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Research Article

The influence of polymer concentration, applied voltage, modulation depth and pulse frequency on DNA separation by pulsed field CE

DNA fragments (0.1–10 kbp (kbp, kilo base pair)) separation by square-wave pulsed field CE in hydroxyethylcellulose (HEC, 1300 K) polymer was performed in this work. The effects of polymer concentration, pulse field strength, pulse frequency and modulation depth were investigated. We found that low HEC (about 0.1%) concentration is suitable for the separation of small DNA fragments (<1 kbp), whereas higher HEC concentration (>0.5%) is appropriated for high-mass DNA molecular (>1 kbp) separation. The mobility of DNA fragments is nearly linearly related to average separation voltage under pulsed field conditions. Higher modulation depth is suited to separate the longer DNA fragments and lower modulation depth favors the resolution of short DNA fragments. Thus, the intermediate modulation depth (100%) and pulse frequency (about 31.3 Hz) are prerequisite for high-resolution DNA separation.

Keywords: DNA separation / Hydroxyethylcellulose / Pulsed field CE
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1 Introduction

Capillary gel electrophoresis (CGE), which is actually capillary polymer electrophoresis, is a versatile tool in biochemical and biological analysis including DNA sequencing for its numerous advantages such as high resolution, automatic operation, short separation time, lower sample consumption, *etc.* [1–4]. In CGE, territorial regime for DNA fragment separation was between 100 and 1000 base pairs so that the Sanger DNA sequencing fragments, that is for DNA sequencing, were well separated. Although the CGE provides high-resolution power for the separation of Sanger fragment, CGE always loses its efficiency for the separation of large DNA fragments under constant field conditions. Theoretical model points out a plateau regime of DNA mobility [5, 6]. For DNA larger than a critical size $N*$, the mobility trends to be a constant independent of the size (N); thus the size-dependent separation of DNA fragments fails [7]. Visualization experiments demonstrated the existence of short-lived polymer/dsDNA entanglement under constant electric field condition [8, 9]. DNA fragment collided with the polymer, elongated and then collapsed back to the random coil configuration. Because long DNA fragments were stretched more than short DNA fragments and entangled at the several points on the polymer, the separation of long DNA fragments became difficult.

In order to extend the upper size limit of CE, Kim and another group introduced ultrasonic vibrations on CE [10, 11]. Experiments demonstrated that DNA fragments migrated faster and the bandwidth was reduced when the capillary was immersed into a sonicator to apply ultrasound vibration during CGE. Other large group methods using periodic variation of the applied electric field were developed since the first work of Carle in 1984 [12], which is named as pulsed field gel electrophoresis. Since then, majority of studies have been launched to improve the separation performance by pulsed field CE (PFCE). Kotaka *et al.* employed sinusoidal field strength for DNA sequencing [13–15]. They indicated that it overcame dynamic self-trapping, consequently yielded band inversion in polymer. However, the number of peaks obtained is sometimes greater than the number of fragments in the DNA sample. Kim and Morris [16] successfully separated the multi-kilo-base length nucleic acids in dilute methylcellulose solutions by delaying the start of field pulsation enforced on the capillary. They [17–19] also tried a variety of pulse protocols for PFCE, such as field inversion CE, chirping mode with increasing frequency and

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Abbreviations: AC, alternating current; **CGE,** capillary gel electrophoresis; **DC,** direct current; **HEC,** hydroxyethylcellulose; **kbp,** kilo base pairs; **PFCE,** pulsed field CE; **TBE,** Tris-borate-EDTA

zero integrated field electrophoresis and the result reveals that zero integrated field electrophoresis can achieve the high resolution; however, the migration times are too long. Chirping mode can be effective; nevertheless, the improvement is limited to a small range of fragment size.

