Journal of Chromatography, 507 (1990) 113–123
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1748

Analysis of oligonucleotides by capillary gel electrophoresis

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SUMMARY

Analysis of oligonucleotides with gel-filled capillary columns is a fast, efficient and automated way to check their purity. Gel-filled columns were optimized to separate oligonucleotides which were 20–50 nucleotides in length. The influence of sample size on resolution is discussed. A comparison between capillary gel electrophoresis and slab gel electrophoresis in terms of analysis time per sample is made. In addition to homopolymeric samples, the separation of heteropolymeric oligonucleotides with one nucleotide difference in size is presented and suggests the possibility of using capillary gel electrophoresis as a tool for DNA sequencing.

INTRODUCTION

The pharmaceutical industry is currently undergoing a major shift in the way new drugs are designed. Whereas in the past, small organic molecules, *e.g.*, molecules with a molecular weight below 1000, made up the vast majority of all drugs, present and future efforts seem to be directed toward peptides and proteins[1]. In this context, biotechnology is rapidly becoming an important part of the pharmaceutical industry. The primary information is stored in the DNA of bacterial or mammalian cells and translated within the cell into the desired protein drug. The demands on analytical methods for biopolymers in general, and proteins, oligonucleotides and DNA in particular, change in parallel with the growth of biotechnology. A new analytical field, which has been termed "analytical biotechnology", is emerging[2].

Analytical biotechnology is, in essence, analytical chemistry dealing with biopolymers. The traditional questions such as structural elucidation, quantitation in a physiological environment, potency, safety and stability are altered by the nature of the samples. The structural elucidation of biopolymers requires information about the sequence of known monomers. Although the sequence determines the primary structure, and therefore the identity of biomolecules, secondary, tertiary and quaternary structure have considerable influence on their activity. The sensitivity in analytical biotechnology must be in the pico- to femtomole range to detect the minute

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0021-9673/90/$03.50

quantities of molecules of interest in the biochemical environment, while the industrial biotechnology environment requires speed, quantitation and automation.

Capillary electrophoresis (CE) is a relatively new analytical technique with the potential of becoming a standard method for analysis of biochemical samples[3–5]. It offers speed, ease, accuracy of quantitation, automation and is compatible with physiological samples.

As with high-performance liquid chromatography (HPLC), the term CE indicates the type of instrumentation and covers several separation modes[6,7]. The most popular techniques today are capillary zone electrophoresis (CZE)[8–14], micellar electrokinetic capillary chromatography (MECC)[15–18], capillary electroendosmotic chromatography[6,7] and capillary gel electrophoresis[19–22]. Gel electrophoresis in the slab configuration is a major separation tool for biochemical samples. Therefore, capillary gel electrophoresis has drawn considerable interest in the biochemical community, since it offers the possibility of transferring routine electrophoretic techniques to the automated capillary format.

Synthetic oligonucleotides are used extensively in biochemistry and molecular biology as probes for gene isolation and diagnostics, as primers for DNA sequencing, site-directed mutagenesis experiments and template amplification, and as linkers and adapters for cloning and gene alteration. In general, purity should not be crucial because only complementary strands will hybridize, but in amplification experiments contaminating oligomers may also be enhanced. Nevertheless, an easy, fast and reliable method for purity assessment of small oligonucleotides is needed to exclude impurities as a possible source of error in biological and biochemical experiments.

Recently, the separation of oligonucleotides on ion-exchange columns has been improved[23,24]. The development of new stationary phases allows the separation of oligonucleotides of up to 70 bases. Because the separation mechanism depends on apparent charge differences, the resolution of larger oligonucleotides becomes increasingly difficult. The same argument holds in CZE where small oligonucleotides can easily be separated by open tube methods. However, no separation could be observed with oligonucleotides of more than thirteen nucleotides. Adding metal ions and sodium dodecyl sulfate (SDS) extended the separation range to about 20 nucleotides[17].

