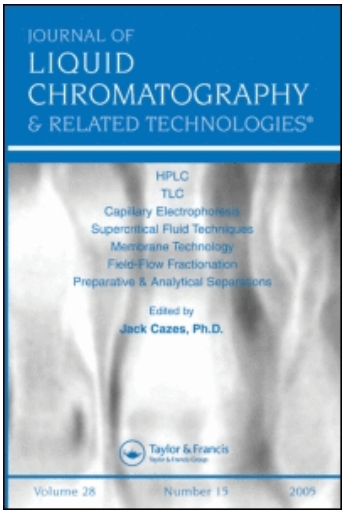

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GEL MATRICES IN N-METHYLFORMAMIDE FOR SEPARATION OF DNA FRAGMENTS

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GEL MATRICES IN N-METHYLFORMAMIDE FOR SEPARATION OF DNA FRAGMENTS

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ABSTRACT

Capillary gel electrophoresis is a powerful method for separation of DNA fragments. Usually, water based poly-(acrylamide) gels are employed. In order to release compressions due to intra-molecular base pairing within DNA fragments, it is often necessary to incorporate denaturing agents (e.g. urea, formamide) and/or to operate at elevated temperatures. In this work we have studied the performance of gels in non-aqueous solvents. Poly(dimethylacrylamide) gels in both formamide and N-methyl formamide were prepared. The formamide based gels showed a poor stability under normal capillary electrophoresis conditions,[1] rapidly developing voids at moderate electric field strengths. However, N-methyl formamide based gels were more stable and could be employed for electrophoretic separations of oligonucleotides. Sieving properties similar to those of regular water-based poly(acrylamide) gels, were observed. Non-aqueous gels without any additives had a considerable denaturing power, in fact, comparable to water-based gels containing 7 M urea. Interesting influences on the separation selectivity when utilizing different concentrations of N-methyl formamide were observed, which are discussed in the paper.

INTRODUCTION

In DNA sequencing by capillary gel electrophoresis (CGE), oligonucleotides differing one nucleotide in length need to be separated. This resolution must be obtained for DNA fragments ranging from a few bases up to several hundred bases in size. Internal base pairing often takes place in G-C rich areas within single stranded DNA. The compressed, secondary structures thus created show an excessive mobility, which results in a disturbing band overlap. To resolve DNA-compressions, a denaturant like urea, formamide, or a combination of both is added to the gel buffer. Another possibility is to perform the separation at an elevated temperature.[1-4] Unfortunately, neither urea nor formamide can be added in large enough amounts to release all compressions. Urea solutions become saturated at room temperature before a concentration, high enough to melt severe compressions in G-C-rich areas of DNA, is reached.[3] Formamide added in high concentrations leads to increased separation times and reduced lifetime of poly(acrylamide) gels.[1,2] Keeping the gel capillary above room temperature during electrophoresis can resolve compressions, especially in combination with a denaturing agent.[1] However, the gels often break down at elevated temperatures.[2] Moreover, the incorporation of a thermostated heating device complicates the CE system.

For denaturing electrophoresis at room temperature, the use of N-methylformamide (NMF) in a gel medium could be an interesting alternative. NMF is a unique organic solvent with a very high dielectric constant (ξ=182), in fact higher than water, leading to a high electrophoretic mobility of analytes. Capillary electrophoretic separations in pure NMF, without added buffer salts, have earlier been described by Jansson and Roeraade.[5] The conductivity of NMF is very low, and therefore, problems with Joule heating hardly exist. UV-detection is restricted to wavelengths above 250 nm due to the strong absorption of NMF at shorter wavelengths. However, this is not a problem with laser induced fluorescence detection of DNA fragments, where the excitation wavelengths are usually > 400 nm.[5] These particular features, as well as the resemblance of NMF to formamide, prompted us to investigate the possibility to prepare gels in NMF and investigate their denaturing abilities. Examples can be found in the literature of monomers that are suitable for formation of gels in mixtures of various organic solvents and water.[6-8] However, to our knowledge, no previous attempts have been made to prepare gels for electrophoresis in NMF.

In this work we have made non-aqueous gel matrices of poly(dimethylacrylamide), [poly(DMA)] in formamide as well as NMF. The sieving behavior of oligonucleotides in the NMF based gels, as well as the denaturing capacity of these matrices has been studied in electrophoretic separations.

