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Optimizing capillary gel electrophoretic separations of oligonucleotides in liquid crystalline Pluronic F127

Yingjie Liu[a], Bruce R. Locke[b], David H. Van Winkle[c], Randolph L. Rill[a,*]

[a]*Department of Chemistry and Institute of Molecular Biophysics, The Florida State University, Tallahassee, FL 32306-4390, USA*
[b]*Department of Chemical Engineering, Florida A & M University and Florida State University College of Engineering, Tallahassee, FL 32306-2870, USA*
[c]*Department of Physics, Florida State University, Tallahassee, FL 32306-4350, USA*

Abstract

Micellar, liquid crystalline solutions of poly(ethylene oxide) (EO)–poly(propylene oxide) (PO)–poly(ethylene oxide) copolymers are alternatives to solutions of random coil polymers as replaceable media for capillary 'gel' electrophoresis. Excellent capillary gel electrophoresis separations of oligonucleotides were obtained in the liquid crystalline phase of 18–30% solutions of Pluronic F127 (approximate molecular formula $[EO]_{106}[PO]_{70}[EO]_{106}$). These solutions are isotropic and flow freely into capillaries at reduced temperatures (0–5°C), but rapidly transform into a gel-like, cubic liquid crystalline phase of spherical micelles at room temperature and above. Separations on Pluronic gels can be tuned because polymer chain packing in the liquid crystalline phase is sensitive to polymer concentration and temperature. Optimum resolution of short oligonucleotides of sizes typically used for DNA sequencing primers or oligonucleotide therapies (≤40 nucleotides) was obtained by capillary gel electrophoresis in 25% Pluronic F127 at 30°C with an applied voltage of 500 V/cm. Decreasing the Pluronic concentration to 20% and increasing the temperature to 50°C substantially reduced analysis time with a modest decrease in resolution. Individual nucleotides in dT_{12-18} and dT_{19-24} standards were separated in 8 min with baseline resolution. The complex dependencies of resolution and peak shape on oligonucleotide size, Pluronic concentration, temperature, and applied voltage are described. © 1998 Published by Elsevier Science B.V.

Keywords: Poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) sieving media; Pluronic F127; Liquid crystalline sieving media; Surfactants; Micelles; Oligonucleotides

1. Introduction

Synthetic oligonucleotides are essential to biotechnology as gene probes and polymerase chain reaction (PCR) primers. Unnatural oligonucleotides proposed for use in antisense, ribozyme or other oligonucleotide-based therapies have potential for treating many life-threatening diseases [1]. Improvements in rapid, automated analyses of oligonucleotide purity and conformational properties are expected to become increasingly important to advances in the Human Genome Project, and oligonucleotide therapeutics. Capillary gel electrophoresis (CGE) is well suited for these purposes [2–9].

Oligonucleotides used as hybridization probes or proposed as therapeutic agents are short, typically 20–30 nucleotides (nt), and seldom longer than 40 nt. High-resolution separations of short oligomers requires a much ''tighter'' sieving matrix than separations for DNA sequencing. Reproducibility in CGE is enhanced if the sieving medium is frequently replaced [10–16]. Unfortunately, most sieving media

*Corresponding author.

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providing high-performance separations of short oligonucleotides are either cross-linked gels or polymer solutions of high concentration and viscosity. Automated refilling of capillaries with these media is difficult or impossible [17].

Gel-like, liquid crystalline solutions of the uncrosslinked copolymer Pluronic F127 were recently introduced as a new class of sieving media for high-performance CGE of oligonucleotides and other nucleic acids [18–20]. Pluronics, commercially available surfactants, are uncharged poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) or $[EO]_x[PO]_y[EO]_x$ triblock copolymers. Like other surfactants, they aggregate to form micelles above some critical concentration (CMC). Pluronic F127 has the approximate molecular formula $[EO]_{106}[PO]_{70}[EO]_{106}$ and molar mass of 13 400. Solutions of Pluronic F127 within a concentration range of 18–30% are low viscosity liquids (<2 poise) at low temperature (0–5°C), but form self-supporting, cubic liquid crystalline gels at room temperature [21–23]. This behaviour is common to many Pluronic copolymers [23]. Capillaries can be filled easily in the cold, then electrophoresis can be conducted in liquid crystalline gels at or above 20°C [19,20].

