Detection of DNA fragments separated by capillary electrophoresis based on their native fluorescence inside a sheath flow

David A. McGregor, Edward S. Yeung*

Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, IA 50011, USA

Abstract

A scheme for the separation and detection of native DNA fragments in capillary electrophoresis is presented. A UV laser at 275 nm excites the intrinsic fluorescence of the fragments, which is greatly enhanced at pH 2.8. To provide a compatible system, methylcellulose-based size separation is performed at the identical pH. A sheath-flow arrangement isolates the detection region from the linear polymer for a reduced background level. The performance is an order-of-magnitude enhancement in detectability over absorption detection. We also uncovered a selective degradation/ligation process at these pH conditions that may be useful as additional selectivity for DNA characterization.

1. Introduction

The separation and isolation of large DNA fragments is a critical preliminary step for DNA sequencing. It is also an important tool for generating physical maps. Recently, DNA fingerprinting has been found valuable in forensic applications. Slab gel electrophoresis (SGE) especially variants of pulsed-field operation (PFGE) [1,2], has been successfully used for these large fragments. One important consideration is detection without additional manipulation, such as the introduction of fluorescent or radioactive labels for visualization. The integrity of the fragile fragments is then preserved. Time, cost, and effort are reduced. The separated fragments are immediately available for use in the subsequent steps, thereby facilitating auto-

mation. More importantly, migration of the DNA fragments will be unaffected by the presence of the label, allowing better control of the separation.

In all current approaches and many proposed approaches to DNA sequencing, some form of tag is attached to the fragments to provide a measurable signal. These can be fluorescent [3,4], radioisotopic [5], isotopic [6], or chemiluminescent labels [7]. Except for isotopic and radioisotopic labels, these modify the DNA fragments sufficiently that the mobility in electrophoresis is affected, leading to potential errors in base calling. The synthesis of special tags can add to the cost of sequencing. These tags also suffer from limited shelf life, can complicate interpretation due to slow kinetics or incomplete reaction, and can generate unacceptable levels of toxic or hazardous waste in a major sequencing effort.

* Corresponding author.

In the last two years, several research groups have shown that capillary gel electrophoresis (CGE) is an attractive alternative to SGE for DNA sequencing and mapping [8–10]. The medium used, buffer composition, separation mechanism, sequencing chemistry, and tagging chemistry for CGE are all derived from proven SGE schemes. A 25-fold increase in the sequencing rate per capillary (per lane) has already been demonstrated. This is a direct consequence of the small internal diameter of the capillary tubes, typically around 50–75 μm, greatly reducing Joule heating associated with the electrical current. Gel distortions and temperature gradients that can affect resolution of the bands are thus virtually absent. More importantly, much higher electric fields can be applied to speed up the separation. For comparison, conventional SGE are limited to field strengths below 50 V/cm while CGE have been successfully used for sequencing up to 500 V/cm [3]. The unique aspect ratio of capillary gels (25–50 cm long) provides uniform field strengths and the large surface-to-volume ratio favors efficient heat removal. These combine to produce much sharper bands than are possible in slab gels.

Detection of native DNA fragments in CGE by UV absorption at 260 nm or at 210 nm has been successfully applied in many situations [11]. However, the short pathlengths available across the capillary tube severely limit the detection power. So, conventional absorption detection schemes are not compatible with the small amounts (1–10 amol) per band in DNA sequencing. It is possible to use photothermal spectroscopy [12] to enhance detection limits in absorption, and this has been demonstrated recently for several nucleotides at the 2 fmol level using pulsed excitation [13]. Since amol detection limits have already been reported for continuous laser excitation of derivatized amino acids [12], application to DNA sequencing should be feasible. What is needed is a suitable excitation wavelength. Recently, ion lasers at 275 nm have become commercially available, with output powers approaching 0.5 W. This is superior to the 248-nm output from excimer lasers [13], which, because of their high peak

powers, cannot be used at the high average powers needed for sensitive detection. The technology has potential for applications to DNA sequencing based on the native (unlabeled) fragments.