EXPERIMENTAL

Materials

Acrylamide, N,N'-methylenebisacrylamide, tris(hydroxymethylamino-methane) (Tris), boric acid, urea, γ-methacryloxypropyltrimethoxysilane and adenosine 5´monophosphate (AMP) were obtained from Sigma (St. Louis, MO). EDTA (Titriplex III) was from Merck (Darmstadt, Germany). N, N-dimethylacrylamide (DMA) was purchased from Fluka (Buchs, Switzerland). N-methylformamide was obtained from Lancaster (Eastgate, Whitelund, Morecambe, England). Formamide was purchased from Aldrich (Steinheim, Germany).

All solutions were filtered through 0.1 μm pore size filters (Millipore, Bedford, MA). Fused-silica capillary tubing (375 μm OD, 50 μm ID) was obtained from Polymicro Technologies (Phoenix, AZ). The custom-synthesized oligonucleotides, $^{5'}$GGG CCC TTT TTT $^{3'}$(1), $^{5'}$GGG TTT TTT CCC $^{3'}$(2) and the $p(dA)_{40\text{-}60}$ were purchased from Pharmacia (Uppsala, Sweden).

Polymerization of Acrylamide Derivatives in Mixtures of Water and NMF

Initially, a screening was carried out, to search for optimum gel formulations and solvent compositions. Thus, simple polymerization experiments, carried out in test tubes, were performed. Buffers in (50% or 90%) NMF were prepared by mixing a water solution of 0.1 M Tris, 0.1 M boric acid and 2.0 mM EDTA with NMF. NMF was also used undiluted, without added buffer salts. A formamide buffer solution of 50 mM Tris and 50 mM boric acid was prepared by dissolving these salts in pure formamide. Acrylamide (0.2375 g) and N, N`-methylenebisacrylamide (Bis) (0.0125 g) were dissolved into 5 mL of buffer solution, to produce 5%T, 5%C monomer solutions. When DMA was used, 0.3312 g of the DMA and 0.0125 g of Bis were added to the same buffer volume, resulting in 6.9%T, 3.6%C solutions.

After filtration through 0.1 μm pore size filters, the monomer solutions were degassed under vacuum at room temperature in an ultrasonic bath for 15 minutes. Polymerization was performed either by chemical initiation, utilizing the ammonium persulfate (APS) and TEMED system,[9] or by γ-radiation.

In the former case, the dissolved monomers were mixed with 10% NMF solutions of TEMED and APS to final concentrations ranging between 0.1% TEMED/ 0.05% APS and 0.4 % TEMED/ 0.2% APS. In the latter case the monomer solutions were exposed to γ-radiation in a ^{60}Co-source for 30 minutes

up to 6 hours (dose 50-600 krad). In some of the γ-ray-initiated reactions the solutions were purged with N_2O-gas for 60 minutes prior to polymerization, in order to provide an extra source of radicals.

Preparation of Gel-Filled Capillary Columns

5%T, 5%C poly(acrylamide) gel columns in (1×TBE, 7M urea, water-based buffer), 6.87%T, 3.64%C poly(DMA) columns in (50% NMF, 90 % NMF or pure NMF), and 6.87%T, 3.64%C poly(DMA) columns in pure formamide, were prepared according to the following procedure: Fused silica capillary tubing was cut to length (3 m), and then rinsed with 0.4 M NaOH for 45 minutes. After a subsequent 30 minutes rinse with deionized water, the capillary inner surface was derivatized by passing a 3% solution of γ-methacryloxypropyltrimethoxysilane in 50/50 water/ethanol (pH adjusted to 3.6 with acetic acid) through the columns for 120 minutes at room temperature. Followed by another 30 minutes rinse with deionized water, the columns were filled with monomer solutions. These solutions were prepared in the same manner as described above. The solutions containing undiluted NMF were purged with N_2O-gas for 60 minutes prior to filling. Polymerization was performed under pressure,[10] while subjecting the columns to γ-radiation from a ^{60}Co source for 30 minutes (dose 50.4 krad) for the poly(acrylamide) gel columns, and for 6 hours (dose 600 krad) in the case of poly(DMA) columns.