The size, hydration, and other properties of micelles in Pluronic liquid crystals change with concentration and temperature [18,23]. These changes are large enough to influence molecular sieving properties of the medium. For example, the average center-to-center distance between spherical Pluronic F127 micelles reportedly decreases from 18 to 16.5 nm when the concentration is increased from 20% to 27%, although the aggregation number stays nearly constant at ca. 54 (corresponding to an aggregate molar mass of 700 000) [23]. We expect that changes in polymer concentration or temperature will permit substantive tuning of gel properties and optimization of CGE separations of specific analytes.

This expectation was confirmed by examining factors that influenced the separation of oligonucleotides standards, principally dT_{12-18}, and dT_{19-24}. Rapid, high-performance CGE separations of oligonucleotides up to 60 nt long were readily obtained in 25% gels of Pluronic F127 [19]. Better understanding of the effects of various operating parameters will allow optimization of separations, thereby assisting design and quality control of synthetic oligonucleotides.

2. Experimental

Pluronic F127 (produced by BASF) and standard oligonucleotide mixtures (dT_{12-18}, dT_{19-24}, and ''Poly U'') were purchased from Sigma (St. Louis, MO, USA). Other oligomers were synthesized using standard phosphoramidite chemistry on an automated DNA synthesizer (Model 391, Applied Biosystems, Foster City, CA, USA). Samples were removed from the resin and deblocked with concentrated ammonium hydroxide, then evaporated to dryness and used without further purification. All oligonucleotides were dissolved in deionized water at concentrations of 1.2–2 mg/ml.

Electrophoresis was primarily carried out with a Beckman P/ACE 5010 unit with temperature control (Beckman Instruments, Palo Alto, CA, USA). Some initial CGE runs reported (Figs. 1 and 2) were performed on a laboratory-built instrument without temperature control. Coated Celect-N columns, 75 μm I.D., were obtained from Supelco (Bellefonte, PA, USA). The total capillary length was 36 cm (30 cm effective length) unless noted otherwise. The UV absorbance detector was set at 254 nm. Resolution was calculated as peak separation divided by average peak width at half-height.

The electrophoresis buffer was 89 m*M* Tris, 89

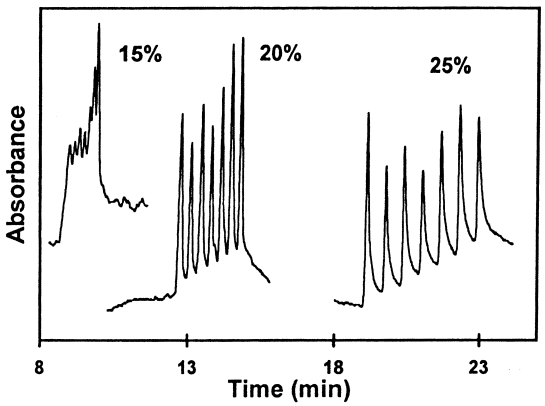

Fig. 1. Dependence of CGE separation of dT_{12-18} on Pluronic F127 concentration (indicated). Conditions were 515 V/cm, 34.8 cm effective column length, ambient temperature.

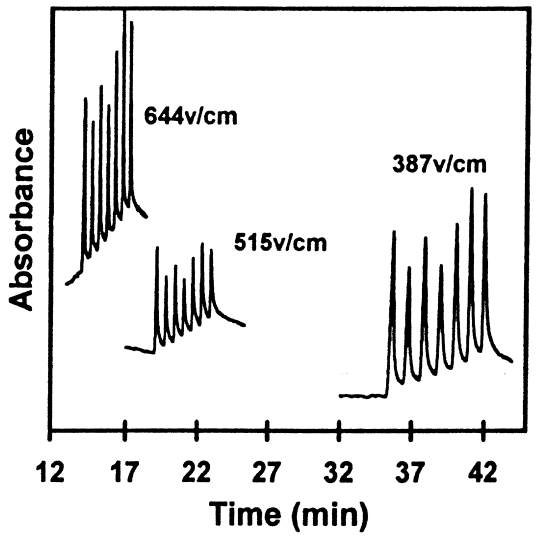

Fig. 2. Dependence of CGE separation of dT_{12-18} in 25% Pluronic F127 on applied voltage. Other conditions were as in Fig. 1 (34.8 cm effective column length, ambient temperature).

m*M* boric acid, 2 m*M* EDTA, pH=8.3 (1×TBE). Pluronic F127 solutions, 15–30% (w/w) in 1×TBE buffer, were prepared and stored in the cold room (4–5°C). Capillaries were filled with Pluronic solution under hand pressure applied by a syringe. Filled capillaries were equilibrated to room temperature for a few minutes before use. Electrophoresis was performed in the reversed polarity mode at controlled temperature. Samples were injected electrokinetically at 10 kV. After each electrophoretic run the capillary was put in the cold room and flushed with 1×TBE buffer with hand pressure applied by syringe before refilling.