In this paper, we explore the separation of DNA ranging from 0.1 to 10 kbp in HEC polymer by square wave PFCE. In order to investigate the DNA motions in the pulsed field conditions, we systematically studied the effect of concentration of a sieving polymer, electric field strength, modulation depth and pulse frequency on the DNA separation performance in the square-wave pulse field conditions. We believe that the experimental results obtained will shed light on the fundamental of complicated motion of DNA in HEC polymer.

2 Materials and methods

2.1 Chemicals

HEC (1300 K) was purchased from Sigma (St Louis, MO, USA). SYBR Green I was obtained from Invitrogen (Carlsbad, CA, USA); 10× Tris-borate-EDTA (TBE) buffer was from Bio-Rad (Hercules, CA, USA). HEC polymer solution containing 1× SYBR Green I was prepared by dissolving in the 0.5× TBE buffer; 0.1 kbp DNA ladder and 1 kbp DNA ladder were bought from Takara (Shiga, Japan). They were dissolved in TBE buffer and mixed to make each DNA ladder concentration 16 μg/mL. Prepared DNA samples were kept frozen at −20°C before being used.

2.2 Apparatus

The separation system in our experiment is similar to that reported in Ref. [20]. A fused-silica capillary (Polymicro Tehchnologies, Phoenix, AZ, USA) with id/od = 75 μm/365 μm was covalently coated with polyacrylamide [21, 22]. Total capillary length was 15 cm and effective length was 8 cm. CE was performed with a system in our laboratory consisting of a microscope with epi-illumination (IX71, Olympus, Tokyo, Japan) and a high-voltage power supply (MODEL 610E, TREK, Medina, NY, USA) for a pulsed field electric field. Waveform and the parameters for a determination of the pulsed field were controlled by the locally programmed LabVIEW software (National Instrument, Austin, TX, USA). The excitation wavelength from a mercury lamp was filtered to be 460–495 nm, which was the wavelength of the excitation maximum of the conjugate of SYBR Green I and the nucleic acid, by the optical filter (U-MWIB-3, Olympus). The fluorescence emission was collected by a 60× objective (PlanApo/IR, Olympus). The fluorescence signal was detected by use of a Photo Multiplier Tube (R928, Hamamatsu Photonics, Hamamatsu, Japan), and the signal was digitized by National Instrument PCI-6024E. Prepared DNA samples were electrokinetically injected at 100 V/cm for 2.0 s. The entire detection system was enclosed in a black box.

3 Results and discussion

3.1 Effect of gel polymer concentration

The concentration of HEC in the separation buffer prepared was based on the optimal molecular mass range of the HEC polymer. In order to investigate the effect of concentration of polymer on the migration of DNA fragments, HEC polymer ranging from 0.1 to 1.0% was employed for the separation of DNA by PFCE. We demonstrated the separation of DNA (0.1–10 kbp) in 0.5× TBE buffer at 100 V/cm average voltage, 100% modulation depth, 50 Hz squared wave PFCE. As shown in Fig. 1, when the concentration of HEC is low (0.1%), only the small DNA fragments (<1 kbp) were separated, while DNA fragments longer than 1.5 kbp lost their resolution. In dilute HEC polymer solution, the longer DNA fragment jostled the sieving polymer without the entanglement enough to diverse its mobility because the polymer formed an astheno physical network [23]. For higher HEC concentrations (0.5 and 0.8% in Fig. 1), the resolution obviously improved. Especially in the case of 0.5%, all of the DNA fragments (<8 kbp) were separated with high resolution within 9 min, which was half the time of DNA separation in agarose/HEC matrices by constant field CE [24].