Another feature of DNA fragments that potentially allows detection without tags is the weak fluorescence after UV excitation. This has been documented many years ago [14,15] but has not found applications because of the extremely low efficiencies. However, laser-excited fluorescence detection in ideal cases can approach the 10^{-21} mole range [16]. So, even giving away a few orders of magnitude in fluorescence efficiency, native DNA fluorescence may still be adequate for sequencing applications.

Recently, we were able to demonstrate sensitive detection of native nucleotides and DNA fragments by laser-excited fluorescence at 275 nm in capillary electrophoresis [17]. The method takes advantage of the shift in fluorescence quantum efficiency associated with a change in the pH of the analyte's environment. By moving above or below the pK_a of fluorescence activation, a significant enhancement in detection limits can be realized. Under these conditions, detection limits are $5 \cdot 10^{-8}$ M, $1.5 \cdot 10^{-8}$ M, $5 \cdot 10^{-5}$ M, and $2 \cdot 10^{-6}$ M for A, G, C, T (monophosphate nucleotides), respectively (concentration at injection). These low pH conditions are however not compatible with separations of DNA fragments in gels or in polymer solutions. In this paper, we describe a procedure for interfacing these techniques to allow sensitive detection.

2. Experimental

2.1. Materials

All buffers were prepared with 0.45-μm filtered deionized water. The buffer consisted of phosphoric acid titrated to pH 2.8 with NaOH (Aldrich, Milwaukee, WI, USA). The linear polymer used in this study was methylcellulose 4000 (Sigma, St. Louis, MO, USA). ΦX174RF DNA-HAE III digest was supplied by United

States Biochemical (Cleveland, OH, USA). The DNA solutions were diluted with running buffer just before injection onto the column.

2.2. Apparatus

The electrophoresis apparatus was built in-house and is described below. The separation was driven by a 30-kV power supply (Spellman, Plainview, NY, USA). The sheath flow was made with concentric capillaries supported by an 1/8-in. (1 in. = 2.54 cm) T-connector (Swagelok, Solon, OH, USA) as pictured in Fig. 1. The sheath buffer is contained by a 1000 μm O.D. 500 μm I.D. quartz capillary (Polymicro Technologies, Phoenix, AZ, USA) supported by PTFE tubing to allow some movement for concentric alignment of the inner separation capillary. The sheath reservoir was gravity fed with the sheath buffer at a height of 10 cm. The capillary used throughout was a 50 μm I.D., 360 μm O.D. DB-1 coated GC capillary with 0.2-μm coating (J&W Scientific, Folsom, CA, USA). The effective and total capillary length was 40
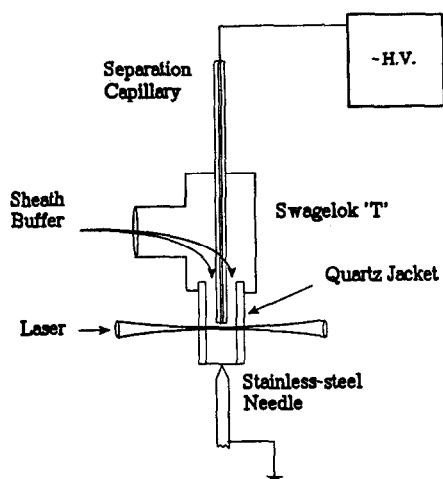

Fig. 1. Experimental setup for sheath-flow detection. The negative high voltage (H.V.) is connected to the separation capillary via a buffer reservoir. Ground is achieved via an electrode placed just under the sheath capillary. Sheath buffer flows through the system by means of gravity flow. The two capillaries are fixed via a "T" connector. The laser light passes through the sheath capillary ca. 0.5 mm below the exit of the separation capillary.

cm. The nucleic acids were excited by a 275 nm (ca. 20 mW) laser line from an argon ion laser (Spectra Physics, Palo Alto, CA, USA). Fluorescence was collected at right angles to the excitation beam by a 20 × microscope objective and directed to a photomultiplier tube at 800 V through two UG-1 filters. The excitation source was directed through the sheath at ca. 0.5 mm below the end of the separation capillary. Alignment was done with fluorescein electrophoretically flowing through the capillary. For comparison, a commercial CE instrument (Spectra-Phoresis 1000, Spectra Physics, Mountain View, CA, USA) was used.