Capillary Electrophoresis

The CE apparatus, built in house, consisted of a Plexiglas box with a high voltage safety interlock and a high voltage supply, +/- 0-30 kV, constructed from a Spellman CZE 100 (Plainview, NY) unit. The oligonucleotides were detected at 260 nm using a UV absorption detector (Linear Instruments, model UVIS 200, Reno, NV). The output signal from the detector was recorded on a PC utilizing a chromatography evaluating software (EZChrom™, Scientific Software Inc., San Ramon, CA).

RESULTS AND DISCUSSION

Gel Polymerization in Mixtures of Water and N-Methylformamide

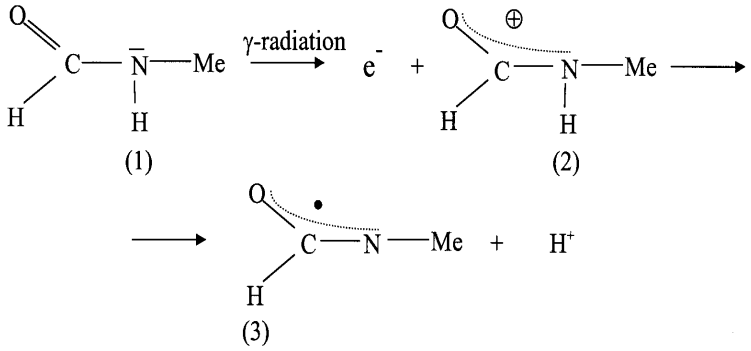
An initial screening procedure was employed to search for an NMF-gel formulation, which would form a matrix suitable for the separation of single stranded DNA. Rheology was tested empirically, by poking in the polymerized

matrix with a steel spatula and then observing the resulting deformation. Gel clarity was determined vide infra. Acrylamide and Bis as well as dimethylacrylamide and Bis monomer solutions were prepared in 50%, 90%, and undiluted NMF. Tris (0.1 M) and EDTA (2.0 mM) in the 1×TBE buffer were not totally soluble in undiluted NMF. Thus, when mixed NMF/ water buffer solutions were prepared, the 1×TBE buffer was first prepared in water, before mixing with NMF.

When γ-radiation from a ^{60}Co-source was applied, an exposure time of 30 minutes (dose 50.4 krad) was needed to obtain polymerization for gels containing 50% NMF. For gels with an increased NMF content, polymerization rates were proportionally slower and extended radiation doses (up to 300 krad for non-aqueous NMF gels) were necessary.

In acrylamide monomer solutions with more than 90 % NMF content, precipitates were generally formed upon polymerization. Thus, experiments with NMF-based poly(acrylamide) gels were discontinued. The problem was not observed with gels based on the more hydrophobic DMA monomer. However, judged from the rheology tests, these formulations yielded brittle and more porous products, which did not show the rigidity of crosslinked poly(acrylamide) matrices, polymerized in water.

We made the assumption that the slow and incomplete polymerization of the DMA monomer in NMF was caused by a lack of the radical species $e^-_{(aq)}$ (solvated electron), $OH^\cdot$ and $H^\cdot$, which attack the monomer double bond and initiate polymerization. These species are radiolysis products of water[11] and are not generated in sufficient amounts when NMF is the dominating solvent and only traces of water are present. However, radical species are also formed from the radiolysis of NMF molecules according to the following reaction :[12]



An electron is liberated from the NMF molecule (1) due to the action of the ionizing radiation. The cation formed (2) subsequently decomposes to form the radical species (3), which can attack the double bond of the DMA monomer. This explains why polymerization is not totally quenched in the undiluted NMF solvent, although small amounts of radicals generated from traces of water are of course also active. To improve the polymerization in NMF, N_2O gas was added to the system as an external source of radicals. Radicals are generated from the reaction of e^- (generated from the radiolysis of NMF) with N_2O:[12]

$$e^- + N_2O \longrightarrow N_2 + \cdot O^- \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} \cdot OH$$

When the DMA and Bis monomer solutions were purged for 60 minutes with N_2O gas prior to the application of γ-radiation, the formation of a gel started much earlier. The gels also showed a more rigid behavior in the above rheology test, suggesting a more complete polymerization reaction. However, for best results, the higher radiation doses were still needed. Consequently reaction times of 3-6 hours were generally employed (doses: 300-600 krad).

