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Polymer solutions as a pseudostationary phase for capillary electrochromatographic separation of DNA diastereomers

The solutions of linear polymers traditionally used for DNA separation have been employed for the capillary electrophoresis (CE) of diastereomers of chemically modified DNA. The selectivity of diastereomeric separation of the phosphorothioate (PS) and 2'-*O*-methylated (2-OMe) PS oligonucleotides depends on the nature of the polymer additive in the CE background electrolyte. The selectivity of separation for different polymers increases in the line: linear polyacrylamide < polyethylene glycol < polyvinyl pyrrolidone. The separation of oligomer diastereomers was shown to be primarily based on the hydrophobic interaction with the polymer network that acts as a pseudostationary phase. While lowering the temperature resulted in improved separation, the addition of organic modifiers such as formamide, methanol or acetonitrile counteracts the solute adsorption on the polymer network, and decreases the selectivity of DNA diastereoseparation. The effect of molecular mass and concentration of the polymer on the separation selectivity was investigated.

Keywords: Phosphorochioate / Diastereomers / Pseudostationary phase / DNA separation

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1 Introduction

The chromatographic separation of enantiomers requires a chiral selector that is used either directly or indirectly. Direct separation is based on the formation of transient diastereomeric complexes between enantiomers and a chiral selector (stationary phase, mobile phase additive). Subtle differences in stability of diastereomeric complexes lead to the enantiomeric separation. Alternatively, an indirect approach utilizes a chirally pure derivatization agent to introduce an additional chiral center to the enantiomeric molecule in order to create a pair of permanent diastereomers. Due to differences in physical and chemical properties of diastereomers, they can be separated on a conventional achiral stationary phase [1, 2].

In the last decade capillary electrophoresis (CE) has established itself as a complementary method to chromatography. CE is particularly suited for certain applications, *e.g.*, size-dependent separation of DNA. The high efficiency of CE is an advantage in the area of chiral separations, where enantiomers are usually separated with low selectivity. Despite numerous reports, only a few papers have been published dealing with the indirect enantiomeric separation by CE [3]. It has been shown that there are few differences in effective electrophoretic mobility of diastereomers, and only in exceptional cases, when the variation in pK_a of the diastereomers affects their ionization, can the baseline separation of isomers be obtained [4–6]. Addition of micellar surfactant is usually necessary to achieve the diastereomeric resolution; separation is driven by different interaction of diastereomers with hydrophobic achiral micelles (micellar electrokinetic chromatography) [3]. Schutzner *et al.* [5, 7] separated diastereomeric derivatives of several amino acids in free-zone CE. They suggest utilizing a nonchiral polyvinyl pyrrolidone (PVP) additive in order to enhance the separation selectivity of the CE system. In the case of relatively hydrophobic (+)-*O,O*'-dibenzoyl-L-tartaric anhydride derivatives of amino acids, the separation of diastereomers improved significantly. However, little or no improvement in separation was observed for more polar (+)-*O,O*'-diacetyl-L-tartaric anhydride derivatives. The authors speculate that the polymer network gives rise to intermolecular interaction with analytes and acts in this way as a kind of pseudophase, similar to chromatography.

Polymer matrices are traditionally used for size separation of DNA. Only the friction forces are considered to play a role in the separation mechanism resulting in size-

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Abbreviations: DMT, dimethoxytrityl; **LPAA**, linear polyacrylamide; **2-OMe**. 2'-*O*-methylated; **PS**, phosphorothioate

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dependent migration of DNA strands [8, 9]. Cross-linked polyacrylamide gel with denaturine additives, such as a urea, works in good agreement with this hypothesis. Nevertheless, separation of short homooligodeoxynucleotides of the same length was described for polyacrylamide (PAA) slab gel [10] with migration times C<A<T<G. Guttman *et al.* [11] found a different order of oligonucleotides, A<C<G<T, using a capillary filled with linear polyacrylamide (LPAA). DeDionisio and Lloyd [12] used a replaceable non-PAA sieving matrix for CE separation of oligonucleotides. They found that short homooligonucleotides of the same length can be easily separated; the order of migration times was C<T<A<G. Generally, migration order differs for different kinds of polymers. This observation cannot be clearly interpreted to be due to either an influence of an ssDNA secondary structure or the differences in effective charge of DNA chains. These results indicate that solute-polymer interaction plays a role in the mechanism of oligonucleotide separation. In comparison, mononucleotide retention at RP-HPLC was found to be C<G<T<A [13]; increased retention correlates with higher mononucleotide hydrophobicity.

The interaction of chemically modified phosphorothioate (PS) and especially 2-*O*-alkyl PS oligonucleotides, which are more hydrophobic, with polymer matrix may explain their anomalous migration observed in CE. We found that the migration time of oligonucleotides of the same length and sequence increases significantly in the following order: natural phosphorodiester < PS < alkylated PS oligonucleotide (Belenky *et al.*, unpublished results). This behavior, observed with LPAA and polyethylene glycol (PEG) sieving polymers, cannot be simply explained by an increase in mass/charge ratio of an oligonucleotide.

Chemical modification of DNA, namely the replacement of a single oxygen with sulfur in phosphate oligonucleotide backbone, was introduced to meet the requirements of oligonucleotide-based drugs: to protect the oligonucleotides from attack by nucleases *in vivo*. However, sulfurization not only increases the stability of DNA *in vitro* ([14, 15] Belenky *et al.*, unpublished) and *in vivo* [16], but also creates a chiral center on the phosphate and changes the physical and chemical properties of the modified DNA molecule. As with many natural molecules, DNA and RNA have built-in *in vivo* stereoselectivity, consisting of three or four chiral centers per mononucleotide (on the carbohydrate moiety), respectively. Nonstereoselective introduction of another asymmetrical center into a chirally pure molecule leads to the formation of a diastereomeric pair. Creation of a new asymmetrical center on the phosphate can be considered to be analogous to the process of derivatization of a chirally pure enantiomer by a racemic derivatization agent. The number of diastereomers dramatically increases with the number of phosphorothioate linkages in oligonucleotide backbone. For example, sulfurization of the 25-mer with 24 internucleotide linkages creates 2^{24} isomers, *i.e.*, 16, 777, 216 diastereomers. The separation of such an extreme number of isomers is not feasible by any present technique.

