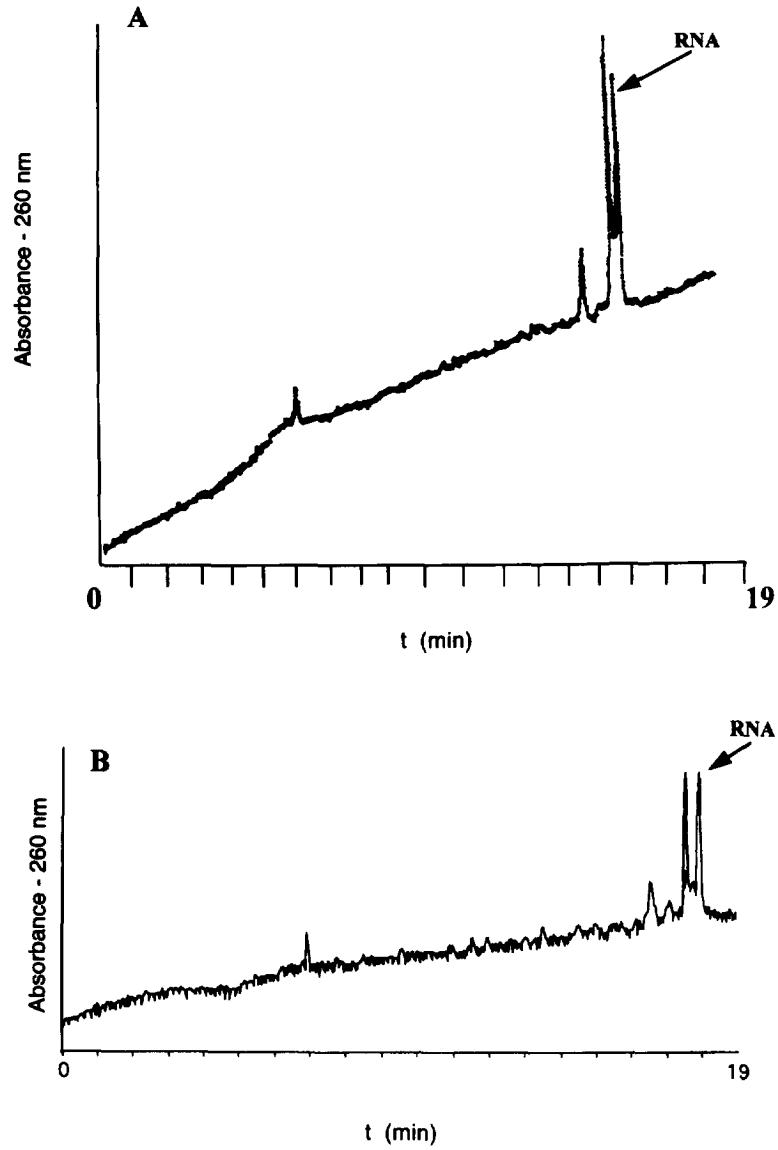

Fig. 15. CGE analysis for an RNase H digestion of a DNA analog/RNA duplex. Buffer conditions and electrophoresis were the same as those used for Fig. 14. Electropherograms were obtained at (A) 0 min at RT and (B) after sample was incubated at 37°C for 24 h.

in a separate analysis by its *RM* relative to an internal standard, $(dT)_{10}$ (data not shown). The experimental conditions for this digestion assay were the same as those used for the ODN/RNA assay of Fig. 14. A control sample consisting of the DNA analog/RNA duplex without RNase H was also run in parallel. This particular antisense DNA analog, did not evoke an RNase-H-mediated hydrolysis of RNA. After 24 h (Fig. 15B), the RNA peak was relatively unchanged. In contrast, the RNA peak completely disappeared for the ODN/RNA digestion assay after only 1 h. (Fig. 14C).

One advantage that CGE has over conventional slab-gel electrophoresis for the analysis of an en-

zyme digestion like the one described here is that samples can be injected directly from the reaction mixture. No sample work-up was necessary since the sample buffer (10 m*M* Tris–HCl–10 m*M* $MgCl_2$) was lower in ionic strength than the running buffer (35 m*M* Tris–5.6 μM H_3BO_3–15% EG). Injecting directly from the reaction vial allows continuous monitoring of the enzymatic assay without the inconvenience of aliquoting samples at various time intervals for a later analysis by slab gel electrophoresis. Conventional PAGE or agarose gel electrophoresis requires samples to be dissolved in formamide or some other suitable denaturant; thus, the digestion buffer must first be evaporated. This requirement introduces the possibility of sample contamination. Due to the ubiquitous presence of ribonucleases in general, RNA can be readily hydrolyzed, and so excessive sample handling, i.e., aliquoting from the reaction mixture into separate Eppendorf tubes, could result in the undesirable hydrolysis of the RNA strand. This CGE analysis, designed to determine if a particular DNA analog could activate an RNase-H-mediated hydrolysis of RNA, could be extended to other enzyme digestion experiments.

6. Conclusions

Although the primary goal for the CGE method described here is to determine the purity of antisense oligonucleotides after their chemical synthesis, this separation technique has potential for a range of applications within the field of antisense technology. The advantage of CGE for the analysis of certain DNA analogs such as SODNs over HPLC lies in its superior resolving power. CGE is able to separate two SODNs that differ in length by 1 nucleotide, whereas HPLC presently cannot. The advantages of CGE over conventional slab gel electrophoresis stem from its on-line detection system which does not require an indirect post-electrophoretic chemical reaction such as staining. Also, the ability for some samples to be injected directly into the capillary gel without prior sample preparation provides another advantage over slab gel electrophoresis.

The electrokinetic injection required for CGE analysis has, in the past, been viewed as an encumbrance [21,22]. We feel that this unavoidable method for sample introduction into the gel can be viewed as an advantage because of the flexibility that the electrokinetic injection provides. The electrokinetic injection has two parameters (voltage and time), that can be adjusted to ensure reproducible peak shape and to discourage the propensity for zone broadening that can occur when samples are not dissolved in pure water. The latitude of the electrokinetic injection permits us to sample crude solutions of synthetic oligonucleotides before they are HPLC purified, and it allows us to inject directly from in vitro enzyme digestion reaction mixtures.

While others have developed similar CGE systems for the analysis of DNA analogs [17], our unique system possesses the potential for more than just providing the purity of synthetic oligonucleotides. Micro-Gel is an entangled polymer that does not require additional polymerization after it has been hydrated and pumped into the capillary. It is stable above pH 8.3 which makes it compatible with a wider range of buffers than polyacrylamide. We have shown that buffers above pH 9.0 are sufficient for the denaturation of single-stranded oligonucleotides that do not possess the tendency to form aggregates (caused by stretches of G within the sequence), and that urea is not necessary to provide baseline resolution for oligonucleotides that differ in length by one nucleotide. Our preliminary data indicates that this system could be used to verify the M_r of oligonucleotides. The linear correlation between the RM of an oligonucleotide and its M_r is evidence that our system has a greater capacity to denature single-stranded oligonucleotides than other electrophoretic systems which do not enjoy this attribute. Results like this give insight into the potential that CGE possesses as the field of antisense therapeutics pushes separation technologies beyond their present limits.

7. Notation

CGE	capillary gel electrophoresis
RM	relative mobility
ODN	phosphodiester deoxyoligonucleotide
SODN	phosphorothioate DNA

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