Since the separation mechanism in column chromatography is not based on size differences, these methods offer no alternative to the resolving power of slab gels, where oligonucleotides of 600 bases can be separated by a one-nucleotide difference. However, the slab gel approach has the disadvantages of being slow, difficult to quantitate, and consists of many time-consuming manual steps. Capillary gel electrophoresis has the potential to combine the resolution power of slab gels with the automation and speed of the capillary instrument[25]. In this paper we examine the separation of various oligonucleotide samples with gel-filled capillaries.

EXPERIMENTAL

Apparatus

The CE system used in this work was similar to those described in the literature[3–5]. It consisted of a fused-silica capillary, a power supply, a detection unit and a data collection system. The fused-silica capillary (Polymicro Technologies,

Phoenix, AZ, U.S.A.), has the dimensions 75-μm inner diameter (I.D.), 360-μm outer diameter (O.D.).

A programmable Spellman power supply UHA 120 W (0–60 kV) (Spellman High Voltage Electronics, Plainview, NY, U.S.A.) was interfaced with a HP series 300 computer system and a HP 3852 data acquisition system (Hewlett-Packard, Palo Alto, CA, U.S.A.). A BASIC program was written to control the power supply during injection and run times and to collect and store the data from the detector output and current readings. The same software also allowed for simple plot and integration routines.

The detector, a Kratos 783 absorbance detector (Applied Biosystems, Ramsey, NJ, U.S.A.), was modified in-house for on-column detection. The HPLC flow cell was replaced by a round disc with a 3-mm hole in the middle upon which grooved apertures of various dimensions were mounted. The capillary was fixed over the aperture with an adhesive. Both ends of the capillary were placed outside the detector housing in 3-ml buffer reservoirs. This arrangement resulted in a minimum length of 30 cm and an effective length (distance from injection point to detection point) of 15 cm, but for convenience a capillary length of 40 cm (20-cm effective length) was used in all experiments. The light reaching the reference cell was reduced by covering it with an aperture having a 50 × 50 μm hole. The average noise level of the detector was determined to be $300 \cdot 10^{-6}$ a.u.

Chemicals

The buffer components, tris-(hydroxymethyl)-aminomethane (Tris), boric acid and urea, the acrylamide–cross-linker mixture (29:1) and the starter of the polymerization reaction, ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were electrophoresis-grade and purchased from Schwarz/Mann Biotech (Cleveland, OH, U.S.A.). Methacryloxypropyltrimethoxysilane was supplied by Petrarch (Bristol, PA, U.S.A.); polyethylene glycol (PEG) 20 000 was obtained from Fluka (Ronkonkoma, NY, U.S.A.); methanol and water were HPLC-grade.

The oligodeoxythymidylic acids pd(T)12–18, pd(T)19–24 and pd(T)25–30 were purchased from Pharmacia (Piscataway, NJ, U.S.A.). Stock solutions of 0.05 A_{260} units per μl or 2 μg/μl were stored at −20°C and diluted by ten for injection. (By convention, one A_{260} unit equals 40 μg of oligomer.) The ladder-type polydeoxythymidylic acids pd(T)10–50 and the heterooligomeric nucleotides used in this study were synthesized with a Beckman System 1 Plus DNA Synthesizer. Reduction of the nucleoside phosphoramidite reagent concentration was automatically programmed at desired synthetic base additions to increase synthesis failure and enhance a given oligomeric size[26].

Procedure

A stock solution of buffer, consisting of 50 m*M* Tris, 50 m*M* boric acid, 3% PEG[27], and 7 *M* urea, was used for the preparation of the gel columns and as the running buffer. All buffer solutions were filtered through a nylon 66 filter unit of 0.2-μm pore size (Schleicher and Schüll, Keene, NH, U.S.A.). To provide an optical detection window, about 2 mm of the polyimide coating were removed with an electrically-heated wire in the middle of an appropriate piece of fused silica, normally about 50 cm long. The capillary was flushed with 1 *M* hydrochloric acid, 1 *M* sodium

hydroxide and methanol, respectively. A mixture of methanol and methacryloxypropyltrimethoxysilane (50:50, v/v) was injected into the capillary and left there for at least 3 h[28,29]. Then 750 mg of the acrylamide–cross-linker mixture were dissolved in 10 ml buffer solution, resulting in a 7.5% polyacrylamide gel. A 10% (w/v) solution of both APS and TEMED was prepared. Depending on the polymerization time, 2–5 μl of each was used to start the polymerization of 1 ml of the monomer solution. The unpolymerized solution was injected into the capillary and left overnight for full polymerization.

