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Research Article

Acetic acid denaturing pulsed field capillary electrophoresis for RNA separation

Based on our previous work of in-capillary denaturing polymer electrophoresis, we present a study of RNA molecular separation up to 6.0 kilo nucleotide by pulsed field CE. This is the first systematic investigation of electrophoresis of a larger molecular mass RNA in linear hydroxyethylcellulose (HEC) under pulsed field conditions. The parameters that may influence the separation performance, *e.g.* gel polymer concentration, modulation depth and pulse frequency, are analyzed in terms of resolution and mobility. For denaturing and separating RNA in the capillary simultaneously, 2 M acetic acid was added into the HEC polymer to serve as separation buffer. Result shows that (i) in pulsed field conditions, RNA separation can be achieved in a wide range of concentration of HEC polymer, and RNA fragments between 0.3 and 0.6 kilo nucleotide are sensitive to the polymer concentration; (ii) under certain pulsed field conditions, RNA fragments move linearly as the modulation depth increases; (iii) 12.5 Hz is the resonance frequency for RNA reorientation time and applied frequency.

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1 Introduction

PFCE has the advantage of separation of long DNA fragments in addition to the advantages of conventional CE, such as high resolution, fast speed, excellent reproducibility and so on, therefore, it is widely employed for the analysis of single and double-stranded DNA [1–3]. In RNA sequential size separation, CE was also employed with denaturing two or more co-existing stable conformers in RNA fragments by carboxylic acid representing acetic acid [4]. Acetic acid denaturing RNA separation was merely a starting point for RNA size separation. Thus, RNA size separation was mostly excited in a conventional slab gel electrophoresis (SGE). A conventional SGE has been fundamental in many methods in the characterization of RNA for several decades [5–8]. In SGE, abundant experiments indicate that SGE suffers from two apparent problems [9]: (i) the separations take many hours, and even days; (ii) like other planar separation techniques, the separated components are hard to quantify. Therefore, it is necessary to develop a more convenient way for RNA analysis.

Katsivela *et al.* first demonstrated the separation of low molecular mass RNA (transfer RNA and 5S ribosomal RNA, 70–135 bases) in CE [10, 11]. Since then there were a few articles published about RNA sequencing by CE. Jarle Skeidsvoll's lab successfully separated the RNA fragments ranging in size from 100 to 1908 bases with formamide for denature [12]. Todorov *et al.* extended the separation of RNA up to 2604 nucleotide with urea as denaturant [13]. However, there are two major drawbacks: (i) RNA samples need to be denatured prior to CE; (ii) formamide is not only expensive but also toxic and carcinogenic, and highly concentrated urea was imperative for RNA denaturant [14]. In order to solve these problems, our lab has developed the "in-capillary denaturing polymer denaturing polymer electrophoresis" [4], which realized denaturing and separating RNA simultaneously in a capillary tube. In addition, numerous researches have been developed on the application of CE for RNA separation from individual cells based on UV detection or laser-induced fluorescence method [15–19].

Another important consideration for RNA separation is to achieve long reads of the RNA bases in a relatively short time. Customary method is to increase the separation voltage. However, early researches show that in high electric fields, nucleic acids become roughly oriented along the field direction and move at a size-independent velocity [20, 21]. It is well established that changing the field direction can

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Abbreviations: HEC, hydroxyethylcellulose; **kbp,** kilo base pair; **knt,** kilo nucleotide; **SGE,** slab gel electrophoresis

disrupt the alignment and recover size-dependent mobility [22]. Thus, PFCE was employed to improve resolutions at DNA chain lengths above 1 kilo base pair (kbp) [23–25]. However, so far although DNA separation by PFCE has been investigated extensively, no group has yet reported the RNA analysis under pulsed field conditions.