We evaluated the mobility of DNA fragments at various concentrations and plotted them in Fig. 2. The mobility was calculated by the following equation [25]:

$$u = L_t L_e / (t(M) * V_{average})$$

where L_t is the total length of the capillary, L_e is the effective length of the capillary, $t(M)$ is the DNA migration time of a molecular size M, $V_{average}$ is the average separation voltage of the pulse electrical field. Fig. 2A illustrates that the migration mobility decreases as a function of HEC polymer concentration over the whole concentration range. Furthermore, there is no great change for the DNA fragment mobility with the concentration of HEC varying from 0.2 to 0.5% and 0.8 to 1.0%. Fig. 2B shows the migration patterns observed for DNA under pulse field conditions. At low concentration (0.1%), a typical segmental curve is observed, in which the curve is divided into three regimes. DNA fragments from 0.1 to 0.6 kbp were well resolved, while the mobility of longer DNA fragments trends to a constant. At higher concentrations, the transition between Regime I and II is difficult to observe. Compared with Ref. [26], the three regimes may be related to Ogston-type sieving, reptation without orientation and reptation with orientation, respectively. However, different from the Regime III in traditional gel, the slope of the curves in Regime III (Fig. 2B) is remarkable, which means effective DNA separation.

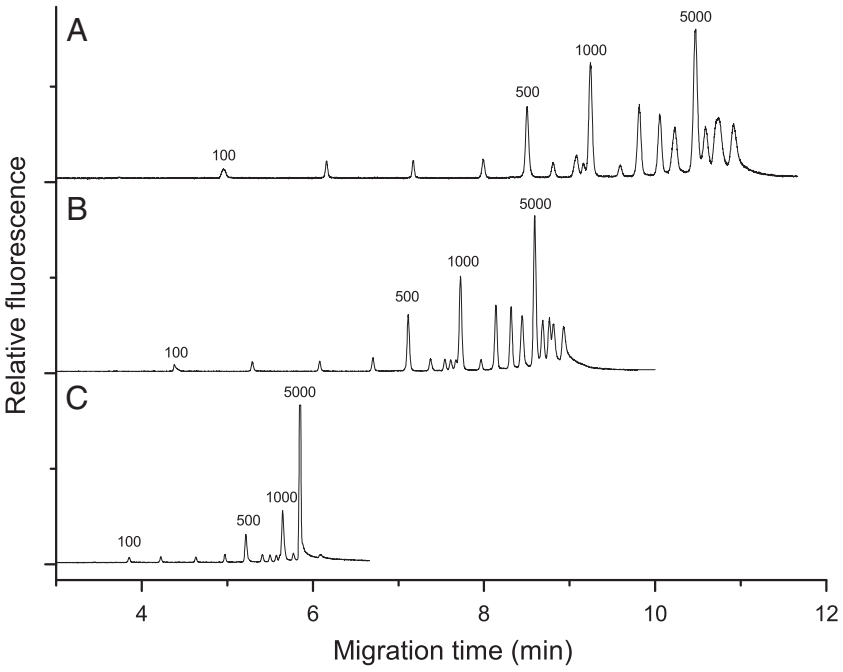

Figure 1. Electrophoretic separation of DNA molecules at various concentrations of HEC (Mw = 1300 K) polymer by square wave PFCE: (A) 0.8%; (B) 0.5%; (C) 0.1%. PFCE conditions: 100 V/cm DC with 100% modulation depth and 50 Hz of pulse frequency. The sample was diluted in 0.5 × TBE, injected at 100 V/cm (2 s) and separated in a coated capillary filled with HEC.