Sample injections were made electrophoretically by switching the cathodic buffer reservoir with a sample vial and applying voltage for a predetermined time. Typical injection conditions were 3–10 s at 1000–2000 V for aqueous solutions of the samples. Fields ranging from 250–450 V/cm were applied (40-cm capillary, 10–18 kV), resulting in a current of 3–7 μA. During operation, the capillary was actively cooled at room temperature with a fan.

Slab gels were prepared with 10% T and 5% C[a] in 0.1 *M* Tris-borate buffer with 2 m*M* EDTA, pH 8.3. The 0.5-mm thick gels were electrophoresed at 1000 V or 55 V/cm for 5 h. Radiolabelling of the oligomers and their detection on photographic film following separation were done according to standard procedures[30].

RESULTS AND DISCUSSION

Separation of homopolymeric oligonucleotides

In contrast to earlier reports[22], where the separations of deoxypolyadenylic acids were carried out on 5% gels, a monomer concentration of 7.5% was used in this work. The electropherogram of the separation of deoxypolythymidylic acids with 12–30 bases (Fig. 1) reveals that the mixture was composed of three different component size groups, each one having a distinct peak pattern. The component containing 12 to 18 nucleotides shows a Gaussian distribution, but for the other two component groups, the relative amount of each oligonucleotide decreases with increasing nucleotide number. The pattern recognition allows an easy check of column performance and resolution.

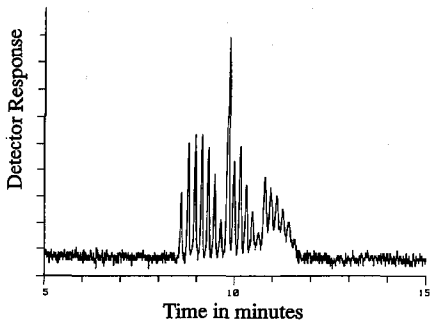

Fig. 1. Separation of deoxypolythymidylic acid pd(T)12–30. Separation conditions: capillary 75 μm I.D., 360 μm O.D., length 40 cm, effective length 20 cm; buffer 50 m*M* Tris, 50 m*M* boric acid, 7 *M* urea; column 7.5% T, 3.3% C; field 375 V/cm; current 5.6 μA; injection 4000 Vs; detection 260 nm UV on-column.

[a] %T = [g acrylamide + g N,N'-methylene bisacrylamide(Bis)]/100 ml solution; %C = g Bis/% T.

In order to check the performance of the gel columns, we synthesized homopolymeric oligonucleotide samples of an exact size by solid phase methods. The sample was a homopolymer to exclude any mobility differences, *e.g.*, the separation mechanism should depend solely on size differences and have an easily recognizable pattern. The synthesis of an oligodeoxythymidylic acid pd(T)50 was carried out in a way that, starting from cycle 15, the repetitive yield of each fifth nucleotide addition was decreased. In this manner, a sample with a ladder-type pattern was obtained. Fig. 2 shows the capillary electropherogram of this sample and the comparable slab gel pattern. Beside the major peak for the pd(T)50, passing the detector at 26 min, seven peaks representing pd(T)15, pd(T)20, pd(T)25, pd(T)30, pd(T)35, pd(T)40 and pd(T)45 can be distinguished. Between pd(T)30 and pd(T)35, and between pd(T)35 and pd(T)40, there are four peaks observed just above the noise level of the detector that originate from the failure rate at each cycle. The migration times increase linearly with the nucleotide number, indicating a separation correlated with size differences for the 15–50 nucleotide chain length. The slab gel electropherogram shows corroboration of the gel-filled capillary pattern. Capillary electrophoresis with gel-filled capillaries, therefore, suggests a more readily quantifiable check of the purity of an oligonucleotide sample.