2.3. Methods

The separation medium was a pH 2.8 phosphoric acid solution which contained 0.5% (w/w) methylcellulose. The preparation of the medium is described in our earlier work [18]. The sheath buffer did not contain methylcellulose. This design was chosen because of possible fluorescence interference and possible quenching of the DNA fluorescence in the presence of the polymer network. Filtration was performed on the polymer solution through a 0.8-μm cellulose acetate filter and the solution was stored at 4°C until needed.

The capillary is first filled with the separation medium and the sheath buffer is allowed to flow. The grounding electrode acts both as a wick that draws the sheath buffer from the system and as an anode. Sheath flow is estimated at ca. 20 μl/min. Once the capillary was pressure-filled it was allowed to equilibrate at running voltages for 5 min to make sure no polymer solution remained in the detection region.

3. Results and discussion

Separation of DNA fragments in the 0–2 kb range in CE in various sieving media is a well established technology [11,18–23]. Invariably, separations are performed at neutral to slightly alkaline conditions. The phosphate groups in the DNA backbone are then fully charged. The

electrophoretic mobilities are then maximized. Wall interaction is also minimized. An additional advantage is that near-physiological conditions can be maintained. For native fluorescence detection [17], it has been observed that GMP shows a 2 orders-of-magnitude increase in fluorescence efficiency at pH 2.7 compared to that in the pH range 5–8. This increase in efficiency is essential to sensitive detection.

One possible approach is to implement DNA size separation under proven conditions [23] and then introduce a pH change from 8.2 to 2.7 in the detection region. This is analogous to post-column derivatization. By using the arrangement shown in Fig. 1, we can introduce dilute HCl as a sheath buffer at the outlet of the separation capillary to produce a pH jump. Normally, sheath flow is designed to focus the core flow to a narrow diameter to minimize the detection volume [24]. There is an insignificant amount of mixing between the sheath flow and the core flow up to and including the observation region. In our case, the observation region is selected to be well beyond the exit of the electrophoretic capillary to assure some diffusional mixing. This region was identified using fluorescein dye in the core flow at a low pH and dilute NaOH in the sheath flow. Since the quantum efficiency of fluorescein increases substantially from pH 4 to pH 8, visual confirmation of mixing under illumination by a hand-held Hg lamp is possible.

When a 275-nm laser is focused into this mixing zone to excite native DNA fluorescence, only a weak signal was recorded. There are two reasons for this observation. First, the ionic strength for the separation buffer was very high (100 mM). The amount of HCl needed to titrate this to a low pH is thus substantial, and may not have been available in the limited mixing region. Second, there exists necessarily dilution associated with mixing. So, one expects the detectability to degrade in such a flow system.

We therefore attempted to separate the DNA fragments at a low pH to provide compatibility with the detection mode. At pH 2.8, the phosphate groups should still dominate over the amine groups to impose a net negative charge on the fragments. In fact, the isoelectric point