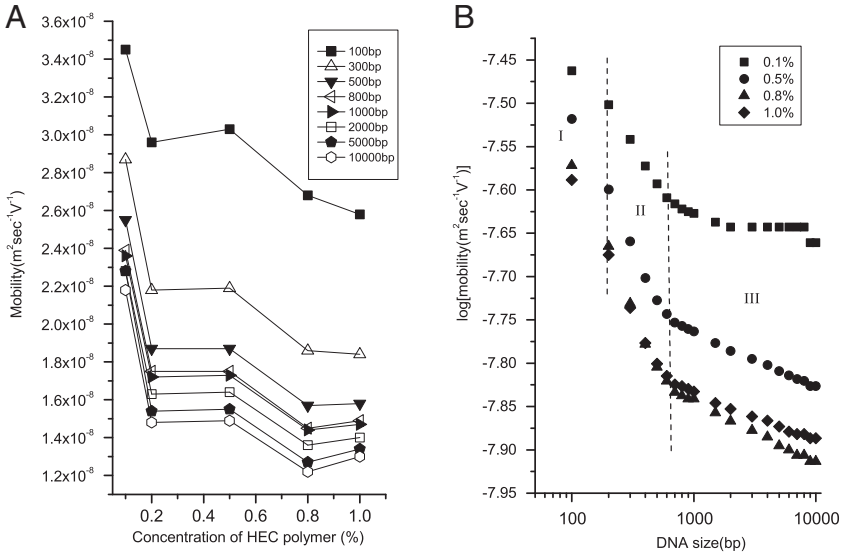

Figure 2. The effect of polymer concentration on electrophoretic mobility of DNA molecules in PFCE analysis: (A) concentration of HEC *versus* motility (B) log(mobility) *versus* DNA size.

3.2 Effect of electric field strength

We carried out the study of DNA separation at different pulsed field electricities with certain modulation depth and pulse frequency by PFCE. Fig. 3 displays the electropherogram of separation of DNA (0.1–10 kbp) in 0.5%HEC polymer at 0.5 × TBE buffer, 100% modulation depth and 50 Hz of pulse frequency by squared wave PFCE. The first five peaks always show high resolution. However, for large DNA fragments ranging from 0.5 to 10 kbp, the resolution declines with the increase of the average electric field strength. For DNA fragments greater than 1 kbp, the resolution shows an increase with the growth of average separation field strength from 67 to 100 V/cm and a marked decrease at higher field strength. We have calculated the mobility of DNA molecule under different electric fields with the formula mentioned above. It demonstrates that the electrophoretic mobility shows a linear relationship with the average separation voltage. DNA fragments move faster when the field voltage increases. This is due to Joule heating, because Joule heating is proportional to the separation voltage. Thus the increase in field voltage will induce the growth of Joule heating in the capillary. Research has shown that the temperature increase in polymer will

yield the decrease of polymer viscosity [24]. Consequently, it strengthens the DNA molecular motion in polymer. Meanwhile, Fig. 3 shows that under 100 V/cm of average separation voltage, nearly all of the DNA fragments from 0.1 to 9 kbp can be better resolved. Therefore, in the following part, we set 100 V/cm as the standard average separation voltage for analysis.

3.3 Effect of modulation depth

Fig. 4 presents the electropherogram of DNA separation by square wave PFCE at 100 V/cm with modulation depth from 10% to 250% in 0.5 × TBE, 0.5% HEC. Modulation depth was the ratio of alternating current (AC) field to DC field. Fig. 4 shows that the separation of large DNA fragments is a strong function of modulation depth. When the modulation depth is relatively low (10%, Fig. 4E), only the small DNA fragments (<1 kbp) were resolved. As modulation depth increases (100 and 50%, Figs. 4C, D), the resolution for DNA fragments between 1 and 5 kbp becomes better. However, the resolution for DNA fragments smaller than 1 kbp was deteriorated. At higher modulation depth (250 and 200%, Figs. 4A, B), the resolution for DNA fragments larger than 1 kbp was greatly improved; yet the resolution for DNA fragments ranging from 0.3 to 1 kbp was decreased. As shown in Fig. 4, the modulation depth influences deeply on the DNA fragments ranging from 0.1 to 10 kbp. It shows that higher modulation depth is appropriate for the separation of large DNA fragments and lower modulation depth favors the resolution of small fragments, which agrees well with the conclusion derived from gel electrophoresis [12, 27, 28]. And this maybe because with the increase of modulation depth, the velocity difference between the adjacent DNA fragments decreased for the short DNA fragments while the velocity difference for the longer adjacent ones increased. In addition, the improvement caused by pulsed field operation may be explained by the additional heating theory proposed by Kim [11]. In pulsed field conditions, heat is proportional to the root-mean-square (RMS) of the DC and AC voltages, which can be expressed as $V_{RMS}^2 = V_{DC}^2 + V_{AC}^2$. Consequently, the heat generated at 250, 200, 100 and 50% modulation depths is 7.25, 5, 2 and 1.25 times of the heat generated at 10% modulation depth, respectively. As observed in Fig. 4, it matches well with Kim's conclusion: additional heating may reduce the resolution for small DNA fragments (<1 kbp) because of the DNA diffusion increase in the run. However, as observed in Fig. 4, it may be only applicable to the small DNA fragments. For larger DNA fragments, this additional heating may strengthen the separation.