Influence of sample size

Using the same separation conditions and the same capillary as in Fig. 2, the capillary column was overloaded by injecting the test sample for 10 s at 5000 V. The resolving power of the column decreases dramatically: only the major peaks can be detected with a distorted and split peak shape and it is not possible to observe the failure sequences (see Fig. 3). These results point out an additional factor relative to

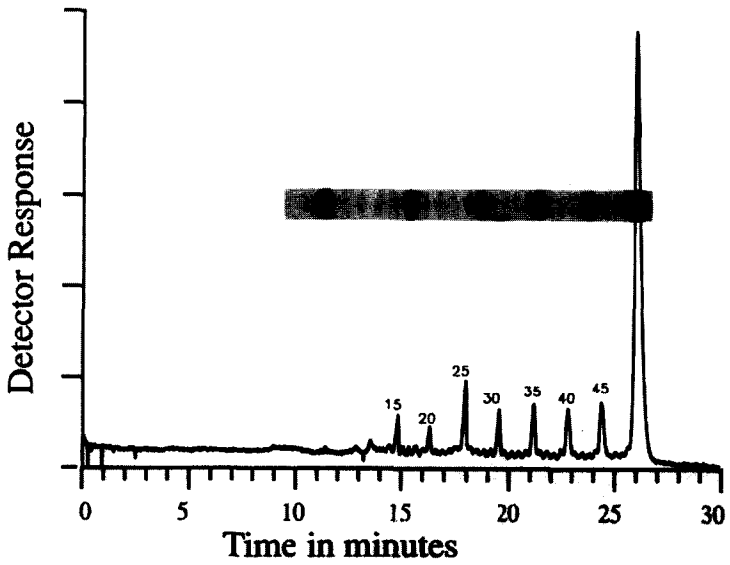

Fig. 2. Separation of deoxypolythymidylic acids pd(T)10–50. Field 250 V/cm; current 5.8 μA; injection 4000 Vs; other conditions same as Fig. 1. Slab gel electrophoresis conditions, see text.

sample injection onto capillaries. It is customary to inject samples dissolved in water or solvents lower in ionic strength than the electrolyte to enhance sample detection by means of zone sharpening. Much of the peak distortion and loss of resolution shown in Fig. 3 may be due to the fact that the sample solvent was water and it was injected into an electrolyte containing 7 *M* urea. The chaotropic effect of urea in the electrolyte is probably inversely related to the size of the aqueous sample slug.

Rose and Jorgenson[31] derived an equation to calculate the volume and quantity of a sample injected electrophoretically onto a capillary column. They used open-tube capillaries and had to correct the electrophoretic mobility for the electroosmotic flow-rates of the system. Since no effective bulk fluid flow is observed when injecting a neutral marker in a gel-filled column, the equation of Rose and Jorgenson simplifies to

$$Q = \frac{\mu_{ep}\pi r^2 V_i t_i}{L} C$$

where Q is the quantity of sample injected, μ_{ep} the apparent electrophoretic mobility, r the radius of the capillary tube, V_i the injection voltage, t_i the injection time, L the overall length of the capillary and C the sample concentration.

However, this equation must be modified to determine the amount injected into gel-filled capillaries. The gel presents several unique factors, some of which are difficult to quantify: the electrolyte displacement by the gel or interstitial space, the geometric obstruction factor[7] pertinent to the solute, and the volume fraction of the electrolyte components. While an estimate of the interstitial space for a gel formulation in a capillary is difficult, it is expected that the volume fraction of 7 *M* urea in the electrolyte solution would be approximately 0.3. These factors are collected as a single term of α of this argument:

$$Q = \frac{\mu_{ep} V_i t_i}{L} C\pi r^2(1 - \alpha)$$

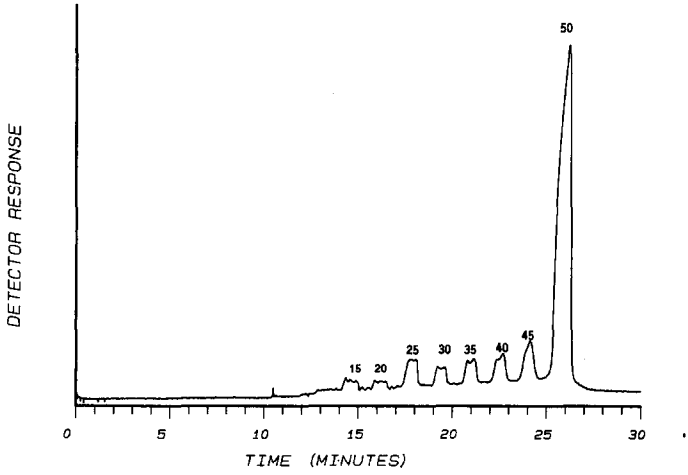

Fig. 3. Same sample and conditions as used in Fig. 2, except injection at 50 000 Vs.

Since these factors were not quantified in this work, only a relative comparison of injections on the same capillary can be made from the mathematical product of the electrical potential or voltage (V) and injection time (t_i) in s. These two product values are 4000 and 50 000 Vs, respectively, or a ratio of 12.5. As stated above, the higher sample loading resulted in a decreased resolution. Therefore, the sample dynamic range for the separation of oligonucleotides is probably less than 10 with the UV detector used in this work. This demonstrates the need for more sensitive detection principles such as fluorescence[32] or radioactivity[33].

Separation of heterooligonucleotides

In order to examine the usefulness of gel-filled capillaries for the separation of heterooligonucleotides, we synthesized a series of four oligonucleotides, two complementary strands with 29 and 30 nucleotides each. The sequence of the 30-mer and its complementary 30-mer strand were the same as the corresponding 29-mers plus an additional nucleotide. Thus, the 29-mer hybridizes with the complementary 29-mer and with one of the 30-mers, whereas hybridization with the second 30-mer is not possible because their sequences are the same.

In Fig. 4, the electropherograms for the 29- and 30-mers are plotted in one diagram. As expected, each separation shows one major component plus a number of smaller failure sequences with higher mobilities than the main product. The samples were used after synthesis without any further purification. Slab gel electrophoresis shows, in general, a smeared spot for impure oligonucleotide samples. Capillary electrophoresis with gel columns can, therefore, be used to check the purity of an oligonucleotide sample.

It is apparent from Fig. 4 that the separation of the failure sequences is not as regularly spaced as with homopolymeric oligonucleotides. This is expected in view of the size difference between the purine and the pyrimidine nucleotides. Nevertheless,

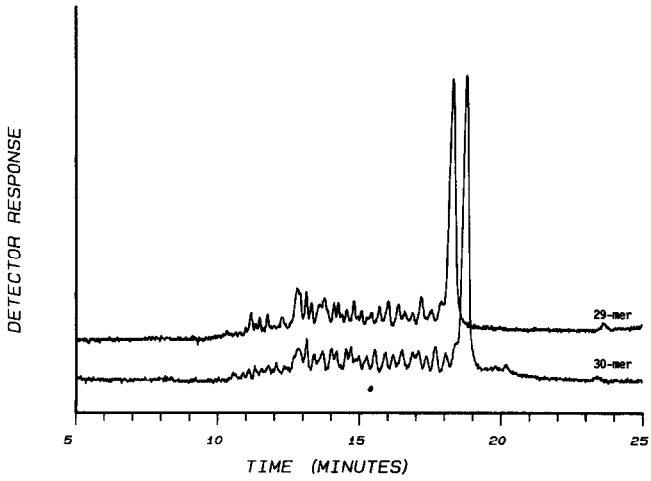

Fig. 4. Separation of crude synthetic oligonucleotides, upper trace, 29-mer: 5'ATGACAGAATACAAG CTTGTGGTGGTGGG3'; lower trace, 30-mer: 5'ATGACAGAATACAAGCTTGTGGTGGTGGGC3'. Each sample was injected separately. Field 250 V/cm; current 4.8 μA; injection 4000 Vs; other conditions same as Fig. 1.