3. Results and discussion

3.1. Effects of Pluronic concentration

The concentration of Pluronic has a significant effect on oligonucleotide run time and resolution (Fig. 1). No liquid crystalline gel phase forms at room temperature when the Pluronic F127 concentration is below 18%. Above this concentration the inter-micelle spacing of the liquid crystalline phase decreases with increasing concentration, while the aggregation number remains nearly constant [23].

Electrophoresis of dT_{12-18} was performed in a 15% Pluronic F127 solution, which remains in the liquid phase, to determine whether the gel phase is essential for oligomer separations. Additional CGE runs were performed in 20%, 25%, and 30% Pluronic gel-filled capillaries. The components of dT_{12-18} were partly separated in the 15% solution-filled capillary, but the peaks badly overlapped. By contrast, all the individual components were well separated in the gel-filled capillaries of ≥20% Pluronic (Fig. 1), demonstrating that the liquid crystalline gel phase is essential for high-performance. While run times increased progressively with increasing Pluronic concentration, resolution did not. Increasing the Pluronic concentration from 20% to 25% improved the average resolution from 1.9–3.2, but a further increase to 30% Pluronic degraded the average resolution to 2.3.

3.2. Effects of voltage

Diffusional band broadening in CGE decreases with increasing voltage because of reduced migration time, but band broadening by the thermal gradient developed across the gel occurs when the voltage is sufficiently high. There is an optimum voltage gradient because of these opposing effects. The highest voltage gradient which can be applied is determined by the conductivity. The conductivity of 20% Pluronic in 1×TBE buffer is about 50% that of a 20% polyacrylamide gel, and 35% of that of a 6% polyacrylamide gel, in the same buffer. Because of the low conductivities of Pluronic solutions, voltage gradients higher than often reported were used in these studies. A voltage gradient as high as 644 V/cm could be applied to 25% Pluronic gel-filled capillaries without external cooling (Fig. 2). All components of dT_{12-18} were detected within 18 min with a resolution between neighboring peaks above 2.8. Decreasing the voltage gradient from 644 V/cm to 515 V/cm and 387 V/cm increased the analysis time and decreased resolution (Fig. 2). (Mobilities did not increase linearly with voltage in these cases due to Joule heating.)

Additional studies of the effects of voltage on resolution were done at constant temperature. The

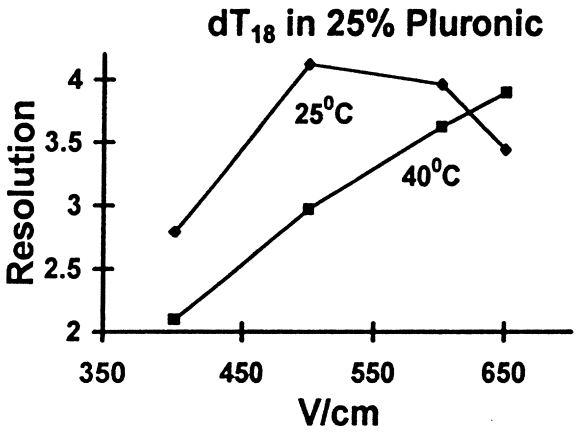

Fig. 3. Dependence of resolution of CGE separation of dT_{12-18} in 25% Pluronic F127 on applied voltage. Temperature: 25°C or 40°C, as indicated; effective column length: 30 cm.

optimum voltage gradient for separation of dT_{12-18} was examined in 25% Pluronic F127 at 25° and 40°C (Fig. 3). The average resolution of all adjacent peaks achieved at 25° increased from 2.8 at 400 V/cm to 4.1 at 500 V/cm. Resolution decreased modestly at higher voltage gradients (R_{av}=3.4 at 650 V/cm). Resolution at 40° increased steadily with voltage, but was poorer for all but the highest voltage than resolution achieved at 25°. The resolution at 40° and 650 V/cm nearly matched the best resolution at 25°, hence the separation time can be reduced with little sacrifice in resolution.