Chemically induced polymerization, utilizing the APS/ TEMED system, was also attempted for DMA and Bis in undiluted NMF. This initiator/ catalyst couple system did not function in undiluted NMF, even when the concentrations were increased to levels above those normally recommended. Thus, the most viable route to polymerization of DMA gels in NMF seems to be by γ-radiation in the presence of N_2O. We believe that this procedure could be generically applicable for the polymerization of other double bond-containing monomers than DMA, and also in other undiluted organic solvents.

Capillary Electrophoresis of Oligonucleotides

The performance of dimethylacrylamide (DMA) gel columns with 50%, 90% and undiluted N-methyl formamide (NMF) were investigated by the separation of the two oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$(1) and $^{5'}$GGG TTT TTT CCC $^{3'}$(2) as originally proposed by Pentoney et al.[1] These two twelve-mers have the same base composition but differ in nucleotide sequence.

As shown in Figure 1, oligo (2) attempts to form a loop-structure due to internal G-C base pairing. This change in conformation simulates a DNA-compression. The resulting decrease in the apparent size of (2) will cause it to migrate faster than (1).

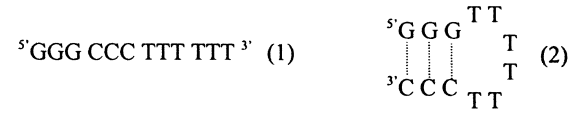


Figure 1. Conformations of the oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$ (1) and $^{5'}$GGG TTT TTT CCC $^{3'}$ (2).

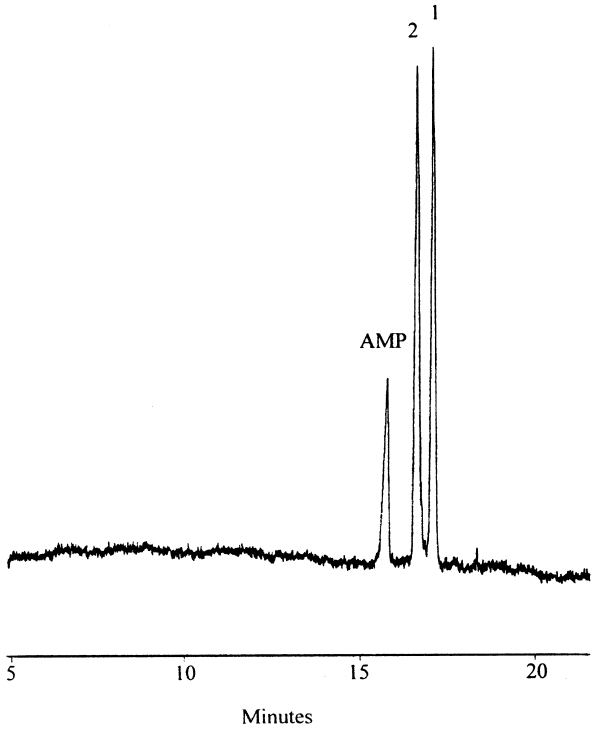


Figure 2. Separation of the oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$ (1) and $^{5'}$GGG TTT TTT CCC $^{3'}$ (2) and AMP (5 μg/ mL each) on a 5%T, 5%C polyacrylamide gel capillary, with the buffer 1×TBE and 7 M urea, at 250 V/cm electrical field strength. The current was 3 μA. Sample injection: 2 sec. at 2 kV. Capillary dimensions: 50 μm ID and 375 μm OD. Total length of the capillary: 52 cm. Length to the detector: 40 cm.

The two oligonucleotides will thus appear as two separate peaks in an electropherogram. If, on the other hand, a denaturing condition is able to break up the loop in (2), the two oligonucleotides should co-migrate and show only one peak.

