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Oligonucleotide analysis by capillary polymer sieving electrophoresis using acryloylaminoethoxyethanol-coated capillaries

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Abstract

Capillary polymer sieving electrophoresis using dynamic sieving polymer solutions provides size-based separations of oligonucleotides. The polymer sieving system described here resolves single-stranded oligonucleotides with single-base resolution up to lengths of 30 bases within 10 min. The effect of temperature on the separation of synthetic oligonucleotide standards was examined between 20°C and 40°C, with optimal performance at 35–40°C. By adding urea to the sieving buffer single-base resolution could be extended to 60 bases in about 40 min. Best performance was achieved with capillaries coated with a new monomer, acryloylaminoethoxyethanol. This coating provides the necessary stability to ensure long lifetimes.

Keywords: Capillary columns; Acryloylaminoethoxyethanol coating; Coated capillaries; Oligonucleotides

1. Introduction

Millions of oligonucleotides are synthesized each year for use as primers or probes in genetic analysis. Although purity requirements for oligonucleotides may vary widely with the application, there has been increasing interest in improved characterization of these synthetic oligonucleotides. Polyacrylamide gel electrophoresis has been widely used to estimate oligonucleotide purity but this does not always have the desired sensitivity and resolution.

Adaptation of polyacrylamide gels to the high-performance capillary electrophoresis format has been limited by difficulties encountered in preparing and using cross-linked gels in capillaries. Capillary electrophoresis in aqueous solutions of dissolved polymers [1] and electrolytes provides separations of DNA and proteins substantially equivalent to polyacrylamide and agarose gel electrophoresis. This consists of dissolving in the appropriate buffer, linear polymers which exert a dynamic sieving on macromolecular analytes just as chemical cross-linked gels do. The effective size range for a particular separation will depend upon the nature of the polymer as well as its concentration and molecular size. This method of capillary polymer sieving electrophoresis (CPSE) is increasingly becoming the method of choice for the high efficiency and rapid separation of a wide range of single-stranded DNA molecules such as synthetic oligonucleotides [2,3] as well as the separation of double-stranded DNA molecules as in polymerase chain reaction (PCR) products and restriction fragments [4].

In this study, we utilize a polymer sieving buffer in capillaries coated with a new hydrophilic, hydrolytically stable, polyacryloylaminoethoxyethanol [poly(AAEE)] on the internal surface [5]. We determined CPSE efficiency, reproducibility, and

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through-put in the rapid analysis of some synthetic oligonucleotides.

2. Materials and methods

Capillary electrophoresis oligonucleotide run buffer, a capillary electrophoresis instrument BioFocus 3000, 75 μm I.D.×375 μm O.D. capillaries internally coated with poly(AAEE), the 8–32mer oligonucleotide standard and urea were obtained from Bio-Rad (Hercules, CA, USA). Homo oligomeric pd(A) 12–18, 19–24, 25–30 and 40–60 were obtained from Sigma (St. Louis, MO, USA). Fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Separations were carried out in 24 cm×75 μm I.D.×375 μm O.D. capillaries installed in User Assembled Cartridges (UAC) from Bio-Rad. Samples were loaded at 10 kV for 4 s and run at 15 kV and 35°C. Samples were detected at 260nm. The capillary electrophoresis oligonucleotide run buffer was degassed by centrifuging the vials for at least 2 min in a microcentrifuge immediately before inserting them into the BioFocus automatic sampler carousels. The sieving buffer was replenished before each run using a 2 min purge cycle at 100 p.s.i. pressure (1 p.s.i. = 6894.76 Pa). Two water rinse cycles were used to remove residual buffer from the capillary and electrode surfaces to prevent buffer carry over into the sample vial.

The oligonucleotide standard consisted of a mixture of 13 synthetic single-stranded oligonucleotides ranging in length from 8 to 32 bases. The oligonucleotide mixture contained approximately 14 μg/ml of each base in TBE buffer (25 m*M* Tris, 25 m*M* borate and 2 m*M* EDTA, pH 8.5).