On the basis of our previous work of ''in-capillary denaturing polymer denaturing polymer electrophoresis'', this study reveals the first demonstration of high molecular mass RNA separation by PFCE. We therefore investigated the influence of polymer concentration, modulation depth and pulse frequency, *etc*. on the separation performance in terms of resolution and migration mobility.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical-reagent grade or of high purity. Acetic acid was obtained from Wako Pure Chemical Industries (Osaka, Japan). 10 × TBE buffer was from BIORAD (Hercules, CA, USA) and was diluted to 0.5 × TBE buffer with sterilized water as the running buffer. Hydroxyethylcellulose (HEC, 250 K) was purchased from Sigma (St Louis, MO, USA). SYBR Green II was bought from Invitrogen (Carlsbad, CA, USA). The sieving buffer consisted of 0.5 × TBE, 3 × SYBR Green II and 2.0 M acetic acid. Perfect RNA^{TM} Markers were 0.1–1 kilo nucleotide (knt) (1000 ug/mL, RNA transcripts: 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 knt) and 0.2–10 knt (1000 μg/mL, RNA transcripts: 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 10.0 knt) (Novagen, USA). These two RNA markers were mixed with the same volume and then the mixture was stirred using a magnetic stirrer at least 1 min to make the sample concentration 500 μg/mL. Thus, a combined marker with molecular mass distributed from 0.1 to 10 knt was obtained, and then it was stored at −80°C before use. In total, 0.1 kbp-DNA ladder and 1 kbp-DNA ladder were bought from Takara (Shiga, Japan). The DNA standard contains 20 double-stranded fragments with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 kbp.

2.2 PFCE

The experimental setup is similar to that described in [4]. High-voltage power supply (MODEL 610E, TREK, Medina, NY, USA) was used to drive electrophoresis. 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 II and the nucleic acid by the optical filter (U-MWIB-3, Olympus, Tokyo, Japan). The fluorescence emission was collected by a 60 × objective (PlanApo/IR, Olympus), and then was detected by a photomultiplier tube (R928, Hamamatsu Photonics, Japan). The applied voltage and data collection were controlled by LabVIEW software (National Instrument, Austin, TX, USA). A certain length fused-silica capillary with id/od = 75 μm/365 μm was covalently coated with polyacrylamide [26, 27]. The total capillary length was 15 cm and effective length was 8 cm. The entire detection system was enclosed in a dark box. RNA samples were electrokinetically injected into the capillary at 100 V/cm for 2.7 s. After each electro-separation, the injection side of the capillary was flushed with sterilized water by pump for 1 min. All separations were performed at 26°C in a clean room controlled by an air-conditioner.

3 Results and discussion

3.1 Separation of RNA by CE and PFCE

The primary motivation for this work was to observe the migration behavior of RNA fragments in pulsed field conditions. Therefore, we have undertaken a comparative study of RNA migration patterns under pulsed field and constant field conditions. Figure 1A illustrates the electrophoretic behavior of RNA in 0.5 × TBE buffer containing 0.8% HEC by square wave PFCE with DC 100 V/cm, 50 Hz of pulse frequency, 167% of modulation depth (Fig. 1A(b)) and 100 V/cm by DC CE (Fig. 1A(c)). As shown in Fig. 1A, all the 13 RNA fragments were completely resolved by both CE and PFCE. Except that for the 10 knt fragment, it cannot be detected in the pulsed field conditions, which was probably due to the extremely small volume of 10 knt RNA fragment. Another interesting phenomenon is that all the RNA fragments move with a higher speed in the capillary by PFCE than by CE, although the same average separation voltages were applied. In addition, because of the structural similarities of RNA and DNA, we carried out the separation of DNA (Fig. 1A(a)) and RNA in the same kind of HEC polymer solutions under the same PFCE conditions. As it is clear in Fig. 1A(a), DNA fragments from 0.1 to 0.5 kbp, 1 kbp to 6 kbp were separated with high resolution. It seems that DNA fragment moves with twice the speed of the corresponding RNA fragment in the polymer, whose details are given in Fig. 1B. Figure 1B plots the migration time *versus* molecular mass for the components in RNA and DNA. Data on Fig. 1B show that the migration trends for RNA and DNA are significantly different in the pulsed field conditions, which is different from the constant field CE conditions as described in [12]. For RNA fragments, the mobility trends are similar but not identical in the capillary by PFCE and CE.