3.3. Effects of temperature

Considering the importance of oligonucleotides to biotechnology and oligonucleotide pharmaceuticals, it is surprising that there are no reports of CGE of short oligomers on crosslinked gels at elevated temperature. Electrophoresis at elevated temperature may have been avoided because polyacrylamide gels are more susceptible to hydrolysis at higher temperature. There are several reasons for doing CGE of oligonucleotides at elevated temperature.

Reduced migration times of analytes are expected, as has been observed in several contexts [24–26]. Higher temperature also helps to prevent oligonucleotide self-association and secondary structure formation, which degrades resolution and compromises the dependence of electrophoretic mobility on length. When linear polymer solutions were used as sieving media, elevated temperature allowed extension of sequence read length due to the markedly improved resolution of longer products [27,28]. Electrophoresis at elevated temperature has found applications in studies of DNA point mutants, where changes in thermal denaturation properties are exploited to detect single base mismatches [5,27,29].

Increasing temperature may also change the micellar liquid crystal structure in ways that are not yet determined. Micelle formation by Pluronics is due to dehydration of $[PO]_{70}$ groups, which increases with increasing temperature. The critical micelle concentrations of Pluronic polymers shift several orders of magnitude within a small temperature range [23]. Pluronic F127 solutions at 18–30% concentrations form cubic liquid crystal phases in the temperature range of about 20 to 100°C. The dependence of micelle size on temperature over this full range is not reported.

Separation times of oligonucleotides dT_{12-18} and dT_{19-24} in 20% Pluronic gels decreased dramatically (60%) when the temperature was increased from 20°C to 50°C. A substantial, but smaller decrease in mobilities was observed for 25% gels. All individual oligomers in dT_{12-18} and dT_{19-24} were separated to baseline in about 8 min by CGE in 20% Pluronic at 50°C (Fig. 4, top and middle). This appears to be the fastest CGE separation with high resolution reported to date for similar size oligonucleotides in a similar buffer system. Only one reported separation [27], which employed a different buffer with less than 1/10 of the conductivity of 1×TBE buffer, was noted which matches the speed and performance of that reported here. Much higher resolution of dT_{19-24} was achieved in 25% Pluronic under optimal conditions (30°C, 500 V/cm), but with a 2.5× increase in separation time (Fig. 4, bottom).

The effect of temperature on resolution of oligonucleotides was not monotonic. In 20% Pluronic the mean resolution between neighboring peaks in dT_{12-18} was a maximum at 30°C (Fig. 5). The mean resolution of dT_n oligomers in the 12–24 nt range was better in 25% Pluronic than in 20% Pluronic gels. Resolution of dT_{12-18} in 25% Pluronic was greater than two at all temperatures from 20 to 50°C, but was best above 30°C (Fig. 5, middle). Highest resolution of dT_{19-24} was obtained in a 25% Pluronic