The sequences for these components are listed below:

1. 8mer GACTGACT
2. 10mer GACTGACTGT
3. 12mer GACTGACTGACT
4. 14mer GACTGACTGACTGT
5. 16mer GACTGACTGACTGACT
6. 18mer GACTGACTGACTGACTGT
7. 20mer GACTGACTGACTGACTGACT
8. 22mer GACTGACTGACTGACTGACTGT
9. 24mer GACTGACTGACTGACTGACTGACT
10. 26mer GACTGACTGACTGACTGACTGACTGT
11. 28mer GACTGACTGACTGACTGACTGACTGACT
12. 30mer GACTGACTGACTGACTGACTGACTGACTGT
13. 32mer GACTGACTGACTGACTGACTGACTGACTGACT

Capillaries were coated using AAEE first described by Chiari et al. [5]. The coating was carried out similarly to the procedure described by Hjerten [7].

3. Results

Capillaries were coated using a novel acrylic monomer, AAEE first described by Chiari et al. [5]. This monomer is more hydrophilic than acrylamide and in addition, the monomer as well as the poly-(AAEE) are significantly more resistant to alkaline hydrolysis than acrylamide [5,6].

The separation of the 8–32mer single-stranded oligonucleotide standard at 35°C using the poly-(AAEE) coated capillary is depicted in Fig. 1. The 8–32mer standard consists of a mixture of 13 synthetic single-stranded oligonucleotides ranging in length from 8 to 32 bases. Although these are synthetic sequences, they contain repeating units of the four bases to more closely resemble naturally occurring sequences. This separation has been optimized with respect to the polymer sieving buffer and is complete within 10 min. Fig. 2 shows the effect of temperature on the separation of this standard. Below 30°C the migration patterns exhibit some irregularities suggesting that there may be some interactions occurring between these oligonucleotides. At the lower temperatures the analysis times are somewhat longer.

Precision data of migration times and area% over twelve runs for the thirteen standards are given in Table 1. The R.S.D. values for the migration times ranged from 0.74% to 0.87% over the 12 runs. For the area%, the R.S.D. values ranged from 0.5% to 5% with an average of 1.5%.