Working with oligonucleotides we found that it is more difficult to separate modified PS oligonucleotides than natural phosphorodiester ones. This was shown to be due to extensive peak broadening of PS-modified oligonucleotides in RP-HPLC as well as in CE with a sieving matrix. Other authors observed similar peak broadening while purifying PS oligomers by ion exchange or RP-HPLC [17], where the partial separation of diastereomers takes place. In the case of short PS oligonucleotides the diastereomeric separation was achieved using RP-HPLC with the C18 stationary phase [18–20]. Differences in hydrophobicity of diastereomers were sufficient to separate the two isomers of dimers, all four isomers of 3-mers, and for certain sequences the separation of all eight isomers of a 4-mer was also achieved.

We used the water-soluble polymers for CE separation of DNA diastereomers as an alternative approach to RP-HPLC. We found that replaceable polymer solutions provide hydrophobic interaction with analytes in a manner similar to CE packed with chromatographic sorbent. Several recently published papers mention the use of either a monolithic polymer bed or replaceable polymers as a stationary phase for capillary electrochromatography (CEC) [21–25]. Replaceable polymers [26] have the potential to make column preparation easier, eliminating the problem with polymer bed shrinkage. The polymer solutions we used do not contain charged species and, thus, do not induce electroosmotic flow (EOF). Therefore, only charged solutes can be separated. Incorporation of charged moieties into the polymer produces EOF, making possible the separation of neutral solutes [26].

We studied the CE separation of PS and 2-*O*-methyl (2-OMe) PS DNA diastereomers using several replaceable polymer matrices. The influence of the nature, molecular mass, and concentration of polymer on separation was investigated. To shed light on the interaction of solutes with the polymer network, the hydrophobicity of background electrolyte was modified by organic modifiers such as formamide, methanol and acetonitrile. The selectivity of separation and retention order of isomers were compared to those obtained by RP-HPLC. Diastereomeric separation by CE was developed to study stereoselective enzymatic digestion of DNA, as well as fast screening of the spontaneous stereoselective oxidation/hydrolysis of phosphorothioate-modified oligonucleotides.

2 Materials and methods

2.1 Chemicals and reagents

Water, acetonitrile, and methanol were HPLC grade (J. T. Baker, Phillipsburg, NJ, USA). Acrylamide, ammonium persulfate, TEMED, and formamide were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA); PEG and PVP were from Aldrich (St. Louis, MO, USA); and Tris and boric acid from Sigma (St. Louis, MO, USA). The PS oligonucleotides (TT, GA, CC, AT, CT, TC, TTC, TTGT; 5' to 3') and 2-OMc PS oligodeoxynucleotides (UU, GAUC; 5' to 3'; see structures in Fig. 1) were synthesized in our lab using an Expedite™ 8909 NASS synthesizer (PerSeptive Biosystems, Framingham, MA, USA), and reconstituted in deionized water. When needed, the dimethoxytrityl protection group was not cleaved from the 5'-end of the oligomer after the last step of synthesis.

2.2 CE

LPAA-filled capillaries were prepared in-house in the following manner: Fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA), 75 μM ID, 365 μm OD, 10–15 cm effective length, and 27 cm total length was bifunctionalized with 3-(methacryloxypropyl)-trimethoxysilane (Petrarch Systems, Bristol, PA, USA). It was then filled with a degassed solution of 15% polymerizing LPAA in 15% v/v formamide media (0.2 M Tris-borate, 2.5 mM EDTA-2Na$^+$ buffer, pH 8.3, containing 7 M urea). Polymerization was achieved using ammonium persulfate/TEMED chemistry. An electric field of 400–500 V/cm was applied, resulting in a current of 10–15 μA [27]. For free-zone CE and CE with replaceable polymer solution, a BioFocus®2000 Capillary Electrophoresis System (Bio-Rad, Hercules, CA, USA) with UV detection was used. For analysis, a 25 cm (20 cm to detection window) coated capillary, 75 μm ID × 375 μm OD (BioCap oligonucleotide from Bio-Rad) was employed. The linear polymers were dissolved in Tris-boric acid buffer, pH 8.3 (0.2 M Tris, 0.2 M boric acid). After each run the polymer was replaced and the capillary was washed with HPLC-grade water. The samples (10^{-5} M) were injected electrokinetically for 1–8 s at 10 kV, and were run at 10 or 15 kV. Separation was performed with a liquid-thermostated column because the decreased temperature was found to improve selectivity of diastereomeric separation. The experiments were performed at 15°C, unless noted otherwise.