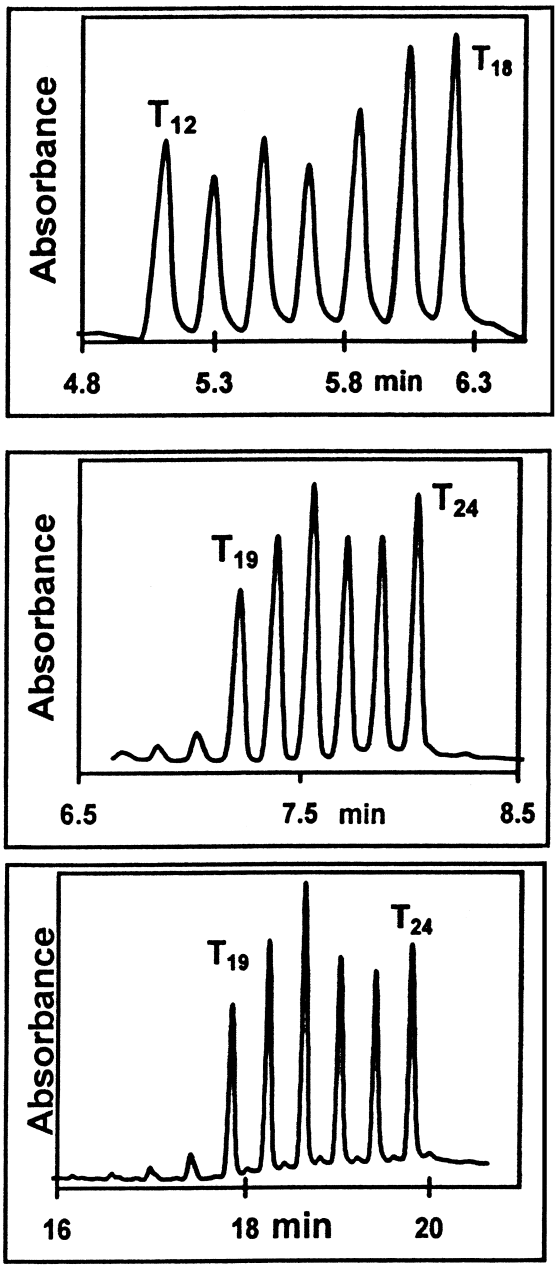

Fig. 4. CGE separations of oligonucleotide standards dT_{12-18} and dT_{19-24} in 20% Pluronic F127 at 50°C (top and middle) and in 25% Pluronic F127 at 30°C (bottom). Voltage gradient: 500 V/cm, effective column length: 30 cm.

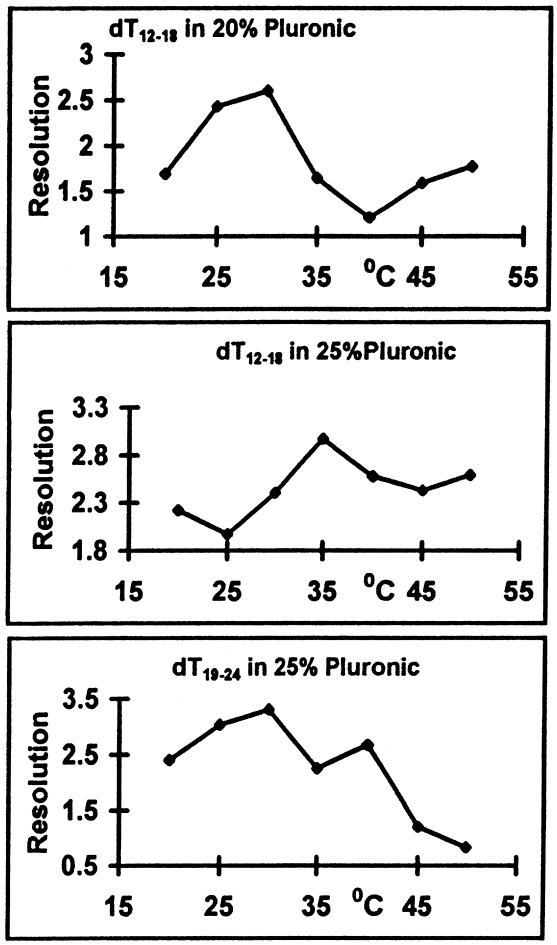

Fig. 5. Dependence of resolution of CGE separations of dT_{12-18} and dT_{19-24} in 20% and 25% Pluronic F127 on temperature. Voltage gradient: 500 V/cm, effective column length: 30 cm. Resolution was calculated as the peak separation divided by average peak width at half height. Data points represent the average resolution between individual components at one temperature.

gel at 30°C (Fig. 5, bottom). Degradation products of individual oligonucleotides, most likely the terminally dephosphorylated forms, were clearly seen in this optimized case (Fig. 4, bottom); which illustrates the importance of attaining symmetric band shapes and better than single nucleotide resolution.

3.4. Factors affecting peak shape

Symmetric peak shapes are especially important for separations of oligonucleotides and their contaminants. Substantially better than single nucleotide resolution is required to detect terminally dephos-

phorylated, incompletely deblocked, oxidized, branched, or otherwise aberrant forms arising during or after chemical synthesis. An example is the CGE separation in 25% Pluronic of a synthetic 22-mer from premature termination products differing by a single nucleotide, plus other products with intermediate mobilities (Fig. 6).

Gels of 25% Pluronic F127 provided higher average CGE resolution of oligonucleotides ≤30 nt long than 20% or 30% gels. Much of the dependence of resolution on Pluronic concentration can be attributed to differences in peak shapes, which were found to be a complex function of polymer concentration, oligonucleotide length, and temperature. For example, the peak shape of T_{18} obtained in 25% Pluronic is very symmetric in the run shown in Fig. 7, while the peak in 20% Pluronic is fronting, and that in 30% Pluronic is tailing.