The 8–32mer standard was analyzed on a poly-

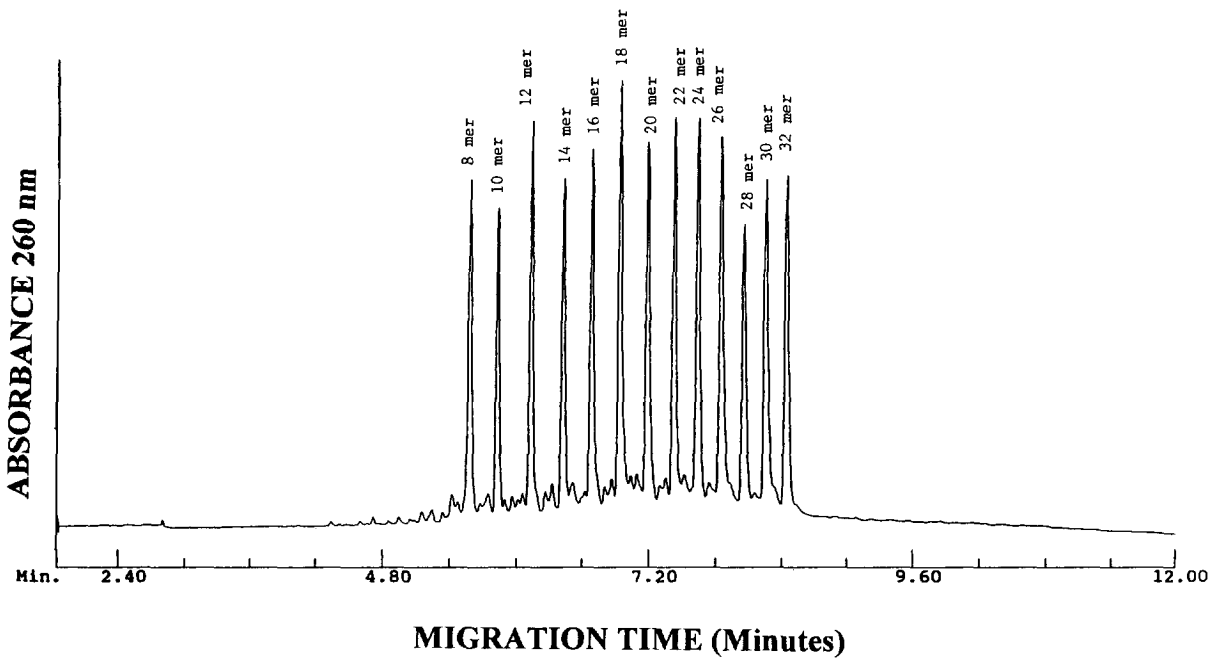

Fig. 1. Separation of 8–32mer standard on a poly(AAEE) coated capillary. The 8–32mer standard was separated on a poly(AAEE) coated capillary at 35°C using the the sieving buffer as described in Section 2.

(AAEE) coated capillary for 100 repetitive runs. Fig. 3 plots the efficiency of the 32mer over the 100 runs demonstrating a high efficiency which remains fairly constant (N~200 000). In Fig. 4(A and B) the electropherograms for the 1st and 100th runs are presented indicating very similar profiles. Fig. 3 also shows the results of a series of analyzes performed on an uncoated capillary. Initially, the uncoated capillary gave a very similar separation to the poly(AAEE) coated capillary. However, after 15–20 runs the separation begins to deteriorate and after 30 runs there is little resolution of the 30mer and 32mer [Fig. 4(C and D)].

The separating power can be enhanced by increasing the capillary length. Fig. 5(A and B) compares the separation of a pd(A) 12–30, 40–60 oligonucleotide standard in 24 cm and 36 cm long poly(AAEE) coated capillaries. The 36 cm capillary was able to give single base resolution in the 12–30 range, but not in the 40–60 range. The resolving power can be further increased by the addition of 6 M urea to the sieving buffer. Fig. 5(C and D) shows the separation of the same pd(A) standards using urea buffer in 24 cm and 36 cm capillaries. These comparisons clearly demonstrate the increased resolving power of the urea buffer. The use of the urea buffer in the 36 cm capillary was able to give baseline resolution of all the components in the standard up to 60 bases in length in about 40 min.

One important application for this analysis system is in the purity analysis of synthetic oligonucleotides. Fig. 6 shows the purity analysis of two different oligonucleotides obtained from a commercial supplier of oligonucleotides. The 26mer appears to be >95% pure while the 30mer contains a number of impurities.

4. Discussion

Acrylamide has been widely used in capillary electrophoresis in gel filled capillaries for the separation of DNA [8]. However, cross-linked gel filled capillaries have a number of drawbacks such as shrinkage of the gel, formation of air bubbles and overall short life span. Gel filled capillaries have recently been replaced by capillaries filled with polymer solutions, a technique first described by Zhu

