Influence of the pH on Separating DNA by High-Speed Microchip Electrophoresis

Tomo OSAFUNE,* Hideya NAGATA,* and Yoshinobu BABA*,***†

**Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, The University of Tokushima, CREST, Japan Science and Technology Corporation (JST), Tokushima 770-8505, Japan*

***Single-molecule Bioanalysis Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Takamatsu 761-0395, Japan*

Various factors are critical in resolving DNA molecules at high speed, including the separation medium, concentration, composition, and pH of the buffer, as well as the electric field strength. To this study, considered the composition of a buffer and the difference in the pH, while paying attention to whether the separation ability changes in the microchip electrophoresis of DNA. DNA separation was particularly affected by both the buffer composition and the pH. Under the optimal microchip electrophoresis conditions that were determined in this study, an improved resolution of a wider range of DNA fragment sizes was achieved. Moreover, the total separation time decreased from 240 s to 100 s. Thus, by simplifying and improving the DNA electrophoresis in the microchip, this technique is now widely applicable to several different scientific fields.

(Received January 14, 2004; Accepted May 17, 2004)

Introduction

Capillary electrophoresis (CE) has been widely utilized because of its ability to separate nucleic acids quickly. Also, it is an effective tool in the analysis of polymerase chain reaction (PCR) products, single nucleotide polymorphisms (SNPs), and DNA sequences.¹⁻⁴ CE played an important role in the Human Genome Project, which was completed in 2001. In recent years, microchip electrophoresis has attracted attention as the next advancement in DNA capillary electrophoresis. Microchip electrophoresis provides high resolution and speed, high throughput, a compact size, and a reduction in the use of reagents and samples. In addition, it is a useful tool in various analysis devices for molecules, such as DNA.⁵⁻⁷ This technology also promises to be easily integrated with other laboratory procedures, *i.e.*, PCR, immobilized enzymatic reactions, and single-molecule dynamics,⁸⁻¹¹ in order to solve complicated enzymatic and biological problems. For the fast and accurate separation of dsDNA fragments on a microchip, linear polymer solutions, for example methyl cellulose (MC) and cellulose derivatives, such as hydroxypropylmethyl cellulose (HPMC) and hydroxyethyl cellulose (HEC), are utilized as sieving matrices. However, for the concentrations currently utilized in the microchip, the polymer is extremely viscous due to its high molecular weight. Therefore, it is difficult to refill the microchip with a polymer after use, and it has remained cumbersome for integration in the micrototal analysis system (μ -TAS) platforms. Thus, recently, low-viscosity polymer solutions have been explored.¹² However, there is a limit to the polymer concentration in a low-viscosity state that can fully separate DNA fragments. Thus, except for

the concentration or type of polymer, it is necessary to further examine the parameters that can be manipulated in microchip electrophoresis that results in a rapid resolution of DNA fragments.

This study examined what has the greatest affects in microchip electrophoresis. In particular, differences in the separation ability due to the buffer composition and pH, which have not been sufficiently explored in microchip electrophoresis, were analyzed. First, the differences in DNA separation due to the polymer concentration and applied field strength were examined. Next, the differences in the buffer composition and pH were analyzed concerning their influence on DNA migration. Lastly, how these differences would be applicable to the resolution of single DNA molecules was tested.

Materials and Methods

Instrumentation

Experiments were performed on a Hitachi SV1100 Microchip CE system (Hitachi Electronics Engineering Co., Tokyo, Japan), which was equipped with a light-emitting diode (LED) confocal fluorescence detector. Excitation was at 470 nm and detection was at 580 nm. SV1100a software was utilized for data analysis. The electrophoresis chip was fabricated from polymethylmethacrylate (PMMA) (Hitachi) with a simple cross-channel of 100- μ m and a depth of 30- μ m. The effective separation length of the chip was 30 mm. The distances from the crossing point to reservoir 1 (buffer), reservoir 3 (buffer), reservoir 2 (sample outlet), and reservoir 4 (sample inlet) were 5.7, 37.5, 5.2, and 5.2 mm, respectively.