3.2 Effect of polymer concentration

Typically, concentration of polymer is another important factor that determines the optimal molecular mass range of separation [28]. In order to investigate the role of HEC

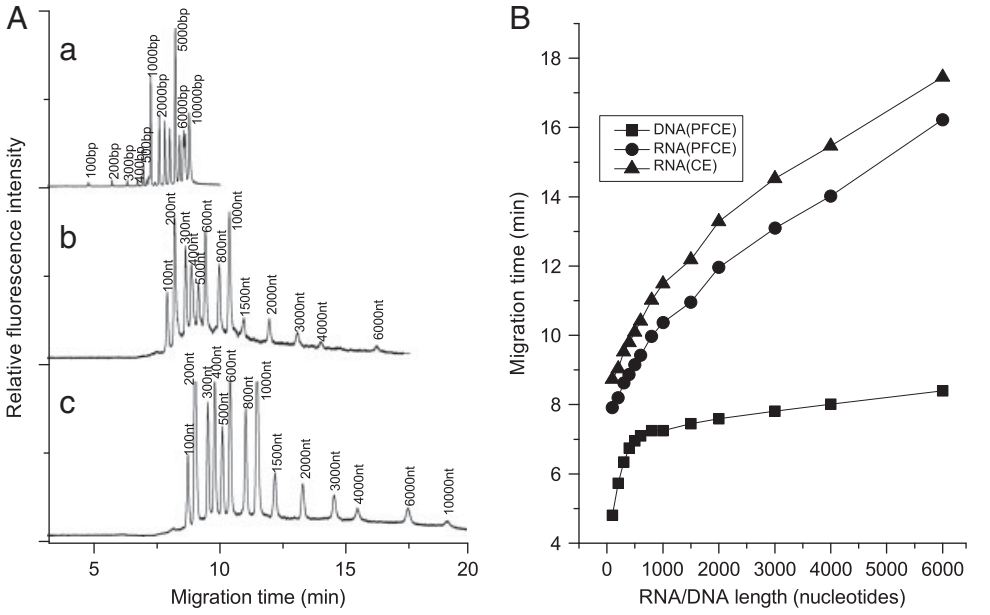

Figure 1. Comparison of RNA and DNA under PFCE and CE conditions. (A) Electropherograms of the separation of (a) DNA by PFCE, (b) RNA by PFCE, (c) RNA by CE. PFCE was performed at 100 V/cm DC with 167% of modulation depth and 50 Hz of pulse frequency. CE was carried out at 100 V/cm DC. All of them are separated in 0.8% HEC (250 K) polymer. Samples are injected at 100 V/cm for 2.7 s. (B) Migration time of RNA/DNA *versus* its length corresponding to (A).