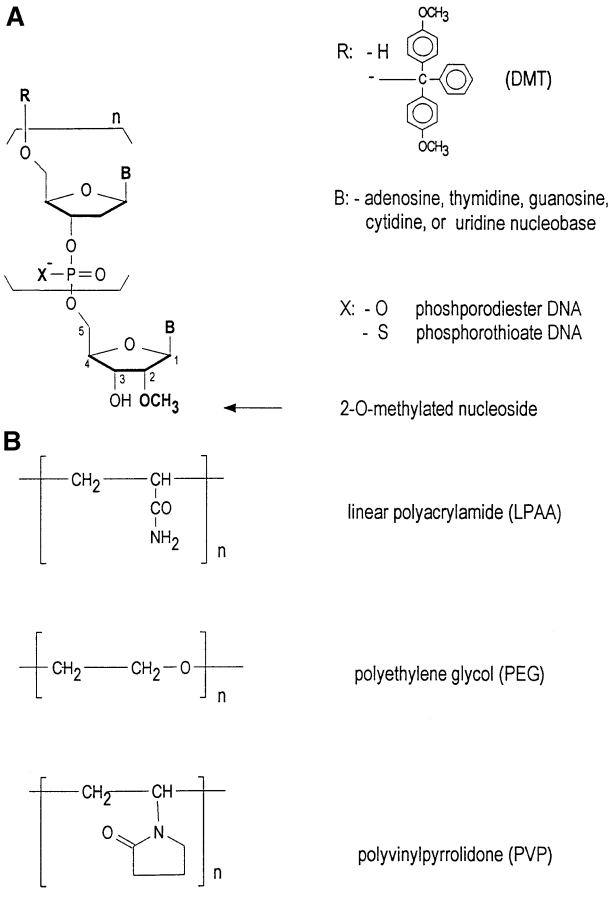

Figure 1. (A) Chemical modification of oligonucleotides and (B) polymer formulas used in this study.

2.3 HPLC instrumentation and separation conditions

HPLC separation of oligomers was performed on a Hewlett Packard 1100 HPLCa system (Palo Alto, CA, USA) composed of a binary pump, degasser, autosampler and diode-array UV-Vis detector. The LichroCart C18, 250 × 4 mm column with 4 × 4 mm C18 guard column (Merck, Darmstadt, Germany) was used for HPLC separations. Mobile phase A contained 0.1 M aqueous triethylammonium acetate, pH 7.1; the acetonitrile was used as mobile phase B. The column was thermostated at 40°C; the mobile phase flow rate was 1 mL. Two different gradients were used for elution of diastereomers; for the mixture of TT, TTC and TTCT (Fig. 3E) the gradient started at 7.5% B, and increased at the rate of 0.15% B/min. For the separation of 2-OMe GAUC diastereomers (Fig. 2E) the gradient started at 5% B and was increased by 0.25% of mobile phase B per min.

3 Results

3.1 Effect of a polymer's additive nature on the diastereomeric separation

As shown in Figs. 2A and 3A, the negligible differences in electrophoretic mobility of modified oligonucleotide diastereomers preclude their separation in free-zone CE. None of the samples investigated (PS oligonucleotides

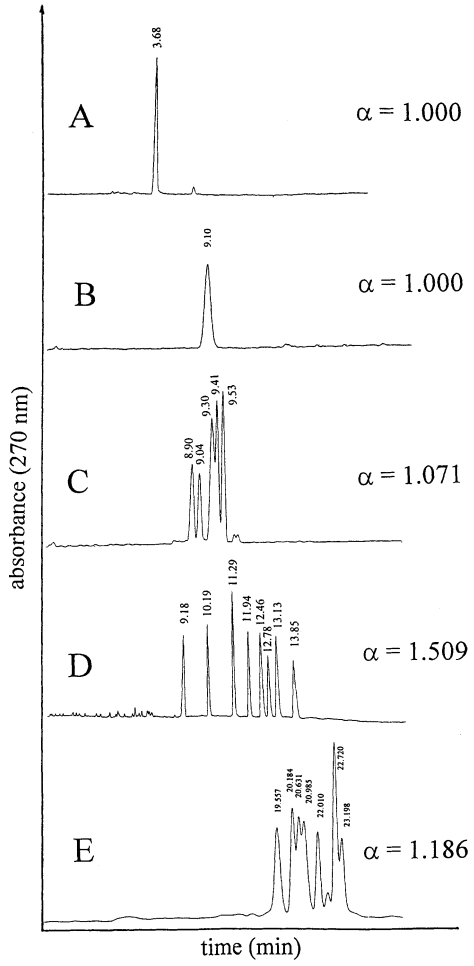

Figure 2. Separation of diastereomers of 2-OMe PS oligonucleotide GAUC in (A) free-zone CE, (B) LPAA matrix, (C) PEG matrix, and (D) PVP matrix. Compare with separation of diastereomers by (E) RP-HPLC. CE separation was performed in (A) 0.2 M Tris-boric acid buffer, (B) the same buffer with 13%T of LPAA, (C) 31.4% PEG 35 000, or (D) buffer containing 4.3% PVP 360 000. For other CE/HPLC conditions see Section 2.2 and 2.3. If the diastereomer peaks in the electropherogram (D) were assigned in (ascending) order of migration time from 1 to 8, their elution order by RP-HPLC (E) would be 1, 5, 2, 4, 6–7, 8, 3, 6.