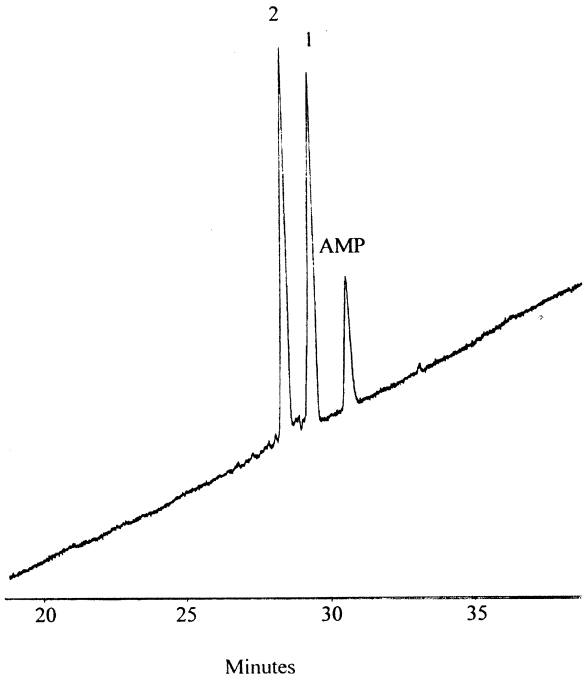


Figure 3. Separation of the oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$ (1) and $^{5'}$GGG TTT TTT CCC $^{3'}$ (2) and AMP (5 μg/ mL each) on a 6.9 %T, 3.6% C DMA gel capillary, with the buffer 0.5×TBE and 50% NMF, at 250 V/cm electrical field strength. The current was 4 μA. Sample injection: 2 sec. at 2 kV. Capillary dimensions: 50 μm ID and 375 μm OD. Total length of the capillary: 52 cm. Length to the detector: 40 cm.

Figure 2 shows the separation of oligos (1), (2) and adenosine 5' monophosphate (AMP) in a reference gel matrix of 5%T, 5%C acrylamide with a 1×TBE buffer with 7 M urea. Figures 3-5 show the same sample separated on 50%, 90% and undiluted NMF/ DMA gel columns respectively. Migration times (t_1, t_2), resolution ($R_{1,2}$) and efficiency (N_1, N_2) data for the separations are presented in Table 1.

Three consecutive separations were performed on each column, except for the 90% NMF column, where only two separations were carried out. As seen in the table, the migration times for the oligonucleotides decrease from run to run in the NMF-based gel columns. This effect is not the result of a gradual temperature rise inside the columns due to Joule heating, as the currents during

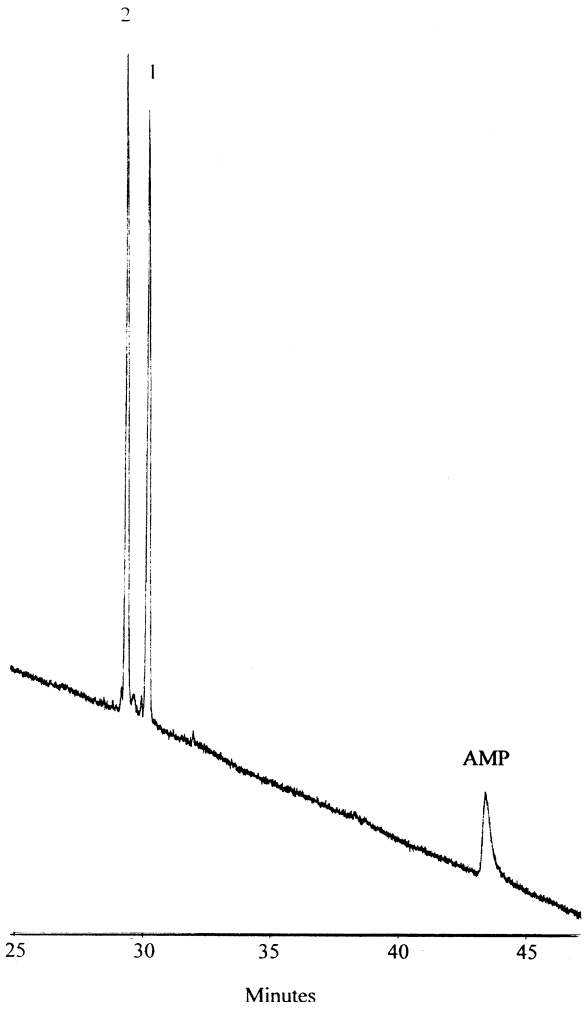


Figure 4. Separation of the oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$ (1) and $^{5'}$GGG TTT TTT CCC $^{3'}$ (2) and AMP (5 μg/ mL each) on a 6.9 %T, 3.6% C DMA gel capillary, with the buffer 0.1×TBE and 90% NMF, at 250 V/cm electrical field strength. The current was 3.0 μA. Sample injection: 2 sec. at 1 kV. Capillary dimensions: 50 μm ID and 375 μm OD. Total length of the capillary: 52 cm. Length to the detector: 40 cm.

electrophoresis in these columns are very low, especially in undiluted NMF (see the texts of Figures 2-5). The cause of this behavior is still unclear to us. It could be, that some kind of slow reorientation of the NMF molecules takes place in the electric field, which would influence the mobilities of the oligos.