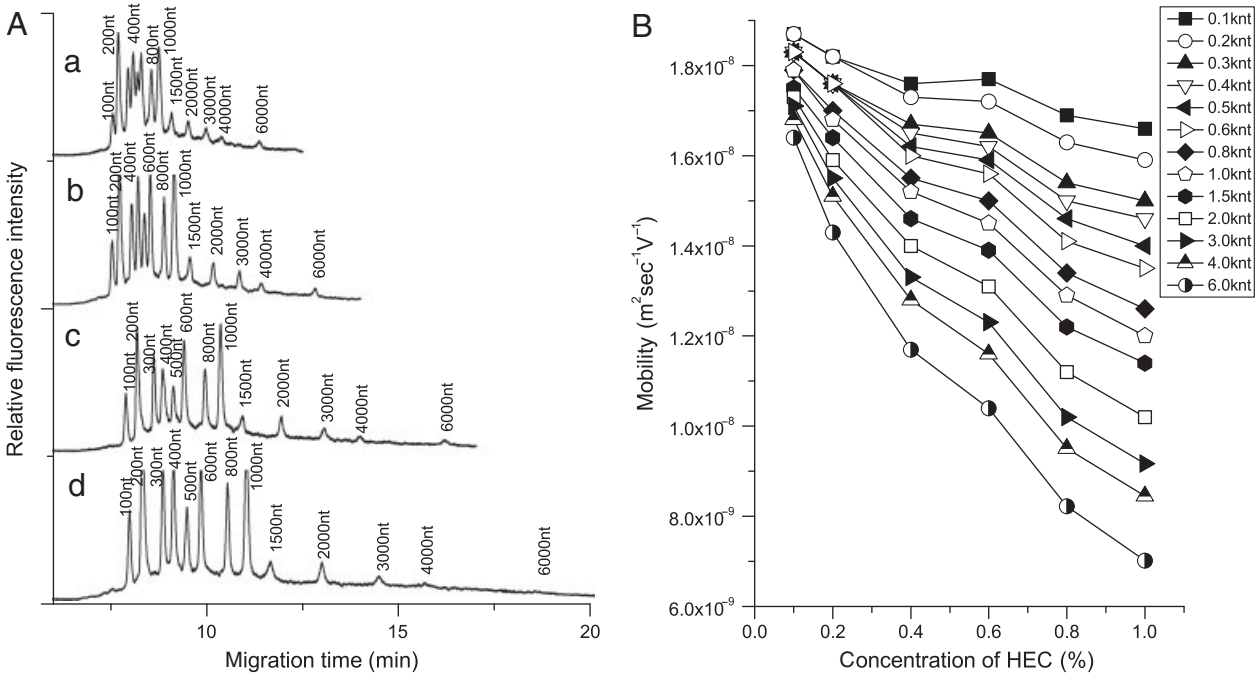

Figure 2. The effect of polymer concentration on RNA separation by PFCE. (A) Electrophoretic separation of RNA molecules at various concentrations of HEC polymer by square wave PFCE: (a) 0.2%; (b) 0.6%; (c) 1.0%. Other conditions are the same as those in (A). (B) Migration mobility of RNA/DNA *versus* polymer concentration under pulsed field conditions corresponding to (A).

concentration in separation buffer by PFCE, we separated the RNA fragments with concentrations of HEC ranging from 0.1 to 1.0% by PFCE. Figure 2A shows an example of the separation of RNA size marker using 0.2, 0.6, 0.8 and 1.0 HEC polymer under pulsed field conditions with 100 V/cm DC, 50 Hz of pulse frequency and 180% of modulation depth. As can be observed in Fig. 2A, when HEC polymer concentration is lower than 0.2%, the resolution obtained was very poor for RNA above 0.8 knt and the bands overlapped for RNA below 0.6 knt. When the concentration of HEC is 0.6%, the resolution for RNA separation improved evidently although the RNA fragments between 0.3 and 0.6 knt were incompletely separated. For 1.0% HEC polymer, all the 14 fragments can be baseline resolved, especially for the RNA fragments between 0.3 and 0.6 knt, at the cost of extended analysis time. In addition, data on Fig. 2A reveal that the baseline was easy to elevate when the polymer concentration is lower than 0.6%. This is

probably that the solution is so diluted that the collision ratio between the RNA and polymer molecule was decreased, thus the RNA fragment migrated together, which enhanced the fluorescence signal. It is also interesting to note that when the polymer concentration is higher than 1.0%, the bands will be wider. This may be attributed to the decrease in migration speed with the increase in HEC polymer concentration. As depicted in Fig. 2B, the mobility of RNA fragments declined linearly with the growth of polymer concentration. Meanwhile, we found that nearly all the RNA fragments from 0.1 to 6.0 knt were resolved with high resolution in 0.8% HEC polymer. Therefore, in the following sections, we are using 0.8% HEC for the analysis.

3.3 Effect of modulation depth and pulse frequency

Previous studies have shown that correct choice of modulation depth is critical to the separation of DNA fragments [29, 30]. Modulation depth (M) is defined as $M = (V_f - V_{DC})/V_{DC}$, where $V_{DC} = (V_f t_f + V_b t_b)/(t_f + t_b)$ is the average separation voltage, V_f refers to the forward electric field strength, V_b is the backward, t_f is the forward time duration and t_b is the backward. Since t_f is equal to t_b in our experiment, M is simplified as $(V_f - V_b)/(V_f + V_b)$. Therefore, in order to force the molecule move backward during t_b, M should be higher than 100% corresponding to $V_b < 0$. A series of experiments in the pulsed regime were performed in 0.5 × TBE/0.8% HEC at 100 V/cm field, 50 Hz of pulse frequency and modulation depth varied from 120 to 200%. Figure 3A demonstrates the electropherogram of four examples of RNA separation by PFCE. For comparison, separation of RNA by CE is shown in Fig. 3A (a). The electropherogram shows that the RNA fragment moves slightly faster with the increase in the modulation depth while the resolution for RNA fragments ranging from 0.1 to 1.0 knt was slightly reduced. This is probably due to the Joule heating, because electric field imposed on the capillary will be increased with the increase in modulation depth and Joule heating is proportional to the separation voltage. Thus, Joule heating follows the modulation depth. Slies' research has shown that temperature increase in polymer will yield a decrease in polymer viscosity [31]. Thus, additional heating may strengthen the RNA mobility in polymer. However, as mentioned in [32], additional heating may also reduce the resolution since diffusion of the RNA bands will be increased during the run. Therefore, Joule heating, which leads to radial temperature gradients and faster axial diffusion, may explain the loss of resolution for the RNA fragments at high modulation depth and short analysis time for RNA. In addition, as shown in Fig. 3B, it seems that the migration mobility of RNA increases linearly with the increase in modulation depth.