Pluronic F127 forms spherical micelles with dehydrated $[PO]_{70}$ blocks in the core, and a layer of hydrated $[EO]_{106}$ blocks on the surface. The $[EO]_{106}$ chains can be thought of as soft bristles on a brush. Pluronic micelles pack into a cubic liquid crystal phase under the conditions used for CGE. Penetration of the hydrophobic, dehydrated $[PO]_{70}$ core by highly charged macromolecules like oligonucleotides is unlikely. The sieving network of Pluronic cubic liquid crystals is determined by the spatial distribution of hydrated $[EO]_x$ chains. The $[EO]_x$ chain

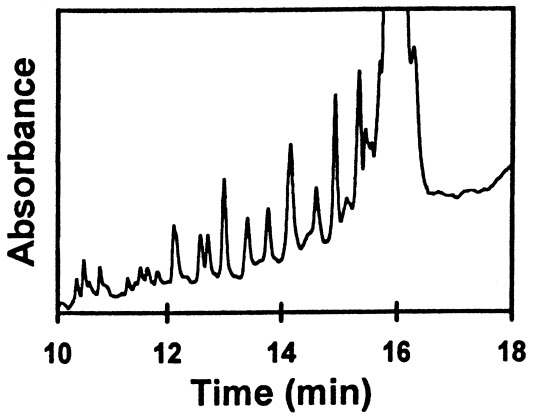

Fig. 6. CGE separation of unpurified products of chemical synthesis of a 22-mer, d(CGATATACAAGCTTATCGATACC), on 25% Pluronic F127 at 30°C (600 V/cm, effective column length 36 cm). The desired product (off scale) appears at about 16 min.

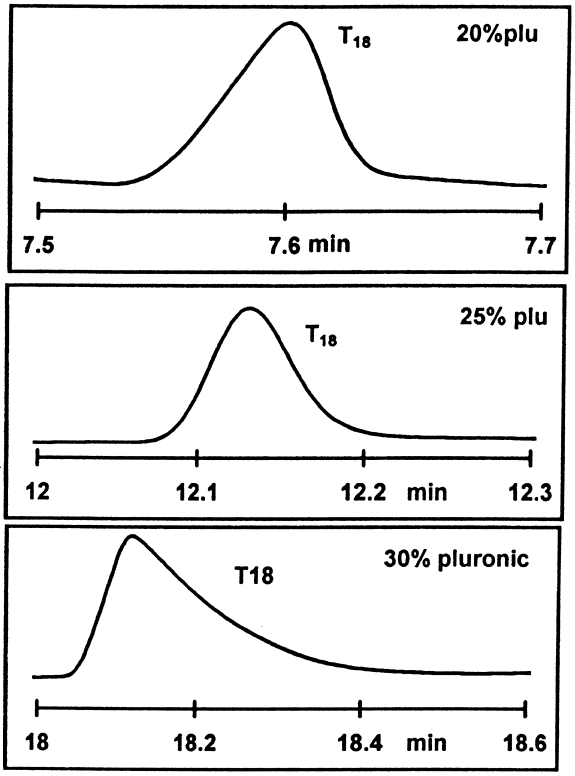

Fig. 7. Dependence of peak shape of dT_{18} on Pluronic concentration. Temperature: 25°C, 500 V/cm, effective column length: 30 cm.

density decreases with increasing distance away from the spherical $[PO]_y$ core within intramicelle domains. In addition there are interstitial spaces between micelles with sizes and shapes determined by the local packing. The chain density in these intermicelle domains should be lower than in intramicelle domains. Thus the pore size distribution of Pluronic gels is complex and unlike the random pore size distribution of crosslinked hydrogels or solutions of entangled linear polymers.

Micelles pack more tightly with increasing polymer concentration by decreasing the average extension of $[EO]_x$ chains. The compressed intermicelle spacing must decrease the mean pore size and substantially alter the pore size distribution. A more subtle effect of this compression is to increase the resistance to passage of a macromolecule through the most constricted ''interbrush'' regions (i.e., between the brushtips of two adjacent micelles at the point of

closest approach). Lower Pluronic concentration (larger pores) produced fronting of the T_{18} peak, while higher Pluronic concentration (smaller pores) produced tailing of the peak. These results suggest that peak shape may be related to oligomer length and pore sizes in the cubic liquid crystal.