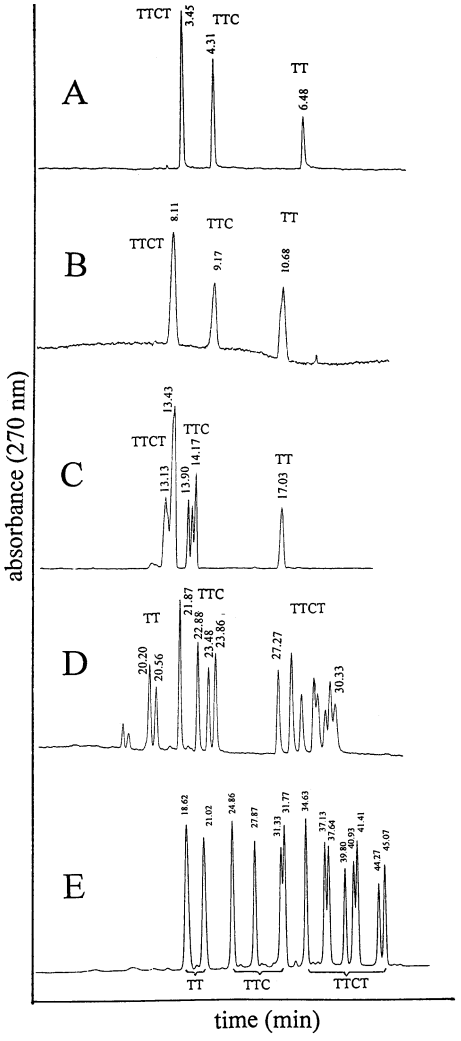

Figure 3. CE separation of diastereomers of PS oligomers: 2-mer TT, 3-mer TTC, and 4-mer TTCT. Separation (A) in free-zone CE using (B) LPAA matrix, (C) PEG matrix, and (D) PVP matrix. Compare with separation and retention order in (E) RP-HPLC. CE separation was performed in (A) 0.2 M Tris-boric acid buffer, (B) the same buffer with 13%T of LPAA, (C) 31.4% PEG 35 000, or (D) buffer containing 14.3% PVP 360 000. For CE/HPLC conditions see Section 2.2 and 2.3. TT diastereomers elute in the same order using (D) CEC or (E) RP-HPLC. The TTC diastereomers changed their retention order from (D) 1, 2, 3, 4 to (E) 1, 4, 3, 2, and the order of TTCT diastereomers in electropherogram (D) changes from 1, 2, 3, 4, 5, 6, 7, 8 to 2, 4, 1, 3, 8, 7, 6, 5 in chromatogram (E). For absolute configuration assignment of diastereomers see [21, 35].

TT, TTC, TTCT, and 2-O-Me GAUC PS oligonucleotide; chemical structures of oligonucleotides are shown in Fig. 1A) were separated into their diastereomers. To achieve the separation of oligonucleotide diastereomers we employed three different polymer solutions. As expected, the separation of diastereomers strongly depends on the nature of the polymer dissolved in CE electrolyte (for polymer structures see Fig. 1B). This fact is demonstrated by the separation of 4-mer 2-O-Me GAUC PS oligonucleotide (Fig. 2). While no separation was observed in the LPAA matrix, some of the diastereomers were separated using PEG, and clearly all eight diastereomers were separated employing PVP solution (Fig. 2D). For comparison, the diastereomeric separation of the same oligonucleotides in the C18 RP-HPLC is shown in Fig. 2E. Separation selectivity α was calculated as a migration time ratio of

the last and the first isomer. Analogous behavior was found for three PS oligonucleotides, as can be seen in Fig. 3. No separation was achieved in free-zone CE, as well as in PAA solution; partial separation of TTCT and TTC diastereomers was found in PEG solution and very good separation of all diastereomers of TT and TTC was achieved using the PVP solution. The eight isomers of TTCT were partially resolved (Fig. 3D) in the PVP-filled capillary.

3.2 Effect of polymer concentration and molecular mass on separation of diastereomers

The concentration of both PVP and PEG was found to have an effect on the selectivity of diastereomeric separation. Seven dinucleotides (TT, AT, TC, CC, GA, CT, 2-O-Me UU) were used for this study. As in the previous experiment, we did not obtain diastereomeric separation of dinucleotides in free-zone CE or LPAA solution. Of the above-mentioned dinucleotides, only partial separation of GA diastereomers and 2-O-Me UU PS diastereomers was obtained in PEG solution. 2-O-Me UU was chosen as a test sample for the experiment summarized in Table 1. Polymer concentration was changed from 7.1 to 31.4% while other parameters of separation were kept constant (sec Section 2.2). The efficiency of CE separation is typically higher than 1 million theoretical plates per meter, which means that selectivity α = 1.010 was sufficient for resolution of approximately R_s = 1.0, and selectivity α>1.015 allow nearly baseline separation. Because of difficulties associated with filling the capillary with viscous matrices, concentration of PEG no higher than 31.4% was used for the experiment.

Good separation of diastereomers was achieved in 7.1% w/v PVP solution (40 000 g/mol molecular mass) for 2-O-Me UU (α = 1.029), GA (α = 1.022), CC (α = 1.021), AT (α = 1.025); TT (α = 1.015), and CT (α = 1.011). All tested dimers were baseline separated by RP-HPLC with the exception of TC. Similarly, only the TC PS 2-mer was not separated into its diastereomers in PVP solution (or any other CE system). Improved separation selectivity of 2-mer diastereomers was achieved using increased PVP concentration (14.3%). However, the TC diastereomers were still not separated. HPLC and CE separation of diastereomers of TC was possible only for TC with attached dimethoxytrityl (DMT), the hydrophobic moiety of which enhances differences between diastereomers [18].

Table 1. Effect of PEG concentration (35 000 g/mol mol. mass) on the separation of 2-O-Me UU diastereomers

[PEG] (%w/v)	MT_1 (min)	MT_2 (min)	Selectivity MT_2/MZ_1
31.4	15.59	15.81	1.014
22.9	11.85	12.00	1.013
14.3	7.75	7.82	1.009
7.1	5.32	5.32	1.000

The changes in separation selectivity of four TTC diastereomers with an increase of PVP polymer concentration are shown in Table 2. Although overall resolution is best at the highest concentration of the polymer, the selectivity calculated as the ratio of migration times of the first and the last migrating isomer MT4/MT1 stays in the range of 7.1–14.3% of PVP constant. The resolution improves because of the shift of the third isomer towards relatively shorter migration time. Complex changes in separation selectivity with the changes in PVP concentration were observed for eight isomers of TTCT, and even a retention switch of some isomers was noticed. The best separation of diastereomers was obtained at the lowest concentration (4.3%) of PVP.