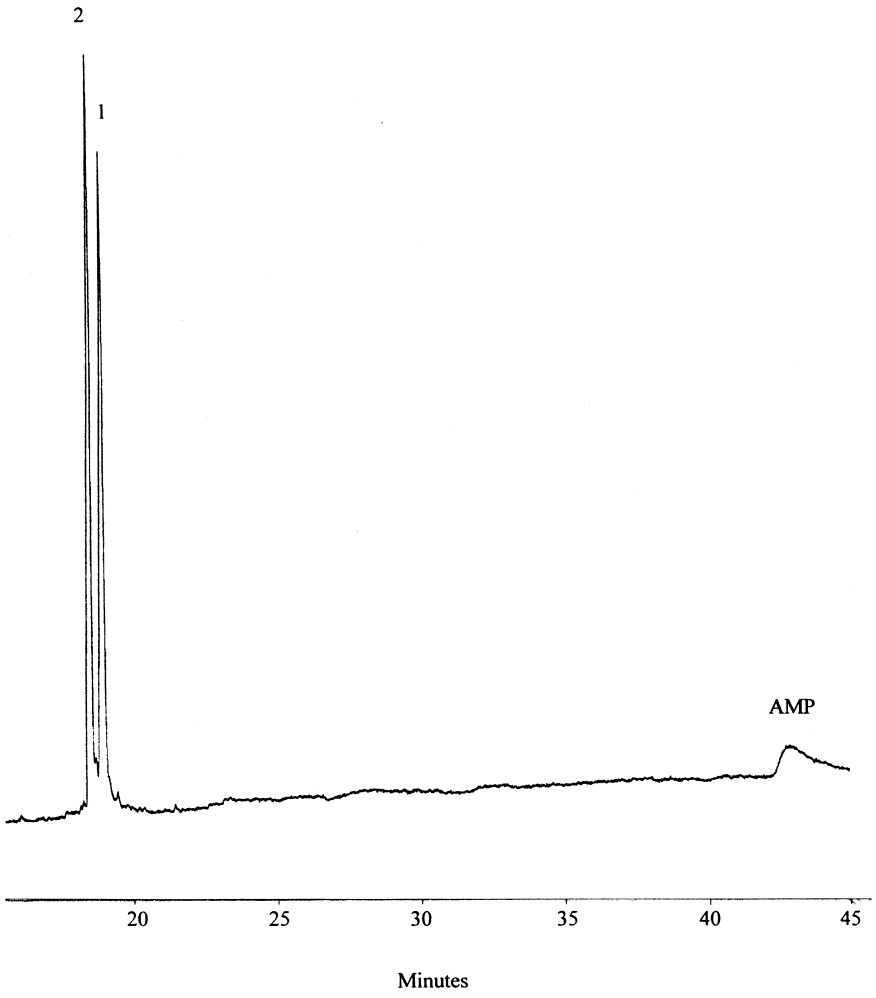


Figure 5. Separation of the oligonucleotides $^{5'}$GGG CCC TTT TTT $^{3'}$ (1) and $^{5'}$GGG TTT TTT CCC $^{3'}$ (2) and AMP (5 μg/ mL each) on a 6.9 %T, 3.6% C DMA gel capillary, with undiluted NMF buffer, at 250 V/cm electrical field strength. The current was 1.0 μA. Sample injection: 4 sec. at 2 kV. Capillary dimensions: 50 μm ID and 375 μm OD. Total length of the capillary: 52 cm. Length to the detector: 40 cm.