3.4 Pulse frequency

Pulse frequency is another factor that is important for the separation performance. Fig. 5 plots the electropherogram of DNA separation by PFCE at 0.5 × TBE buffer, 0.5% HEC, 100 V/cm average with 100% modulation depth. Fig. 5 displays that the pulse frequency influences greatly on the DNA fragments above 0.6 kbp. Through Fig. 5 we find that at lowest frequency (20 and 5 Hz, Figs. 5D and E), full separation is achieved for small DNA fragments smaller than 0.6 kbp and DNA fragments between 1 and 5 kbp, while large DNA fragments (>5 kbp) are incompletely separated. At relatively higher frequency (33.33 and

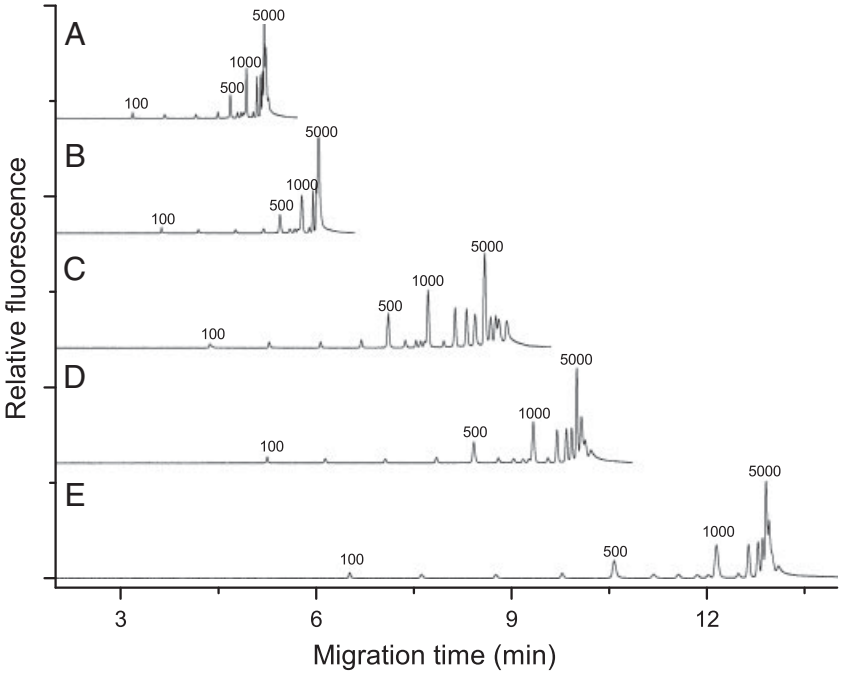

Figure 3. Electrophoretic separation of DNA molecule in 0.5% HEC at various average separation voltages by PFCE: (A) 133 V/cm (B) 117 V/cm; (C) 100 V/cm; (D) 83 V/cm; (E) 67 V/cm. Other conditions are the same as those in Fig. 1.