Fig. 4 indicates that the separation of the 29- and the 30-mers is possible with this system. It should be noted that an absolute comparison of migration times cannot be made for the experiments described here. Since this CE system was not equipped with an adequate temperature control unit, current changes from 7 to 4 μA were seen over the course of 5 to 10 h of repeated operation. Also, as seen by inspection of the capillary under a microscope, bubbles formed at the injection end of the gel-filled capillary. Therefore, the capillary was cut by a few mm when the current dropped below 3 μA. After cutting the capillary, the current readings went back to former values and the separation power of the capillary was restored.

For the reasons stated above, single-base resolution of heterooligonucleotides can only be proven by injection of a mixture of oligonucleotides, differing by one nucleotide in length. Fig. 5 shows the result of this experiment. Because the 29- and the 30-mers were mixed and the absolute concentration of oligonucleotide in the sample volume decreased by a factor of two, the amount injected is too small for detection of the failure sequences. But the 29- and the 30-mers show baseline resolution. This experiment confirms that capillary electrophoresis has the potential for DNA sequencing[34]. Extending the separation range of gel-filled capillary columns to base numbers of several hundred and using Sanger-type sequencing mixtures will require a more sensitive fluorescence detector for CE, as well as certain improvements in the instrument (temperature control) and optimization of gel columns.

The use of 7 *M* urea in both the capillary and the buffer inhibits hybridization of complementary single-stranded oligonucleotides. With homopolymeric, non-complementary samples, hybridization does not occur. However, hybridization may occur if the complementary 29-mer and 30-mer are mixed in water at room temperature, rather than in urea and above the melting point of the double-stranded DNA fragment. In Fig. 6, the electrophoretic separation reveals three different species: two peaks between 18 and 19 min corresponding to the 29- and 30-mer and an additional peak at 20 min.

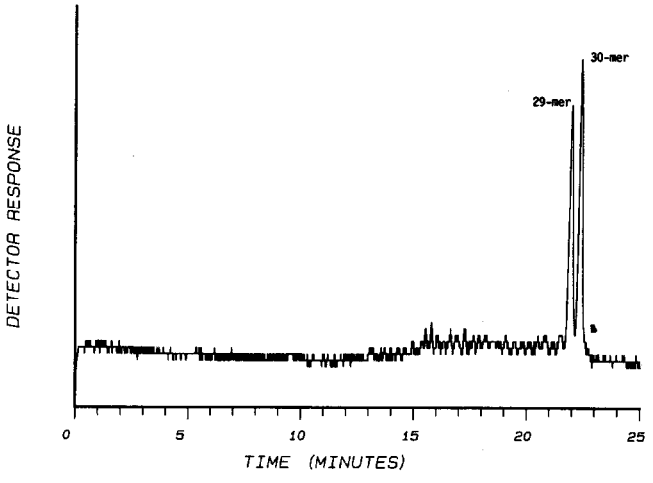

Fig. 5. Separation of a mixture of the two oligonucleotides described in legend to Fig. 4 under the same conditions.