As can be seen in Table 1 and Figures 2-5, the migration times for the two oligonucleotides (1) and (2) change considerably as the NMF concentration in the buffer is increased. The shortest migration times are obtained on the 5%T, 5%C acrylamide column with 1×TBE and 7M urea buffer. When the 6.9%T,

Table 1

Migration Times, Resolution and Efficiency Data from Separations of the Oligonucleotides $^{5'}$GGG CCC TTT TTT$^{3'}$ (1), $^{5'}$GGG TTT TTT CCC$^{3'}$ (2), and AMP

	t_1 (min)	t_2 (min)	$\Delta t_{1,2}$ (min)	$R_{1,2}$	N_1 ($x10^5$)	N_2 ($x10^5$)
5%T, 5%C (AA, Bis)						
1xTBE, 7M urea						
Run 1	17.1	16.7	0.5	1.9	0.9	0.7
Run 2	17.2	16.8	0.5	2.1	1.1	0.8
Run 3	17.2	16.7	0.5	2.4	1.3	1.2
6.9%T, 3.6%C (DMA, Bis)						
0.5xTBE, 50% NMF						
Run 1	29.7	28.7	1.0	2.8	1.1	1.2
Run 2	29.2	28.3	1.0	2.8	1.1	1.2
Run 3	29.1	28.1	1.0	3.1	1.3	1.4
6.9%T, 3.6%C (DMA, Bis)						
0.1xTBE, 90% NMF						
Run 1	31.7	30.8	0.9	3.9	2.9	3.1
Run 2	30.1	29.3	0.8	4.3	3.6	3.7
6.9%T, 3.6% C (DMA, Bis)						
Undiluted NMF						
Run 1	20.1	19.6	0.5	2.0	1.0	1.1
Run2	19.8	19.3	0.5	2.0	0.9	1.0
Run 3	18.9	18.4	0.5	1.9	1.0	1.1

3.6%C DMA and Bis column with 0.5×TBE and 50% NMF buffer is employed, a substantial increase in the migration times of the oligonucleotides is observed. A maximum is reached when the same gel composition and a 0.1×TBE buffer containing 90% NMF is utilized. In the undiluted NMF gel, the migration times decrease again. The observed mobility shifts could stem from a variation in the effective charge of the analytes with the NMF content of the buffer.

In capillary zone electrophoresis (CZE), utilizing bare fused silica tubes, the electroosmotic mobility has been found to vary with the NMF concentration, with a minimum in mobility around 50% NMF content.[5] As the magnitude of the electroosmotic mobility is a function of the effective charges of the silanol

groups on the capillary inner wall, these charges should also vary with the NMF concentration. Similarly, in the above CGE-experiments, the effective charges of the DNA molecules could also vary with the amount of NMF added to the buffer. However, since the minimum in DNA mobility in the DMA gels is observed at 90% and not at 50% NMF content, it is likely that other factors like the pore size of the gel matrix are also relevant.

It is evident that the pore size of the gel matrix affects the overall DNA migration as well as the efficiency and resolution that can be obtained in a separation. As can be seen in Table 1 the separation in a 90% NMF gel column is slow but yields a high number of theoretical plates. The low mobility could be due to an increased friction in a tighter gel mesh. Although the molar concentrations of monomer and crosslinker are identical in the acrylamide and DMA formulations utilized, the pore sizes could be quite different in the various NMF and water mixtures, due to differences in the polymerization process and a different swelling behavior of the polymerized gels.

AMP was used as a migration time marker. The migration time for the AMP peak increases drastically as NMF is added to the gel. This is not the case for the oligonucleotides. This behavior could possibly be explained as follows: The conformation of single stranded DNA is affected by hydrogen bonds between the bases of adjacent nucleotides on the DNA strand.[13] However, AMP can interact more freely with surrounding NMF molecules and this could result in shifts in the pK_a values of the adenine base and the 5'phosphate group, which, as a consequence could lead to a reduced effective charge and a reduced mobility of the AMP molecule.

Table 1 also shows the difference in migration time (Δt), between oligonucleotides (1) and (2) separated on an acrylamide gel column with 7M urea, as well as on DMA gel columns with 50%, 90% and undiluted NMF. The Δt values obtained with the undiluted NMF matrix are similar to the Δt values in the acrylamide and 7M urea reference gel.

We suggest that Δt is related to the denaturing effect of the two gel matrices, although the possibility of a pore size effect can not be excluded from the discussion.

Figure 6 shows a separation of a $p(dA)_{40\text{-}60}$ oligonucleotide sample on an undiluted NMF/ DMA gel capillary. The excellent separation of DNA fragments as large as 60 bases indicates that DNA sequencing using these columns could be feasible. The drifting baselines in Figures 3, 4, and 6 are anomalies and are probably due to an electrical disturbance, caused by the high voltage power supply.