Because the PEG matrix did not give us reasonable separation of 2-OMe GAUC diastereomers (Fig. 2C), two different approaches were used to improve diastercoseparation. Either more hydrophobic polymer additive (PVP) was employed, or the hydrophobicity of 2-OMe GAUC was increased. We used the hydrophobic properties of the DMT group, which serves as a 5'-end protective moiety in DNA synthesis. In the last step of DNA synthesis the deprotection procedure was deliberately omitted in order to keep the DMT group attached to 2-OMe GAUC 4-mer and thus increase its hydrophobicity. Using separation conditions identical to those in Fig. 2C, significantly enhanced separation of diastereomers was obtained (Fig. 4). Separation can further be optimized by varying the concentration of PEG solution. Unlike the 2-mers and 3-mers, the diastereomeric separation of 4-mer improves when decreasing the PEG concentration (see results in Fig. 4).

In addition to concentration, the molecular mass of a polymer influences the selectivity of diastereoseparation. This effect is more pronounced for 4- and 3-mers than for

Table 2. Effect of PVP concentration (360 000 g/mol mol. mass) on the separation of TTC diastereomers

[PVP] (% w/v)	Selectivity MT_2/MT_1	Selectivity MT_3/MT_2	Selectivity MT_4/MT_3	Selectivity MT_4/MT_1
4.3	1.036	1.026	1.006	1.069
7.1	1.039	1.030	1.011	1.086
14.3	1.044	1.025	1.016	1.086

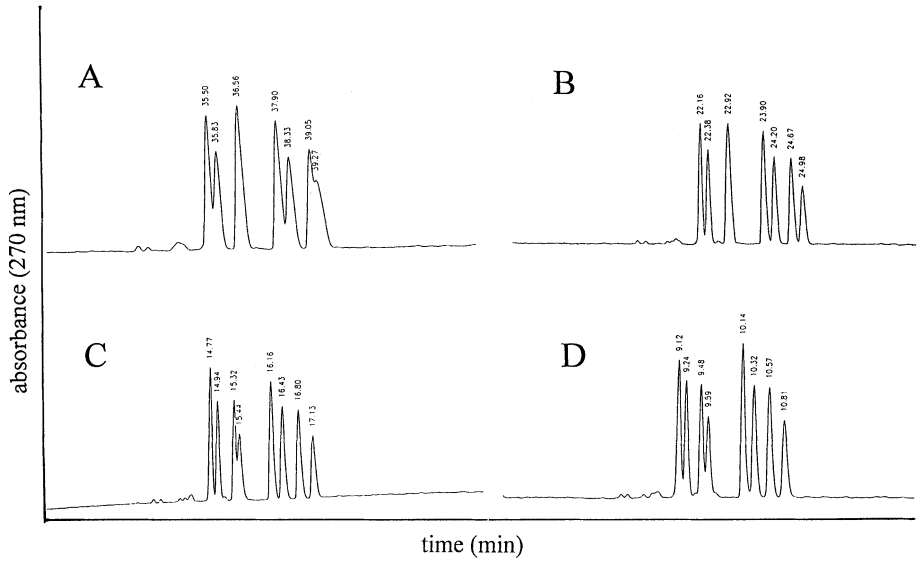

Figure 4. Selectivity of CE diastereomeric separation of 2-OMe GAUC 4-mer (with DMT group) improve with decreasing PEG concentration in buffer (PEG 35 000 g/mol). Selectivity $\alpha_{8/1}$ is calculated as a ratio of migration times of last and first eluting diastereomer MT_8/MT_1: (A) 31.4% PEG, $\alpha_{8/1}$ = 1.106; (B) 22.9% w/v PEG, $\alpha_{8/1}$ = 1.127; (C) 14.3% PEG, $\alpha_{8/1}$ = 1.160, (D) 7.1% PEG, $\alpha_{8/1}$ = 1.185.

2-mers. The 31.4% PEG matrices were prepared using polymers with three different molecular masses. Results are shown in Table 3. The effect of molecular mass (10 000, 40 000 and 360 000 g/mol) of PVP on resolution of TTC diastereomers is shown in Fig. 5. The improvement in separation of TTC and TTCT diastereomers in 7.1% PVP shows a similar trend (see Table 3).

3.3 The effect of organic modifiers on diastereoseparation

Table 4 shows the effect of methanol or acetonitrile in polymer matrix on the diastereoselectivity of CE separation. Three oligonucleotides with the highest selectivity of diastereoseparation were used for this experiment. Another modifier we tested, formamide, has an even weaker ability to counteract oligonucleotide interactions with polymer matrix than methanol (data not shown).

4 Discussion

4.1 Polymer matrix, background electrolyte, and solute hydrophobicity effects on DNA diastereoseparation

The diastereomeric separation of modified oligonucleotides using HPLC, mostly by RP-HPLC, was described in the literature [18–20, 28]. Short oligonucleotides (2-, 3-, and 4-mers) can be separated into their diasteromers due to hydrophobic interaction; triethylamine is often employed as ion-pair agent in the RP-HPLC system. We employed CE for separation of diastereomers (Fig. 2 and 3). From Fig. 2A and 3A it is evident that differences in electrophoretic mobility of isomers are not large enough to permit diastereomeric separation in free-zone CE. In

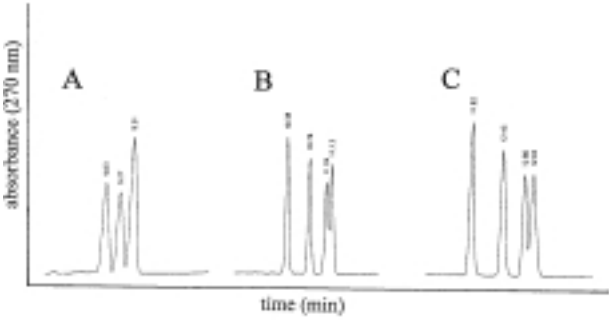

Figure 5. Effect of molecular mass of PVP on separation of TTC diastereomers. (A) PVP 10 000 g/mol; (B) PVP 40 000 g/mol; (C) PVP 360 000 g/mol. Concentration of PVP was 7.1% w/v in each case. For selectivity of diastereomeric separation see Table 3.