Reagents and samples

Methylcellulose (MC), tris(hydroxymethyl)aminomethane

† To whom correspondence should be addressed.
E-mail: ymttbaba@fc.ph.tokushima-u.ac.jp

Fig. 1 Effect of the TBE and phosphate buffer on the migration time and intensity. The experimental conditions consisted of an E_a of 180 V/cm, a MC concentration of 0.2%, and a buffer pH of 8.6.

(Tris) were purchased from Sigma (St. Louis, MO, USA). Boric acid was obtained from Katayama Chemical Co. (Osaka, Japan). Sodium dihydrogenphosphate, dihydrate, disodium hydrogenphosphate 12-water were obtained from Wako (Osaka, Japan). DNase- and RNase-free water used for sample preparation was from ICN Biomedicals (Aurora, OH, USA). DNA ladder (100-bp) was purchased from TOYOBO Co. (Osaka, Japan). The dye chosen for this study was ethidium bromide (EtBr) (Nippon Gene (Tokyo, Japan)).

Procedures

DNA ladder (100-bp) was diluted to 70 ng/ μ L in DNase- and RNase-free water and stored at -20°C until utilized. A polymer solution was prepared by adding MC to a boiling buffer solution ($1 \times$ TBE or 20 mM phosphate buffer) to generate a final concentration of 1% MC. The solution was stored at 4°C overnight until the solution appeared to be homogeneous and transparent. EtBr was added to the polymer solution at room temperature and mixed completely by vibration. An EtBr concentration of 0.5 $\mu\text{g/mL}$ was utilized because there was no obvious change in the peak heights of the 100-bp DNA ladder fragments with EtBr concentrations ranging from 0.5 $\mu\text{g/mL}$ to 3.0 $\mu\text{g/mL}$. The polymer solution was filled from reservoir 3 into the microchannels of the chip using a specific Hitachi syringe. Using a pipette, reservoirs 1 and 2 were filled with a polymer solution and reservoir 4 was filled with the sample. By applying 300 V to reservoir 2 for 20 s, the sample filled the microchannel, while the other reservoirs maintained their grounded state. Unless sample separation was indicated, sample separation was performed at a field strength of 180 V/cm by applying 810 V at reservoir 3, and 140 V at reservoirs 2 and 4, and by utilizing reservoir 1 as a ground for 120 s.

Results and Discussion

Effect of the composition of the buffer on DNA migration in the microchip

Generally, in electrophoresis the TBE buffer is utilized because of its stability and high buffering ability, and also the TBE, itself, rarely affects the experiment after electrophoresis. However, it is unclear as to which buffer has the better buffering capacity in microchip electrophoresis. In addition to the conventional TBE buffer, phosphoric acid buffer was prepared and the separation ability of these two buffers was examined. To optimize the composition for the separation matrix and applied field strength, the resolving power and analysis time for DNA separation were examined while varying

Fig. 2 Effect of the buffer pH on the separation of the 100-bp DNA ladder. The separation buffer was 20 mM phosphate buffer. All other conditions were the same as in Fig. 1. Three different pH values were tested: pH 2.6, 8.6, and 12.6, which are indicated by different colored lines.

both the concentration of methyl cellulose from 0.2% to 1.0% and the field strength from 150 V/cm to 180 V/cm. Consequently, the combination of a 0.2% solution of methyl cellulose and 180 V/cm field strength is suitable for the high-speed, high-resolution separation of DNA. These separation conditions were used to examine the effect of the pH on the separation of DNA. Interestingly, the migration time in the phosphate buffer was shorter than that in the TBE buffer, and the separation ability of the phosphate buffer was across a broader range (Fig. 1). Differences in the separation ability of the buffers were due to a combination of the effect of the dielectric constant and the flow in the buffer on the migration of the DNA molecules. When the dielectric rate was compared between the two buffers, the value of the dielectric rate of the phosphate buffer was higher than that of the TBE buffer. Although the dielectric rate is determined by various factors, it is thought that the charging quantity mainly influences this rate. Hence, the migration time will decrease if a solution with a high dielectric rate is utilized. However, if the dielectric rate becomes high, the amount of Joule heat generated becomes large, and substantially effects the buffer solution. While examining the flow of the phosphate buffer, when the flow velocity was observed simultaneously, it was earlier than that of the TBE buffer (data not shown). In addition, the current value of two buffer solutions was compared. Since the current value of the TBE buffer solution was 14.0 μA and the phosphate buffer solution was 17.5 μA at pH 7.6, it was shown that the phosphate buffer solution tends to let a current pass; the current value of the phosphate buffer solution was about 3 μA larger than that of the TBE buffer solution for each pH. Therefore, it is easier to be drawn rather than the TBE buffer solution by an electric field; as for the phosphate buffer solution, the phosphoric acid molecule strongly drawn by the electric field also affects the water element, and the flow velocity is considered to be faster than that of the TBE buffer solution. Overall, the phosphate buffer has a better separation ability than the TBE buffer. This experiment showed that DNA can be resolved in the phosphate buffer at high speed and resolution.