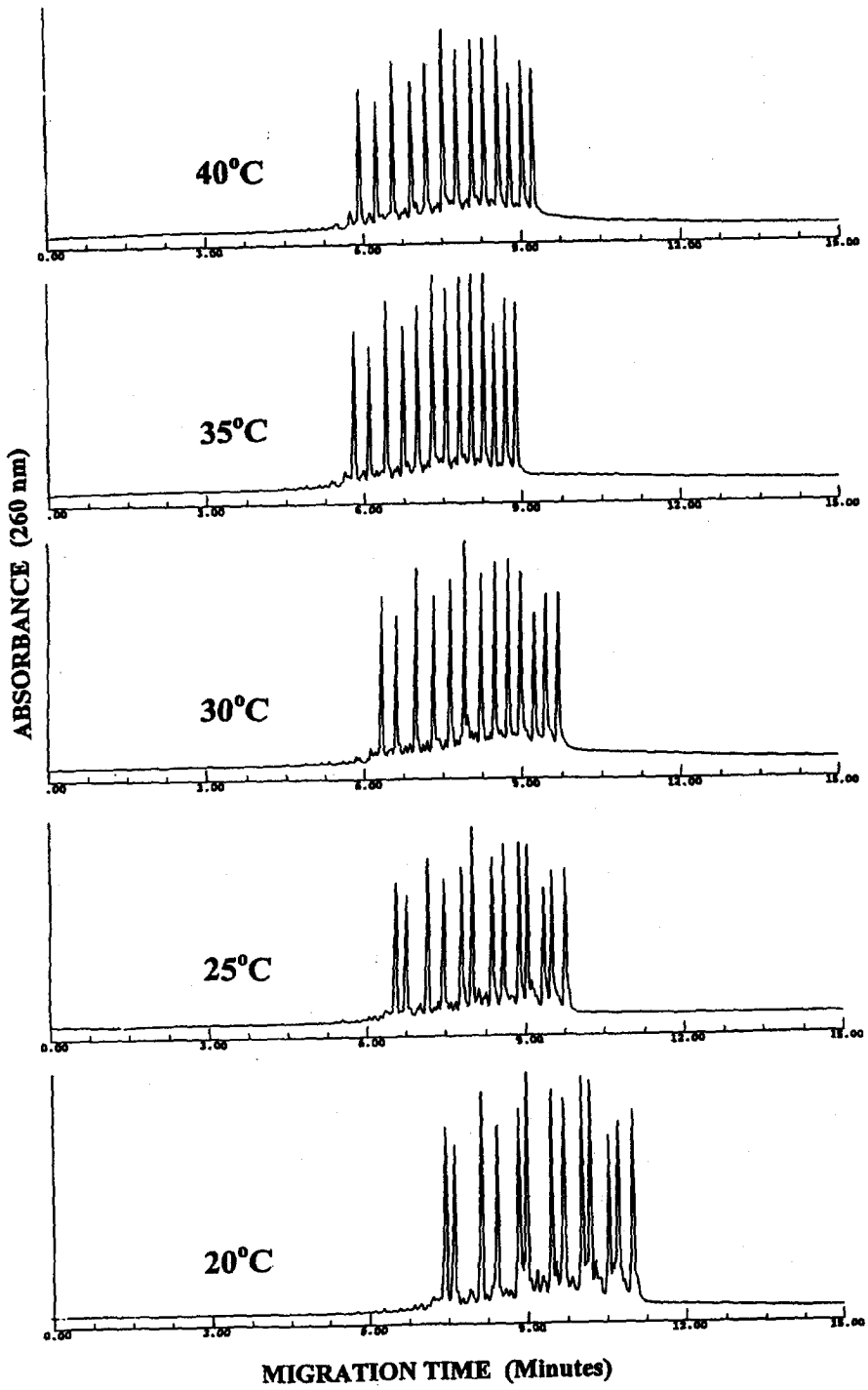

Fig. 2. Effect of temperature on oligonucleotide separation. The analysis of the 8–32mer standard was performed at the indicated temperatures using the oligonucleotide sieving buffer on poly(AAEE) coated capillaries as described in the Section 2.

Table 1
Precision data on migration time and area% for the 13 oligonucleotide standards

	Migration time (min)												
	8mer	10mer	12mer	14mer	16mer	18mer	20mer	22mer	24mer	26mer	28mer	30mer	32mer
S.D.	0.0430	0.0462	0.505	0.5033	0.565	0.0574	0.0604	0.0626	0.0653	0.0673	0.0703	0.0727	0.0745
AVER	5.8017	6.0533	6.3500	6.6400	6.9008	7.1433	7.3850	7.6167	7.8217	8.0242	8.2217	8.4100	8.5875
R.S.D.	0.74%	0.76%	0.79%	0.80%	0.82%	0.80%	0.82%	0.82%	0.84%	0.84%	0.86%	0.86%	0.87%
av R.S.D.													0.82%
	Area%												
	8mer	10mer	12mer	14mer	16mer	18mer	20mer	22mer	24mer	26mer	28mer	30mer	32mer
S.D.	0.1150	0.0543	0.0616	0.0443	0.0591	0.1836	0.0717	0.2889	0.0889	0.0642	0.0453	0.2400	0.1413
AVER	6.5742	8.1025	7.1542	5.5983	11.1617	8.0383	9.3850	5.7392	6.1967	7.9042	6.8883	10.2325	7.0308
R.S.D.	1.75%	0.67%	0.86%	0.79%	0.53%	2.41%	0.76%	5.03%	1.43%	0.81%	0.66%	2.35%	2.01%
av R.S.D.													1.54%