should be around pH 2.0. We are however not aware of any previous attempts of size separation of DNA fragments at these low pH conditions. The results from a commercial instrument are shown in Fig. 2a. Several features can be noted. First, the migration times are generally longer than previous experiments at the same field strength, even after correction for column length. This is expected due to the lower net charge on the fragments. Second, the separation efficiencies are very high, in excess of 100 000 theoretical plates for all peaks. This shows that the polymer medium is intact under these buffer conditions. Third, the expected fragment peaks in the HAE III digest are present, but other peaks are also clearly seen. This separation pattern is reproducible over many runs. The HAE III fragments are identified based on the relative peak heights as well as comparison with other DNA samples with different fragment sizes run in the same medium. We note that if the
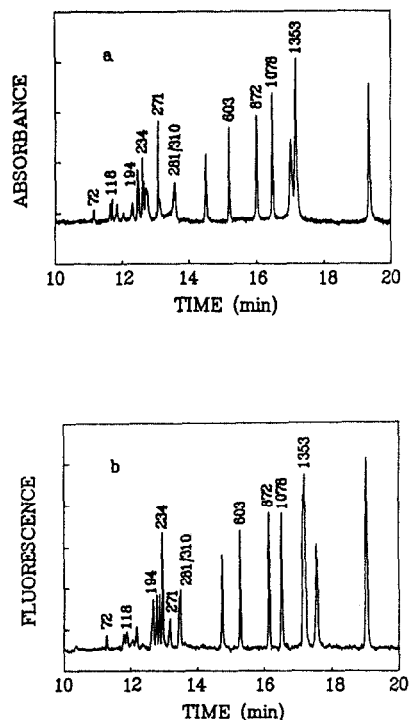

Fig. 2. Separation of HAE III DNA fragments at 24 μM/ml injected for 5 s at 500 V/cm. (a) Absorption detection and (b) fluorescence detection, both in arbitrary units.

DNA samples are placed in a pH 1 buffer for a short time, significant degradation can be observed as a change in the peaks in the electropherogram in location as well as in intensities. At pH 2.8, apparently there is some change in the sample in the 1-h period for sample preparation, but not to the extent that the utility for DNA fingerprinting is lost. Both higher- and lower-molecular-weight fragments are found, indicating the presence of both ligation and/or degradation of the fragments. Since discrete peaks rather than a broad distribution of products are present, sequence-specific interactions are involved. Since the patterns are reproducible, peak assignment is possible based on these electropherograms and those for other DNA size standards (data not shown). Further investigation into such processes may lead to the development of additional specificity in DNA analysis.

When the same sample is studied by laser-excited native fluorescence, essentially the same patterns are obtained, as shown in Fig. 2b. Except for the extra peak at 17.6 min in Fig. 2b, there is a one-to-one correspondence. The origin of this extra peak at present is not clear. The separation efficiency in Fig. 2b is also very similar to that in Fig. 2a, confirming that the sheath flow did not degrade the performance of the column. There are subtle differences between the magnitudes of the signals in the two detection modes. In absorbance, all four bases contribute, although not identically. In fluorescence, only the guanine moiety provides a significant response [17]. For a fragment with hundreds of bases, however, the net fluorescence signal is still roughly proportional to the fragment size.

The main difference between absorption and fluorescence detection of DNA fragments is depicted in Fig. 3. At a low injected concentration, the absorbance electropherogram (Fig. 3a) barely shows a few features. Moreover, there is a significant perturbation between 12 to 14 min which can be attributed to absorbing materials in the sample matrix. For the same sample, the fluorescence electropherogram (Fig. 3b) shows decent signal-to-noise levels, revealing all of the fragment peaks that are present in Fig. 2b. The limit of detection is roughly 10 times lower for
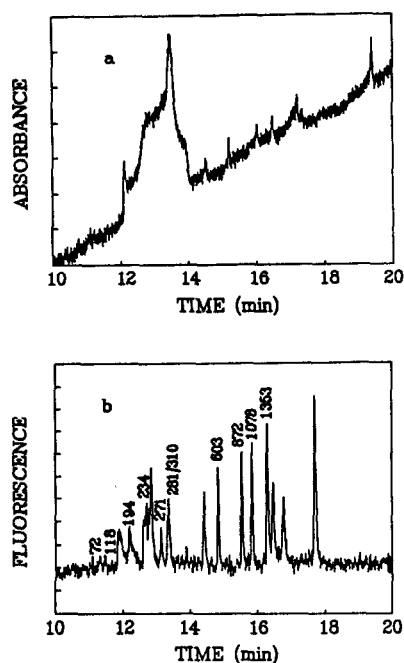

Fig. 3. Separation of HAE III DNA fragments at 1.6 μM/ml injected for 5 s at 500 V/cm. (a) Absorption detection and (b) fluorescence detection, both in arbitrary units.

fluorescence detection. Also, the impurity interference in the absorbance plot is absent.