Influence of the pH on the DNA separation behavior

The microchip separation of 100-bp ladder DNA fragments was performed using a phosphate buffer ranging from pH 2.6 to pH 12.6. Figure 2 shows that the separation ability changed tremendously along with changes in the buffer pH. Thus, we

Fig. 3 Plot of the DNA mobility vs. various pH conditions (a) using the TBE buffer, and (b) the phosphate buffer. The mobilities of the 100, 500, 1000, and 2000 bp fragments are indicated by unique lines. The conditions were the same as in Fig. 1, except for the pH of the buffer.

Fig. 4 Dependence of the DNA fragment resolution on the pH of the electrophoresis buffer. The experimental conditions are described in Fig. 3. The x-axis is divided into 1 pH units, ranging from pH 2.6 to 12.6. The y-axis shows the resolution divided into 0.5 R_s units. The resolution of the 100/200 and 500/600 bp fragments is indicated with circles and squares, respectively. (a) Resolution in TBE buffer. (b) Resolution in 20 mM phosphate buffer.

predicted that the separation ability changed according to the ionization state of the composition of the buffer. To test this, the following experiment was performed. First, the pH extremes were investigated utilizing HCL and KOH for any pH adjustments (Fig. 2). At pH 2.6, the DNA was not completely separated. The DNA fragments greater than 700 bp were inseparable, particularly the 1500-bp and 2000-bp fragments. Next, the polymer solution was adjusted to pH 12.6 and the experiment was repeated. At pH 12.6, the DNA ladder fragments were separable with a shorter migration time, in contrast to their separation at pH 2.6. In addition, 1000-bp and 1500-bp fragments were partially separable. Similarly, the migration time did not become shorter when the experiment was repeated while utilizing the TBE buffer solution at a basic pH, and the migration time in the TBE buffer was slower than that in the phosphate buffer. Thus, the phosphate buffer may be more beneficial than TBE in microchip electrophoresis.

In addition, the DNA migration behavior was examined. Figure 3 shows the relationship between the relative electrophoretic mobilities of the DNA 100-bp ladder and the pH of the TBE buffer and the phosphate buffer. The DNA migration in the phosphate buffer was similar to its migration in the TBE buffer. At a higher pH, the DNA mobility increased. However, in the TBE buffer, a decrease in the DNA mobility occurred at pH 6.6, a neutral pH for TBE. It is thought that this migration difference was due to the composition of the buffers. TBE is composed of boric acid and Tris, which is a weak acid substance that presumably causes the electric charge state of the buffer solution to change dramatically at pH extremes (*i.e.*, pH

2.6 and 12.6). At a strong basic pH, almost all buffers are ionizing and the current flows easily through the buffer. Since the DNA molecule is also charged, it is similarly influenced by the current. In this state, if a voltage is applied, a current tends to flow easily and the DNA is drawn to the current, thus increasing the DNA mobility. The DNA mobility also increases along with an increase in the pH in the phosphate buffer. However, as for a fixed degree of DNA mobility being acquired, unlike the TBE buffer, in the phosphate buffer the ratio of ions to non-ions is nearly regular in every pH unit, because the phosphate buffer consists of both weak acid and weak base substances. From the results with the TBE and the phosphate buffer, the ratio of ions to non-ions in the electrophoresis buffer is important for DNA separation. The increase of the DNA mobility caused by an increase in the pH indicates the dissociation of the buffer solution.