order to enhance the selectivity of separation we utilized replaceable polymer matrices. It was found that the nature of the polymer is important for successful separation. Figures 2 and 3 demonstrate the increase in diastereoselectivity with increasing hydrophobicity of the polymer employed. A highly polar solution of LPAA gave us no distinguishable separation of diastereomers. A less hydrophilic PEG matrix separates some of the isomers or causes significant peak broadening (Figs. 2C, 3C). A relatively more hydrophobic PVP matrix allows for good diastereoseparation even in the case of numerous diastereomers of 2-OMe GAUC or TTCT (Figs. 2D, 3D).

Despite the sieving properties of PAA and PEG matrices, the migration order of the 2-, 3-, and 4-mer oligomers follows the same migration pattern as in free-zone CE (Fig. 3A). Migration of short oligomers is determined by the ion mobility (mass/charge ratio) rather than size-dependence.

Table 3. Effect of molecular mass of a polymer on the separation of oligonucleotide diastereomers

Polymer matrix (g/mol)	2-O-Me UU Selectivity MT_2/MT_1	TTC Selectivity MT_2/MT_1	TTC Selectivity MT_3/MT_2	TTC Selectivity MT_4/MT_3	TTC Selectivity MT_4/MT_1	TTCT Selectivity MT_8/MT_1
31.4% PEG 3000	1.012					
31.4% PEG 12 000	1.013					
31.4% PEG 35 000	1.014					
7.1% PVP 10 000		1.027	1.026	1.000	1.053	1.068
7.1% PVP 40 000		1.037	1.026	1.008	1.072	1.106
7.1% PVP 360 000		1.043	1.030	1.011	1.086	1.136

Table 4. Effect of organic modifier content in CE polymer matrix on the separation of oligonucleotide diastereomers

Polymer (g/mol)	% w/v	Org. modifier[a)]	Oligonucleotide 2-O-Me UU Selectivity MT_2/MT_1	Oligonucleotide TC DMT Selectivity MT_2/MT_1	Oligonucleotide GAUC Selectivity MT_4/MT_1
PEG (35 000)	22.9	No	1.013	1.053	-
PEG (35 000)	22.9	50% MeOH	1.012	1.020	-
PEG (35 000)	22.9	50% ACN	1.000	1.007	-
PVP (360 000)	7.1	No	-	-	1.315
PVP (360 000)	7.1	50% MeOH	-	-	1.124
PVP (360 000)	7.1	50% ACN	-	-	1.073

a) The buffer concentration in the matrix was kept constant; 50% of water volume was replaced by organic solvent.

Evidently, the polymer matrix pore size was not small enough to force the length-dependent separation of oligonucleotides, as is the case for longer (> 6–8-mer) single-stranded DNA fragments. Although polymer solutions are traditionally used as a matrix for separation of oligonucleotides based on the sieving mechanism, we do not expect the sieving to take place in the separation of diastereomers. Different conformations (secondary structure) of diastereomers may ultimately lead to a different shape (size) of molecules or isomers, but it is unlikely that these differences will be so substantial as to allow sieving-based separation. We repeat: small oligonucleotides are not separated by a sieving mechanism (see Figs. 3A, B, C). Polymer solutions used in our experiment do not allow for the sieving-based separation of oligonucleotides shorter than 8-mer.

The reversed migration of 2-, 3-, and 4-mer phosphorothioate oligonucleotides was found in PVP solution (Fig. 3D). This order is comparable to the retention observed in RP-HPLC on the C18 stationary phase (Fig. 3E). This comparison of TT, TTC, and TTCT retention in 14.3% PVP solution leads us to speculate that a similar mechanism, *i.e.*, hydrophobic interaction of oligonucleotides with polymer network, plays a significant role in CE diastereoseparation. This hypothesis is supported by the following experiment. TT, TTC and TTCT phosphorodiester oligomers (without a chiral PS center) were separated using the same CE conditions as in Fig. 3D. Although they have identical sequences, the less hydrophobic phosphorodiester oligonucleotides are not as well retained in 14.3% PVP and migrate according to mass/charge ratio, *i.e.*, TTCT first, followed by TTC and TT.

The comparison of Figs. 2D and 2E as well as 3D and 3E suggests that the PVP network serves as a pseudostationary phase, retaining and separating analytes in the same way as a chromatographic stationary phase. Despite overall similarity, however, the retention order of TTCT (TTC) and 2-OMe GAUC diastereomers is not in exact agreement with CE. The reinjection of the fractions collected from HPLC in the CE revealed differences in retention order of some diastereomers (see Fig. 2, and Fig. 3 captions). This finding is not surprising; polymer network provides different interactions (H-bonds) than the alkyl C18 phase, which results in different separation selectivity.

The differences in separation selectivity using different polymers suggest that the separation of oligonucleotide diastereomers is, at least partially, based on hydrophobicity of the polymer matrix. To test this hypothesis we prepared PVP and PEG matrices containing 50% of two different organic additives known to be strong eluents in RP-HPLC. Although these additives do not change the physical sieving properties of polymer solutions, we found that they significantly reduce the ability of polymer solutions to separate oligonucleotide diastereomers (Table 4). Acetonitrile totally suppresses the separation of 2-OMe UU in PEG solution and considerably degrades the diastereoseparation of TC DMT. Similarly, acetonitrile is more effective in reducing the diastereoseparation of GAUC in PVP solution than methanol, although neither inhibits the separation completely (Table 4). Migration time of solutes in acetonitrile solution decreased as expected due to decreased adsorption of oligonucleotides to polymer network.