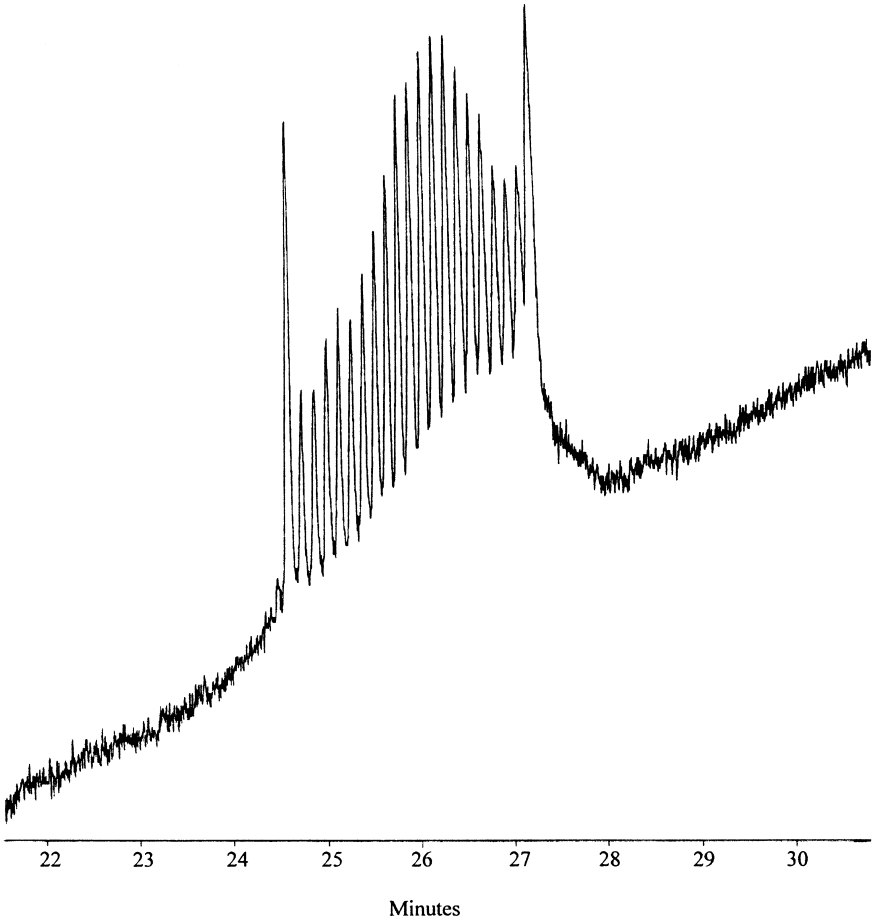


Figure 6. Separation of $p(dA)_{40\text{-}60}$ oligonucleotides (1 μg/ mL) on a 6.9 %T, 3.6% C DMA gel capillary, with undiluted NMF buffer, at 200 V/cm electrical field strength. The current was 1 μA. Sample injection: 4 sec. at 4 kV. Capillary dimensions: 50 μm ID and 375 μm OD. Total length of the capillary: 44 cm. Length to the detector: 35 cm.

CONCLUDING REMARKS

A 6.87%T, 3.64%C DMA and Bis gel has been prepared in undiluted NMF, which seems to have similar denaturing ability as a 5%T, 5%C acrylamide gel with 7M urea. The NMF based gel columns described in this study could be utilized for separations of oligonucleotides by capillary electrophoresis (e.g. for purity control after ss-DNA synthesis). For DNA

sequencing, further optimization of this gel matrix will probably be necessary. The pore size of the 100% NMF/ DMA gel could be increased by reducing the monomer and/ or crosslinker content, to optimize the matrix for separation of large DNA fragments. This will be studied in further detail.

The stability of 100% NMF/ DMA gels is quite limited at high electric field strengths. The gel matrix is recommended to be used at electric field strengths not exceeding 250 V/cm and below. As a result of the high boiling point of NMF (180°C), the solvent evaporation from column ends exposed to air is slow, which is a useful feature. Another possible application of NMF gels could be size-based separation of hydrophobic proteins (e.g. membrane proteins), which are difficult to separate by electrophoresis in water based systems.[8]

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