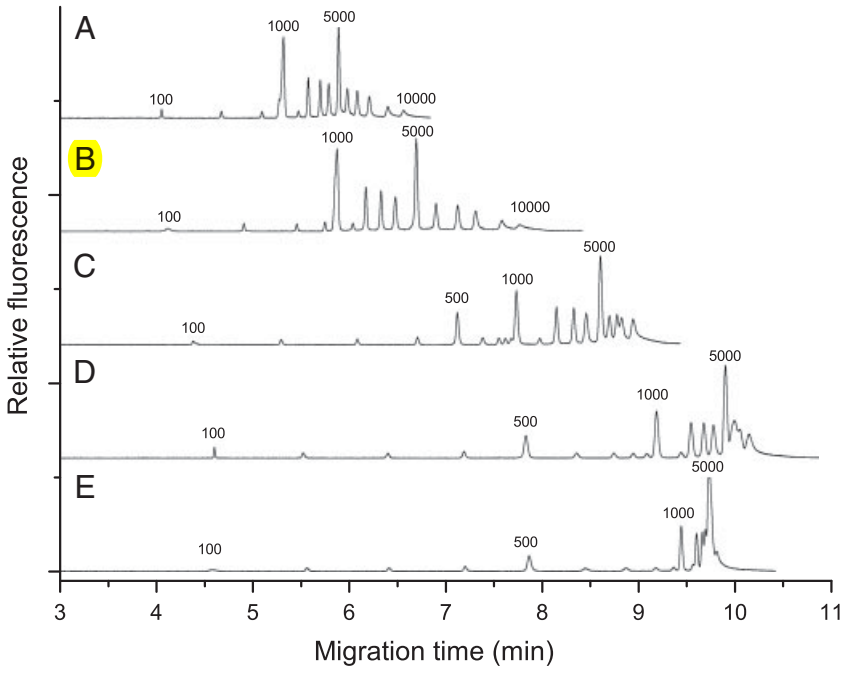

Figure 4. Electrophoretic separation of DNA molecule in 0.5% HEC at different modulation depths by PFCE: (A) 250%; (B) 200%; (C) 100%; (D) 50%; (E) 10%. Other conditions are the same as those in Fig. 1.

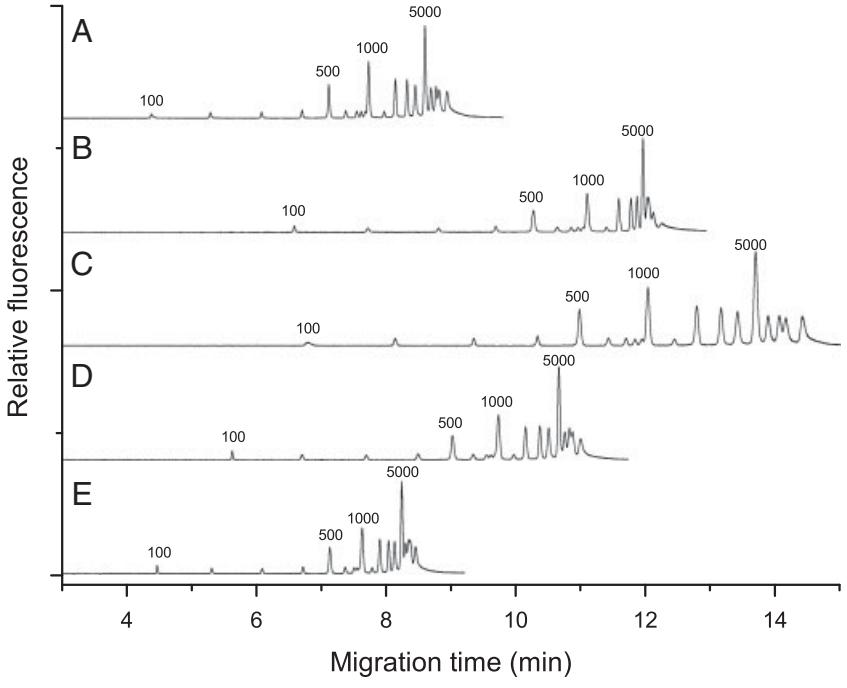

Figure 5. Electrophoretic separation of DNA molecule in 0.5% HEC at various pulse frequencies by PFCE: (A) 50 Hz; (B) 33.3 Hz; (C) 31.3 Hz; (D) 20 Hz; (E) 5 Hz. Other conditions are the same as in Fig. 1.