In summary, we have demonstrated improved detection of native DNA fragments based on UV-excited fluorescence. A sheath-flow arrangement isolates the linear polymer from the optical region to minimize the background signal. Separation at pH 2.8 provides an increased fluorescence yield while maintaining a high separation efficiency. This approach may find use in the manipulation and purification of larger DNA fragments, when staining and then destaining with fluorophores may complicate the process.

Acknowledgement

search, Office of Health and Environmental Research.

References

[1] D.C. Schwartz and C.R. Cantor, *Cell*, 37 (1984) 67.
[2] E. Lai, B.W. Birren, S.M. Clark, M.I. Simon and L. Hood, *BioTechniques*, 7 (1989) 34.
[3] H. Swerdlow, J.Z. Zhang, D.Y. Chen, H.R. Harke, R. Grey, S. Wu, N.J. Dovichi and C. Fuller, *Anal. Chem.*, 63 (1991) 2835.
[4] L.M. Smith, J.Z. Sanders, R.J. Kaiser, P. Hughes, C. Dodd, C.R. Connell, C. Heiner, S.B. Kent and L.E. Hood, *Nature*, 321 (1986) 674.
[5] M.D. Biggin, T.J. Gibson and G.F. Hong, *Proc. Natl. Acad. Sci. USA*, 80 (1983) 3963.
[6] H.F. Arlinghaus, N. Thonnard, M.T. Spaar, R.A. Sachleben, F.W. Larimer, R.S. Foote, R.P. Woychik, G.M. Brown, F.V. Sloop and K.B. Jacobson, *Anal. Chem.*, 63 (1991) 402.
[7] C. Martin and I. Bronstein, Poster P60, *Second DOE Contractor-Grantee Workshop on Human Genome, Santa Fe, NM, 1991.*
[8] A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith, and B.L. Karger, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 9660.
[9] H. Drossman, J.A. Luckey, A.J. Kostichka, J. D'Cunha and L.M. Smith, *Anal. Chem.*, 62 (1990) 900.
[10] G.M. Church and S. Kieffer-Higgins, *Science*, 240 (1988) 185.
[11] D.N. Heiger, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.
[12] M. Yu and N.J. Dovichi, *Anal. Chem.*, 61 (1989) 37.
[13] K.C. Waldron, J.A. Miller and N.J. Dovichi, Paper 009, *38th Canadian Spectroscopy Conference, Peterborough, Ontario, Canada, 1992.*
[14] S. Udenfriend and P. Zaltzman, *Anal. Biochem.*, 3 (1962) 49.
[15] H.C. Börresen, *Acta Chem. Scand.*, 17 (1963) 921.
[16] D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang and N.J. Dovichi, *J. Chromatogr.*, 559 (1991) 237.
[17] R.E. Milofsky and E.S. Yeung, *Anal. Chem.*, 65 (1993) 153.
[18] M. Zhu, D.L. Hansen, S. Burd and F. Gannon, *J. Chromatogr.*, 480 (1989) 311.
[19] M. Strege and A. Lagu, *Anal. Chem.*, 63 (1991) 1233.
[20] W.A. MacCrehan, H.T. Rasmussen and D.M. Northrup, *J. Liq. Chromatogr.*, 15 (1992) 1063.
[21] A.M. Chin and J.C. Colburn, *Am. Biotech. Lab.*, 7 (1989) 16.
[22] K.C. Chan, C.W. Whang and E.S. Yeung, *J. Liq. Chromatogr.*, 16 (1993) 1941.
[23] D.A. McGregor and E.S. Yeung, *J. Chromatogr. A*, 652 (1993) 67.
[24] N.J. Dovichi, J.C. Martin, J.H. Jett, M. Trkula and R.A. Keller, *Anal. Chem.*, 56 (1984) 348.