Methods: the 8–32mer oligonucleotide standard was analyzed at 40°C as described in Section 2 for 12 runs. The sieving buffer was changed after 6 runs.
Number of runs (N), 12.
Area%, relative peak area of each peak.
S.D., standard deviation.
AVER, average.
R.S.D., relative standard deviation.

et al. [1]. In this technique incorporation of a hydrophilic polymer in the electrophoresis buffer causes a molecular sieving effect in which the nucleic acids are separated. The polymer solutions can be forced out of the capillary after each run and fresh polymer solution introduced into the capillary. This offers the advantages of easy preparation and allows the capillaries to be reused for long lifetime.

In the present paper, a non-acrylamide water soluble polymer has been utilized for the rapid

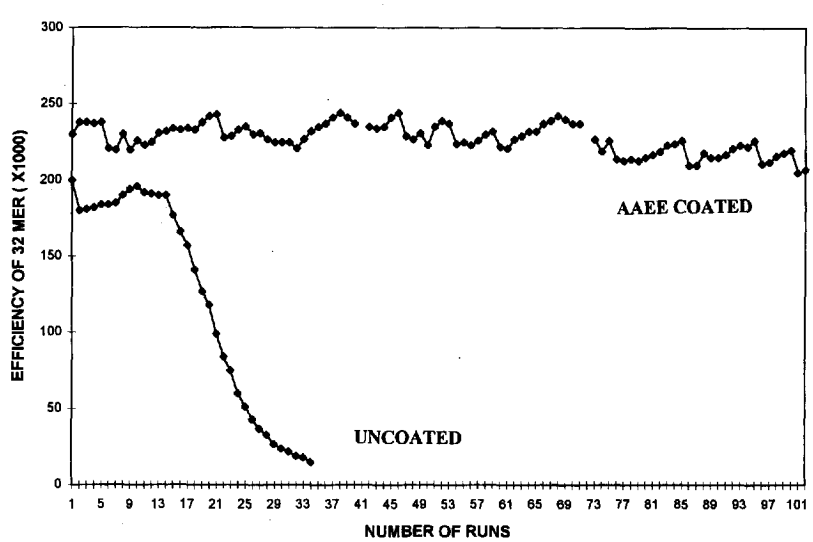

Fig. 3. Lifetime studies on poly(AAEE) and uncoated capillaries. The 8–32mer standard was analyzed for 100 consecutive runs on a poly(AAEE) coated capillary and for 33 runs on an uncoated capillary. The sieving buffer was replaced after every 40 runs. The results are expressed as the efficiency of the 32mer peak.