Methanol acts in a similar fashion, although as a weaker "mobile phase". Migration time of solutes in polymer solutions containing methanol, however, was significantly extended. This is likely to be caused by the change in the mobile phase dielectric constant, which affects the electrical double layer, and reduces the apparent migration velocity of analytes. Formamide, often used as an additive in sieving matrices, was found to have a similar capacity to counteract the interaction of solutes with a polymer. The effect of formamide was, however, even weaker than that of methanol (data not shown).

As mentioned above, the PS TTCT, TTC, and TT exhibit a retention order opposite from that directed by mass/charge ratio (Fig. 3D). We believe that this behavior can be explained by stronger adsorption of 4-mer than 3- and 2-mer, respectively, in the 14.3% PVP solution. By addition of acetonitrile (which counteracts the adsorption of oligomers to the polymer pseudostationary phase) to the 14.3% PVP solution, the migration order was reversed. In addition to the reversal migration order (*i.e.*, MT 4-mer<3-mer<2-mer), the diastereomeric separation of oligomers was entirely suppressed. This observation clearly supports the hypothesis that adsorption of solutes to the polymer pseudostationary phase is responsible for separation of DNA isomers. Our conclusion agrees with the report describing the CE separation of TGCA 4-mer from its TG(OMe)CA, and TG(OBu)CA alkylated analogs, based on the hydrophobicity of the PVA pseudostationary phase [29].

The hydrophobicity of the polymer pseudophase is important for the separation of oligonucleotide diastereomers. However, diastereomers of highly hydrophobic oligonucleotides were also separated with rather hydrophilic PEG. CE diastereoseparation and retention in PEG solution are enhanced if a hydrophobic DMT group is attached to the oligomers (Fig. 4, Table 4). The trityl group significantly increases the retention and improves separation of 2-OMe GAUC diastereomers (Fig. 4) compared to "DMT off" 2-OMe GAUC sample (Fig. 2C). Of the seven different 2-mers, only alkylated 2-OMe PS UU was well separated into diastereomers in CE with PEG solution. We speculate that because of the higher hydrophobicity of 2-O-Me UU the diastereomeric separation is enhanced compared to nonalkylated PS. When 14.3% PVP was used instead of PEG, the interaction of tritylated oligomers with the polymer network was so strong that none of the DMT oligomer was detected for up to 90 min. Similarly, the migration time of longer (>4-mer) PS oligonucleotides dramatically increases. The 25-mer PS oligonucleotide did not migrate from the capillary for up to 90 min, although the phosphorodiester oligonucleotide of the same length and sequence reached the detector in 8.7 min.

4.2 Effect of polymer concentration and molecular mass on separation

As expected, the concentration of the polymer pseudophase influences the CE separation of diastereomers. An increase of PEG concentration improves the separation of 2-OMe UU isomers (Table 1). Improved separation of 2-OMe GAUC diastereomers with a 5'-attached DMT moiety was obtained by decreasing the concentration of PEG polymer solution (Fig. 4). We found that separation of 2-mer diastereomers is generally enhanced at higher polymer concentration, while there is no clear effect of polymer concentration on diastereoseparation of 3-mers and 4-mers. Table 2 highlights the increase in the effect of PVP concentration on the selectivity of TTC diastereoseparation. Although at a concentration of 14.3% all four isomers are nearly baseline-separated, this improvement of resolution is caused by the shift of the third peak

towards shorter migration time, which leads to a decrease of separation selectivity MT3/MT2 and a reciprocal increase of the MT4/MT3 ratio. The overall separation selectivity MT4/MT1 remains unchanged in the range of 7.1–14.3%. Even more complex was the separation of TTCT diastereomers. We obtained the best resolution of eight isomers at the lowest PVP concentration of 7.1%. A further increase in concentration has a mixed effect; while resolution of certain isomers improves, the separation selectivity of others diminishes. Such behavior can possibly be explained as a combined effect of hydrophobicity and H-bonding interactions between an oligonucleotide and the PVP pseudostationary phase, which are affected differently by variations of polymer concentration. The separation selectivity calculated for the first and last TTCT isomer, MT_8/MT_1, was 1.123, 1.136, and 1.112 for 4.3%, 7.1%, and 14.3% PVP solutions, respectively.

Besides concentration, the molecular mass of polymers has an interesting effect on diastereomeric CE separation. Generally, the higher the molecular mass of the polymer, the better the separation. This effect is less pronounced for short oligomers (2-O-Me UU; Table 3); however, this trend is distinct for TTC diastereomers separated in PVP solutions (Fig. 5). This observation might be partially related to the broader range of molecular masses of PVP (10 000–360 000 g/mol) than PEG (3000–35 000 g/mol) used in the experiment. Better performance of longer polymers is in agreement with the separation performance of polymers for DNA sequencing [30, 31}, where a higher molecular mass of polymer permits the reading of a longer length of DNA sequence ladder. We do not believe that the improved separation of oligonucleotide diastereomers in CEC is attributed to the mechanics of sieving interaction. In addition to the higher efficiency of separation, the use of the high molecular mass of polymer pseudophase changes the diastereomer separation selectivity. We do not have a clear explanation for this phenomenon.