31.3 Hz, Figs. 5B and C), all of the DNA fragments can be baseline resolved. At high frequencies (50 Hz, Fig. 5A), resolution for DNA fragments smaller than 1 kbp deteriorated; yet the resolution for DNA fragments larger than 1 kbp was improved. As depicted in Fig. 6, two migration patterns were observed by PFCE: (i) when pulse frequency is lower than 31.3 Hz, the mobility of DNA fragments decreases with the increase of pulse frequency; (ii) for pulsed field greater than 31.3 Hz, the mobility increases with the growth of the pulse frequency. Thus, the lowest mobility at 31.3 Hz probably indicates that 31.3 Hz may be the resonance frequency for DNA reorientation time and applied frequency. Because at high frequencies (>50 Hz), DNA cannot reorient quickly enough to keep up with the field inversion, while at lowest frequencies (<20 Hz), the pulsed field did not take effect and large DNA fragments are easy to aggregate in linear polymer solution. Therefore, the data above match well with the theory that DNA reorienta-

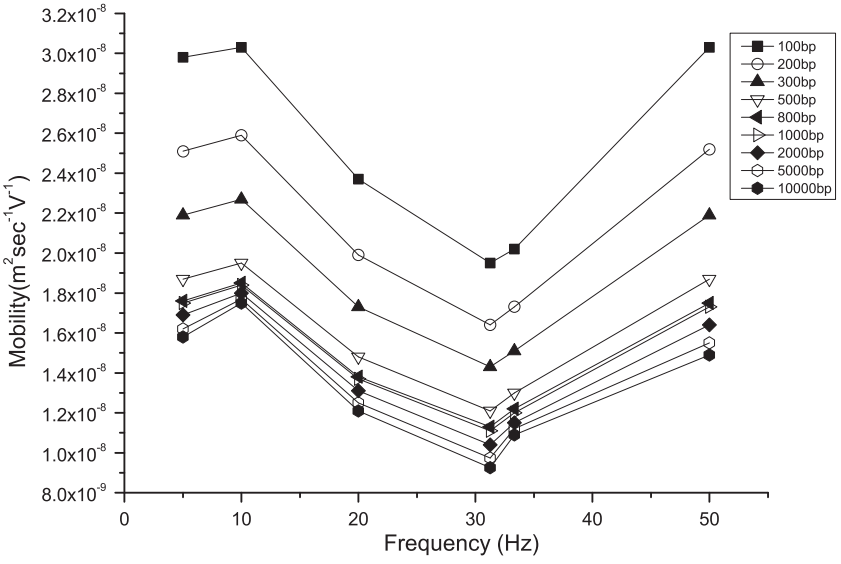

Figure 6. The effect of pulse frequency on the electrophoretic mobility of DNA molecules in PFCE analysis.

tion time in the gel is a strong function of DNA size [29, 30]. Hence, an intermediate frequency is necessary for the high resolution of DNA over the whole range.

4 Concluding remarks

This paper systematically studied the separation of DNA ranging from 0.1 to 10 kbp by square wave PFCE. We have investigated the factors that may affect the separation performance. These factors include concentration of polymer, average separation voltage, modulation depth and pulse frequency. Result shows that 0.5% HEC polymer, 100 V/cm DC, 100% of modulation depth and 50 Hz of pulse frequency is the optimal condition for separation of DNA fragments from 0.1 to 10 kbp. Meanwhile, modulation depth and pulse frequency are found to be very sensitive for the separation of DNA by PFCE.

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The authors have declared no conflict of interest.

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