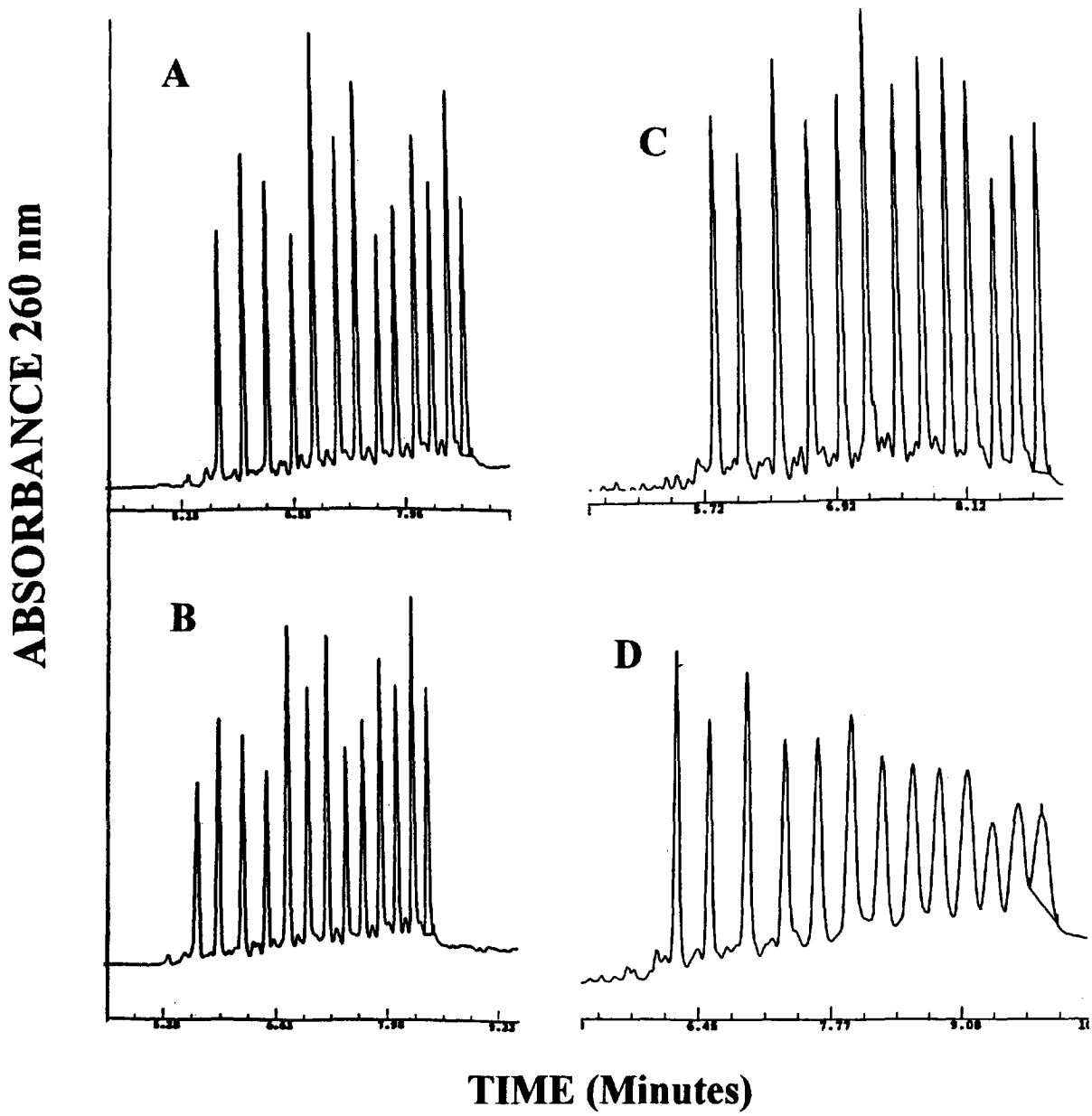

Fig. 4. Oligonucleotide separations on poly(AAEE) coated and uncoated capillaries. (A and B), the 8–32mer standard was analyzed for 100 consecutive runs on a poly(AAEE) coated capillary. Fresh vials of sieving buffer were utilized every 40 runs. The 1st (A) and 100th (B) runs are shown. (C and D), the 8–32mer standard was analyzed for 33 consecutive runs on an uncoated capillary. The 1st (C) and 30th (D) runs are shown.

separation of single-stranded oligonucleotides in the 4–30 base range in about 10 min using a 24 cm×75 μm I.D.×375 μm O.D. coated capillary. Although the polymer sieving buffer has a viscosity of greater than 60 centipoises, it was easily replenished between each run using a short 2 min purge at 100 p.s.i. pressure. This was important to maintain high efficiencies and to ensure reproducibility in the