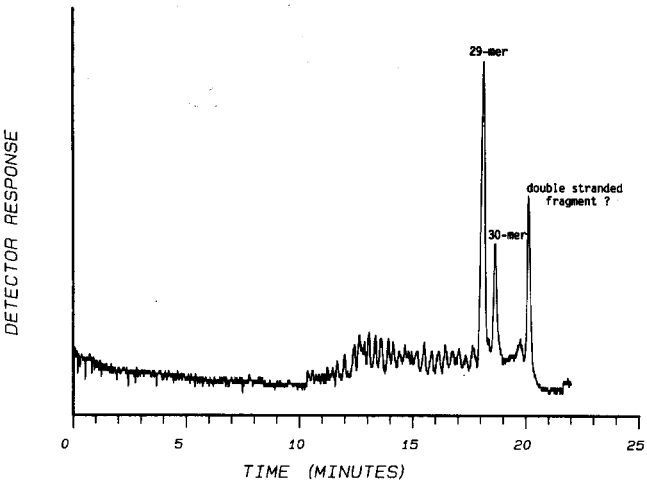

Fig. 6. Separation of a mixture of two crude, synthetic oligonucleotides, 29-mer: 5'GCCCACCACCAC AAGCTTGTATTCTGTCA3', and 30-mer: 5'ATGACAGAATACAAGCTTGTGGTGGTGGGC3'. Separation conditions were the same as described in legend to Fig. 4.

It can be speculated that even if the separation medium contains urea, this is not sufficient to break up all hydrogen bonds in the double-stranded DNA. The appearance of a third peak depends on the time that the sample was left to hybridize. It is expected that there is approximately 8% decrease in renaturation rate for each mole increase in concentration[35]. The separation shown in Fig. 6 was obtained by injecting the sample 10 min after mixing. If the same mixture is injected 40 min after mixing, the peak at 20 min increases in height, whereas the peak for the 30-mer at 18.7 min decreases. After 70 min, only two peaks can be observed. This would be expected, since one oligomer is present in excess of the other. After hybridization, the mixture consists of a double-stranded DNA fragment and a single-stranded, unhybridized oligonucleotide. If the separation time is sufficiently short compared to the hybridization time, it may be possible to measure the hybridization kinetics under different conditions by this method. The analysis time can be shortened by increasing the field strength in one of two ways: by an increase in the voltage drop at a constant capillary length or by a decrease in the capillary length at a constant voltage.

Comparison of slab gel electrophoresis and capillary gel electrophoresis for the separation of oligonucleotides

When comparing capillary gel or column electrophoresis to slab gel or open-bed electrophoresis, two variations of the same basic separation technique are contrasted. In general, the advantages of a column-operated technique are on-line sample detection, easy quantitation and automation and the separation medium can be reused for a number of runs. The disadvantage of this technique is that only one sample at a time can be analyzed. In contrast, several samples can be analyzed at the same time with slab gels.

To compare analysis times of the two techniques, the run time per sample has to be determined. In capillary gel electrophoresis, run times are typically 30 min or less.

No additional time is needed for detection and data reduction; sample preparation is the only manual step and accounts for 2 min per sample. On a slab gel, 18 samples can be loaded on one plate. Total estimated analysis time for slab gel electrophoresis consists of 2 h for gel preparation and casting, 5 h of run time, 1 h of gel transfer time to the photographic plate and film development, and 16 h of autoradiogram development. The total analysis time is 24 h, or 80 min per sample, more than double the time required with CE instrumentation. Also, technician time adds up to 3 h total, or 10 min per sample, and is considerably more than with CE.

CONCLUSIONS

Capillary gel electrophoresis can be used to check the purity of a synthetic oligonucleotide. The separation by CE is completed within 10 to 30 min and is much faster than slab gel electrophoresis. A comparison of the analysis time for both techniques is made. When the differences in mobilities are taken into account, the method also gives easy and accurate quantitation, which should be useful to optimize synthesis programs using an automated DNA synthesizer. The columns in this work were stable and were used for 50 consecutive injections (more than 20 h of run time).

Single-nucleotide resolution can also be achieved with oligonucleotides composed of an arbitrary sequence. CE with gel-filled columns seems to be an alternative to slab gel electrophoresis to obtain sequence information from DNA fragments. Further developments in column design are directed to the resolution of longer oligonucleotides with nucleotide numbers between 100 and 500.

ACKNOWLEDGEMENTS

We gratefully acknowledge the considerable and expert technical assistance of Theresa Cash in the synthesis of the oligonucleotides used in these studies. We thank Nebojsa Avdalovic for suggesting the sequence of the 29- and 30-nucleotide heterooligomers.

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