If this holds true, the following suggestions are also expected to be true. Since the voltage gradient affects neither the Pluronic gel structure nor the conformation of oligonucleotides, changing the voltage gradient should not affect the peak shape. To test this, T_{18} was subjected to electrophoresis in 25% Pluronic gels at four different voltages. Although the peak width decreased slightly with increasing voltage gradient, the peak shape remained essentially the same (Fig. 8). Secondly, if pore size is important, then oligonucleotides of different sizes are expected to show different peak shapes. Results with T_{18} indicate that, for a given Pluronic concentration, reducing the oligomer size should favour fronting, while increasing the oligomer size should favour tailing. To test this, a mixture of dT_n covering the size range from dT_4–dT_{28} was subjected to electrophoresis in 25% Pluronic gel. The peak shapes were as predicted (Fig. 9). Symmetric peaks were obtained for oligomers dT_{16} and dT_{20}, like T_{18}. The larger oligomer (dT_{28}) gave a tailing peak, and the smaller oligomers (dT_4–dT_{12}) gave fronting peaks. Fronting became more severe as the oligonucleotide became smaller.

Because of the lack of detailed knowledge about the effects of temperature on the Pluronic cubic liquid crystal gel structure, the peak shape at different temperatures is more difficult to predict. Peaks for T_{24} in 25% Pluronic gels at different temperatures are shown in Fig. 10. The dT_{24} peak was very symmetric at 30°C, but tailed at all the other temperatures studied. Tailing was most severe at higher temperature.

The effect of electrophoretic migration on the concentration distribution in free zone electropho-

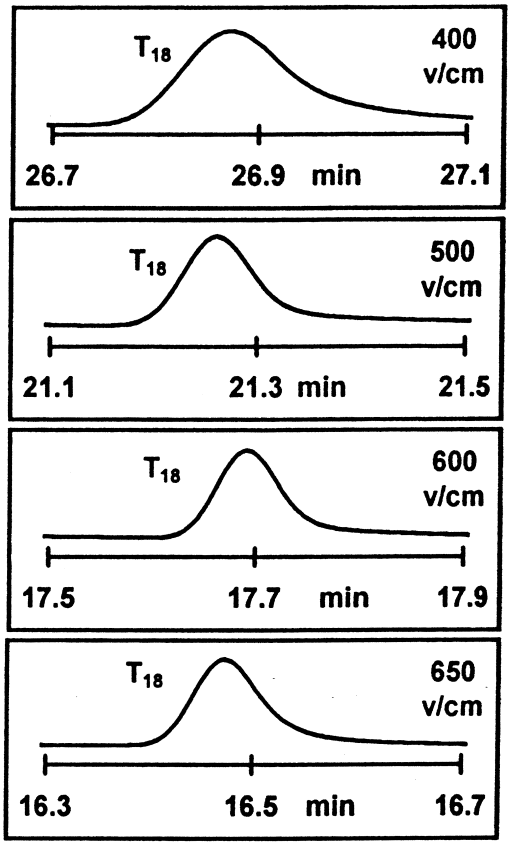

Fig. 8. Dependence of peak shape of dT_{18} on voltage in 25% Pluronic. Temperature: 25°C, effective column length: 30 cm.

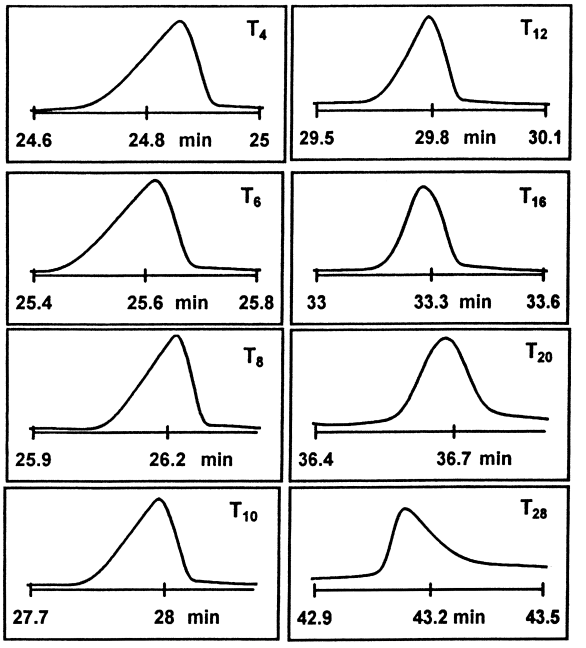

Fig. 9. Dependence of peak shape of dT_n on its size in 25% Pluronic. Temperature: 25°C, voltage gradient: 500 V/cm, effective column length: 50 cm.