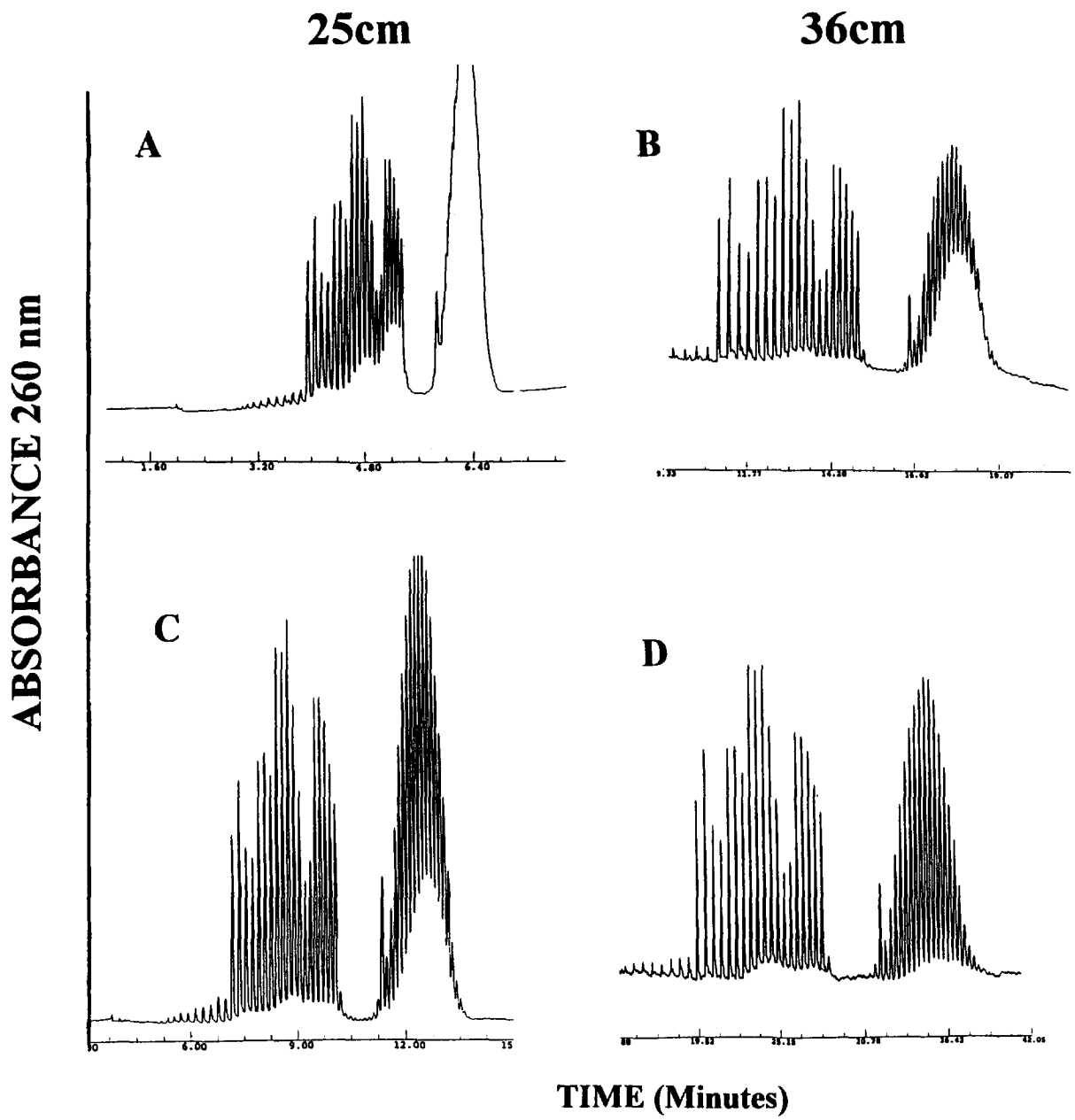

Fig. 5. Effect of capillary length and addition of urea to the sieving buffer. (A) Separations of a pd(A) standard (12–30, 40–60) were carried out in 24 cm×75 μm I.D.×375 μm O.D. capillaries using the sieving buffer. (B) Separations of a pd(A) standard (12–30, 40–60) were carried out in 36 cm×75 μm I.D.×375 μm O.D. capillaries using the sieving buffer. (C) Separations of a pd(A) standard (12–30, 40–60) were carried out in 24 cm×75 μm I.D.×375 μm O.D. capillaries using the sieving buffer containing 6 *M* urea. (D) Separations of a pd(A) standard (12–30, 40–60) were carried out in 36 cm×75 μm I.D.×375 μm O.D. capillaries using the sieving buffer containing 6 *M* urea.