While polymer solutions may successfully serve as pseudostationary phases for separation of small molecules, the analyte-polymer hydrophobic interaction may be undesirable for DNA sequencing. A recently published paper [32] describes the use of PVP for DNA genotyping and sequencing. The authors deal with major "impurity" in fluorescein-labeled DNA primer. In light of our experiments, we suggest that the separation of two positional isomers of fluorescein occurred in the PVP matrix, splitting the primer into two peaks. This separation can be achieved by RP-HPLC and in CE with hydrophobic PVP, while CE separation of primer in poly(ethylene oxide) (PEO) and PAA gives only a single peak. We expect that the hydrophobicity of PVP may complicate the DNA sequencing due to extensive differences in CE mobility shift for the four different sequencing dyes.

4.3 PS peak-broading mechanism in CE with polymer matrices

The ability of PVP solution to distinguish between phosphorodiester and PS oligomers was used to separate the 25-mer oligomers differing in the number of PS modifications. Figure 6 shows the separation of four oligonucleotides (25-mers) of identical sequence in 14.3% PVP (without organic modifier additive). The first peak, labeled 24PO (24 phosphorodiester internucleotide linkages), is completely separated from the same oligonucleotide containing a single PS linkage at the 3'-end (23PO/1PS). The 25-mer containing two PS linkages (22PO/2PS) is partially separated into its diastereomers. Two of four expected isomers coelute. The eight diastereomers of the

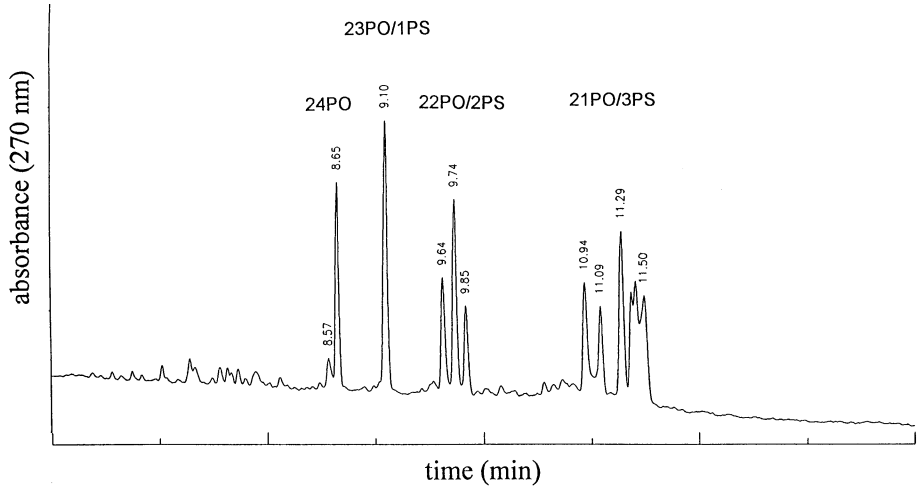

Figure 6. Separation of four 25-mer oligomers (25 bases long, *i.e.*, 24 internucleotide linkages) of identical sequence but differing in the number of PS internucleotide modifications. Retention time increases with the number of backbone modifications. Partial separation of diastereomers was observed for 2S/22O and 3S/21O oligomers. 13.4% PVP 360 000 g/mol matrix in 0.2 M Tris-boric acid buffer, pH 8.3, was used for the separation. For other CE conditions see Section 2.2.

21PO/3PS oligomer are also partially separated. The mechanism of peak broadening can be explained by extrapolating these results to longer PS oligonucleotides. For example, the 10-mer oligonucleotide has 512 diastereomers that are only partially resolved by CE with polymer solution. In fact, the width between the first and the last diastereomers represents the actual peak width. Partial diastereoseparation is the reason why sieving matrices, although they work well for natural phosphorodiester oligonucleotides, generally give unsatisfactory results for PS (alkylated PS) oligonucleotides. The peak broadening was found even in PAA sieving matrices and was usually explained as an effect of the more complicated secondary structure of PSs [12, 15]. To the best of our knowledge, this is the first experimental demonstration of the mechanism of PS peak broadening described in the literature. Typical CE separations of oligomers using replaceable polymers show a decrease in the separation efficiencies in the line phosphorodiester >PS>2-OMe PS (Belenky *et al.*, unpublished results). In light of our results, it is now clear why elevated temperature and addition of formamide or ethylene glycol into the sieving matrix is the right strategy for improving peak shape and thus separation of PS oligonucleotides [33].

5 Conclusions

The separation of multiple PS diastereomers was obtained employing polymer solutions as a pseudostationary phase for CE. We found that CE separation of diastereomers is enhanced when relatively hydrophobic polymers such as PVP are added to BGE. Alternatively, the moderately hydrophilic PEG solution can be used to separate diastereomers of strongly hydrophobic oligomers with the attached DHT group. Similarly to RP-HPLC, the organic additives such as methanol or acetonitrile reduce adsorption of oligonucleotides to the polymer network, which leads to a decrease in selectivity of diastereomeric separation. We also tested the influence of polymer concentration and molecular mass on separation. Our experiments lead us to conclude that polymers with higher molecular mass give us generally better selectivity of diastereomeric separation than lower molecular mass poylymer matrices. The polymer concentrations should be adjusted individually for each solute: diastereomers of 2-mers are better separated at a higher concentration of polymer; the separation of eight isomers of 4-mers is enhanced in matrices with relatively low concentrations of polymer. We demonstrate that the stereogenicity of PS backbone has an impact on the separation of PS oligonucleotides by polymer sieving matrices. Partial separation of numerous diastereomers that takes place in the CE (CGE) separation leads to the undesirable peak broadening of PS oligonucleotides. The proposed method for the diastereomeric separation is useful for studies of stereoselective degradation of oligonucleotides by nucleases, as well as the study of spontaneous stereoselective oxidation of PS internucleotide linkages. This method is suitable for diastereomeric purity control of oligonucleotides and possibly other molecules, *e.g.*, peptides, drugs and agrochemicals.

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6 References

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