Next, the relationship between the resolution and pH was investigated. Figure 4 shows that a different effect was seen in each buffer. In the case of the TBE buffer (Fig. 4a), resolving 100 bp/200 bp and 500 bp/600 bp fragments was unaffected by pH changes. It is thought that the increasing the temporary resolution near the neutral region of the TBE buffer is due to the slower mobility of each DNA molecule. Although there was no big difference in the resolution of the 500 bp/600 bp fragments in the case of the phosphate buffer (Fig. 4b), as for the 100 bp/200 bp fragments, the resolution decreased as the pH became more basic. It is thought that the fall of this separation ability is due to more than the actual migration. Since the decrease of the fluorescent intensity and the broadening of the peak intensity

occurs as the pH becomes high, other aspects of the process, such as the rate of fluorochrome labeling or the sensitivity of the measurement apparatus, may contribute to the lower resolution of the DNA. Nevertheless, the phosphate buffer is useful for separating DNA, since it has a resolution nearly equivalent to that of the TBE buffer, and has a faster resolving power.

Conclusion

This study considered which factor would have the greatest influence on microchip electrophoresis. It was especially shown that a phosphate buffer can be utilized for separating and resolving DNA by PMMA microchip electrophoresis, because it provides a higher resolution and speed, as compared to that of the TBE buffer. In addition, the impacts of the buffer composition and the pH were investigated, which showed that both parameters strongly influenced the DNA separation. Summarizing this influence, it is thought that it is a large factor that a DNA molecule rides the changing flow of the phosphate buffer by pH changing from this experiment. However, not only this, but also the influence of the salt of the buffer solution and the influence of the ionic strength may be considered, and may have had some influences on the DNA. Experiments and examinations are now also taking place including the action of a single molecule. These influences are due to be considered from now on. In this study, although separation of 100-bp to 2000-bp sized DNA fragments had previously taken approximately 240 s, when these sized fragments were dissociated under the optimal conditions, it became possible to dissociate the DNA fragments within 100 s. An important key factor is not only the molecular sieve effect of the polymer, but also the states (composition, pH, *etc.*) of the electrophoresis buffer for DNA separation. By using the polymer separation medium, itself, in this study can also work as an EOF exclusion agent in microchip electrophoresis. In this study, it was shown that DNA separation can be performed at high-speed without reducing the resolution by changing the state of the buffer solution. Since the preparation of the polymer and buffer is

simple, these improvements to the μ -TAS system can easily be applied to future experiments.

Acknowledgements

This work is partially supported by the CREST program of the Japan Science and Technology Corporation (JST), a Grant from the New Energy and Industrial Technology Development Organization (NEDO) of the Ministry of Economy, Trade and Industry, Japan, a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Technology, Japan.

References

1. I. Kheterpal and R. A. Mathies, *Anal. Chem.*, **1999**, *71*, 31A.
2. M. N. Albarghouthi and A. E. Barron, *Electrophoresis*, **2000**, *21*, 4096.
3. Y. Baba, N. Ishimaru, K. Samata, and M. Tsuchiko, *J. Chromatogr.*, **1993**, *653*, 329.
4. Y. Baba, *J. Chromatogr. B*, **1996**, *687*, 271.
5. D. J. Harrison, A. Manz, Z. H. Fan, H. Ludi, and H. M. Widmer, *Anal. Chem.*, **1992**, *64*, 1936.
6. S. C. Jacobson and J. M. Ramsey, *Electrophoresis*, **2001**, *22*, 763.
7. E. T. Lagally, I. Medintz, and R. A. Mathies, *Anal. Chem.*, **2001**, *73*, 565.
8. Z. Ronai, C. Barta, M. Sasvari-Szekely, and A. Guttman, *Electrophoresis*, **2001**, *22*, 294.
9. M. Ueda, T. Hayama, Y. Takamura, Y. Horiike, and Y. Baba, *Electrophoresis*, **2002**, *23*, 2635.
10. N. Kaji, M. Ueda, and Y. Baba, *Biophys. J.*, **2002**, *82*, 335.
11. Y. Hirano, Y. Baba, Y. Matsuzawa, and A. Mizuno, *Appl. Phys. Lett.*, **2002**, *80*, 515.
12. F. Xu, M. Jabasini, and Y. Baba, *Electrophoresis*, **2002**, *23*, 3608.