oligonucleotide separations. Between 20–40°C the optimal temperature for this separation was determined to be 35°C–40°C. The standard employed for this analysis is a mixture of 13 synthetic single-stranded oligonucleotides ranging in length from 8 to 32 bases. This system provides reproducibility of

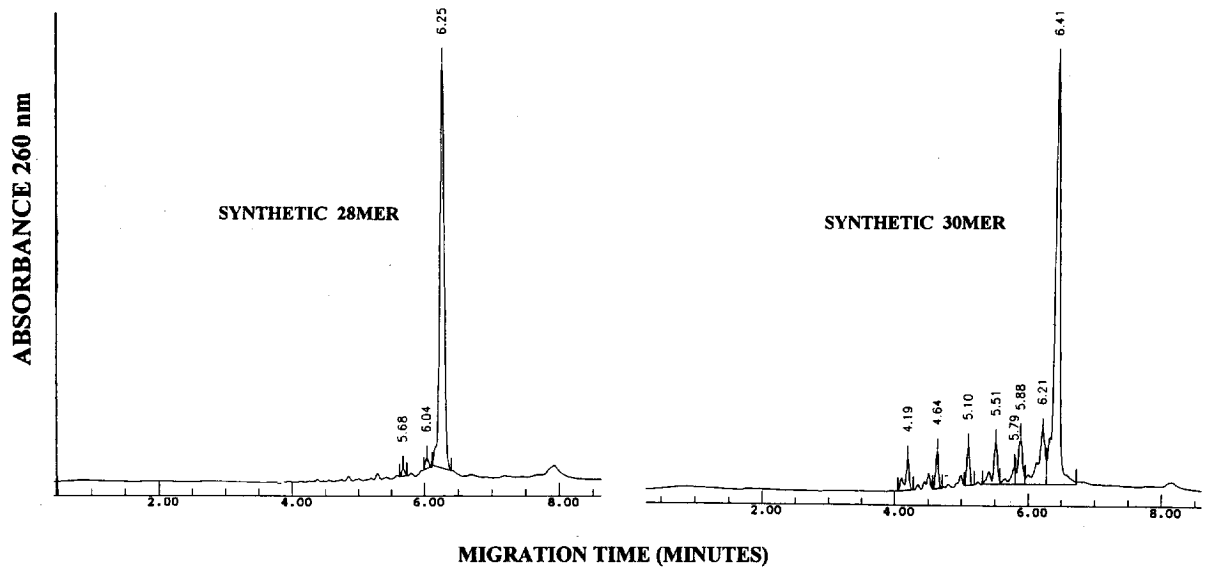

Fig. 6. Purity analysis of synthetic oligonucleotides. Two synthetic oligonucleotides obtained from a commercial vendor were separated on poly(AAEE)-coated capillaries using the oligonucleotide sieving buffer.

migration times of less than 1% and peak area of less than 5% R.S.D.. This analysis system can be applied to determining the purity of synthetic oligonucleotides with quantitative estimation of the amounts of product and failure sequences. For the efficient separation of larger oligonucleotides several experimental variables have been examined. For this analysis an oligomeric pd(A) standard was employed which went up to 60 bases. Increased resolving power was achieved by increasing the capillary length to 36 cm and by incorporating urea in the sieving buffer. Using the standard sieving buffer increasing the capillary length from 24 cm to 36 cm resulted in baseline separation of single bases up to the 30mer, with little resolution of the larger oligonucleotides. The analysis time was increased to about 15 min. Adding 6 *M* urea to the polymer sieving solution allowed baseline separation in the 60mer range with an analysis time of about 40 min using the 36 cm×75 μm I.D.×375 μm O.D. coated capillaries. Urea is probably acting by minimizing the interactions between the oligonucloetides and the sieving polymer.

An important component for the repeated use of a capillary in this analysis is the use of capillaries coated internally with a new polymer [poly(AAEE)]. This uses a novel acrylic monomer first described by Chiari et al. [5,6] which offers a high resistance to hydrolysis at the alkaline pH values. This is particularly important for nucleic acid analyses which are typically performed around pH 8.3. The use of poly(AAEE)-coated capillaries is a crucial component for the long life performance in terms of high reproducibility of efficiency and retention times.

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