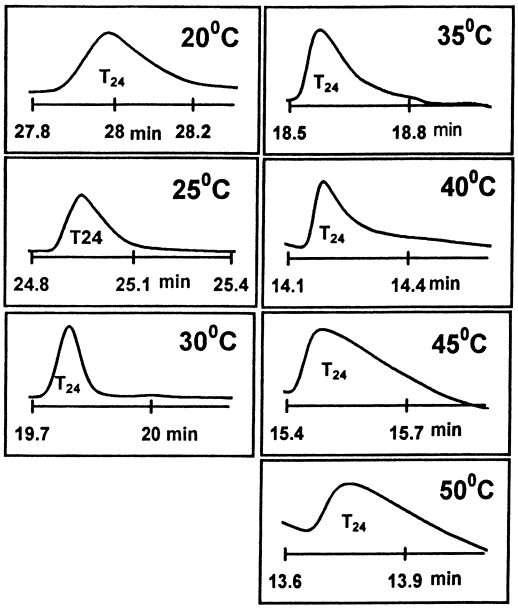

Fig. 10. Dependence of peak shape of dT_{24} on temperature in 25% Pluronic. Voltage gradient: 500 V/cm, effective column length: 30 cm.

resis has been studied using a non-diffusional model [28]. It was shown that sample constituents having a mobility higher than that of the carrier constituent migrate with a concentration distribution that is diffuse at the front and sharp at the rear of the zone. The reverse holds for sample constituents that have a mobility lower than that of the carrier constituent. This analysis might be related to our observations on peak shapes, but this would mean that the small oligonucleotides have higher mobility than the carrier ion, which in this case is $H_2BO_3^-$. The charge-to-mass ratio of dT_4 is much smaller than that of $H_2BO_3^-$. A smaller charge-to-mass ratio and strong sieving effect of the Pluronic gel are expected to make even the smallest oligonucleotide, T_4, migrate slower than the carrier ions. Further, temperature changes are not expected to significantly alter the relative mobilities of dT_n oligomers compared to the carrier ions. Comparable mobilities between sample ions and carrier ions do not seem likely as causes of the oligomer size, temperature and Pluronic concentration dependence of peak shape.

4. Conclusions

Exceptional separations of oligonucleotides can be obtained by CGE in liquid crystalline, gel-like solutions of Pluronic F127. Solutions sufficiently concentrated to form the gel phase are essential for high-performance. Electrophoretic mobilities decrease progressively with increasing Pluronic concentration, and increase progressively with increasing temperature or voltage.

Resolution is a more complex function of these variables, largely because of changes in peak symmetry, which range from fronting to tailing. Peak shape varies progressively with oligonucleotide size. The observed correlations of peak shape with oligonucleotide size and polymer concentration indicate that peak shape is related to pore size distribution and chain packing in the Pluronic liquid crystalline phase. Because of these effects Pluronic gels can be tuned to provide exceptional resolution over a narrow analyte size range by optimizing polymer concentration and temperature.

Highest resolution of dT_{12-24} oligomers was attained in 25% Pluronic F127 at 30°C with an applied voltage of 500 V/cm. Analysis times can be reduced substantially without severely degrading resolution by decreasing the Pluronic concentration, or increasing the temperature or voltage. Voltage gradients of at least 650 V/cm can be applied because of the low conductivity of Pluronic gels. Oligonucleotide mixtures dT_{12-18} and dT_{19-24} can be separated to baseline within 8 min at 50°C in 20% Pluronic. Pluronic F127 is a convenient, easily repaceable medium which seems especially well suited for high-performance CGE of oligonucleotides in the size range of primers for DNA sequencing and PCR, and of oligonucleotide therapeutics.

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phoresis equipment. This work was supported in part by NSF grant BES-951381 and the FSU Center for Materials Research and Technology (MARTECH).

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