Pulse frequency is another important factor for the separation of DNA fragments by PFCE [9, 25, 33]. Here, we have performed the study of RNA separation at certain

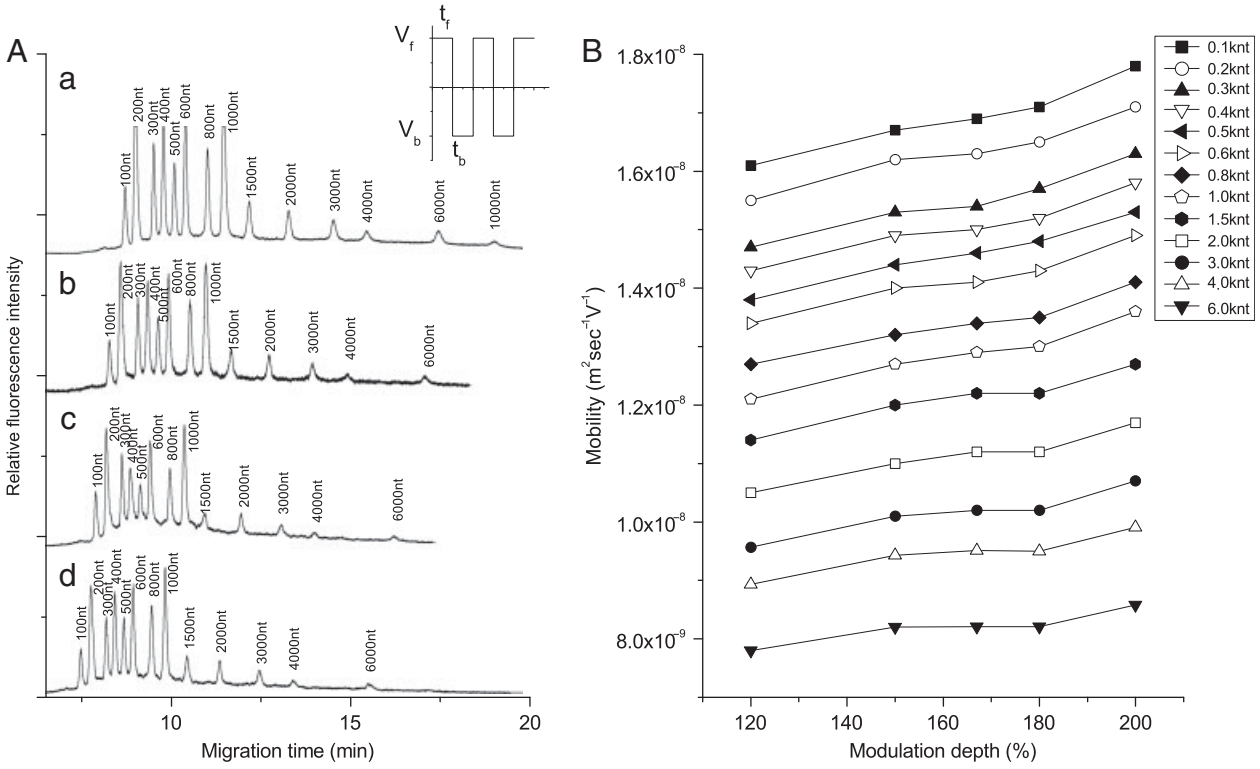

Figure 3. The effect of modulation depth on RNA separation by PFCE. (A) Electrophoretic separation of RNA molecules in 0.8% HEC at different modulation depths by PFCE: (a) 0(DC); (b) 120%; (c) 167%; (d) 200%. Other conditions are the same as those in Fig. 1A. (B) Migration mobility of RNA/DNA *versus* modulation depth under pulsed field conditions corresponding to (A).

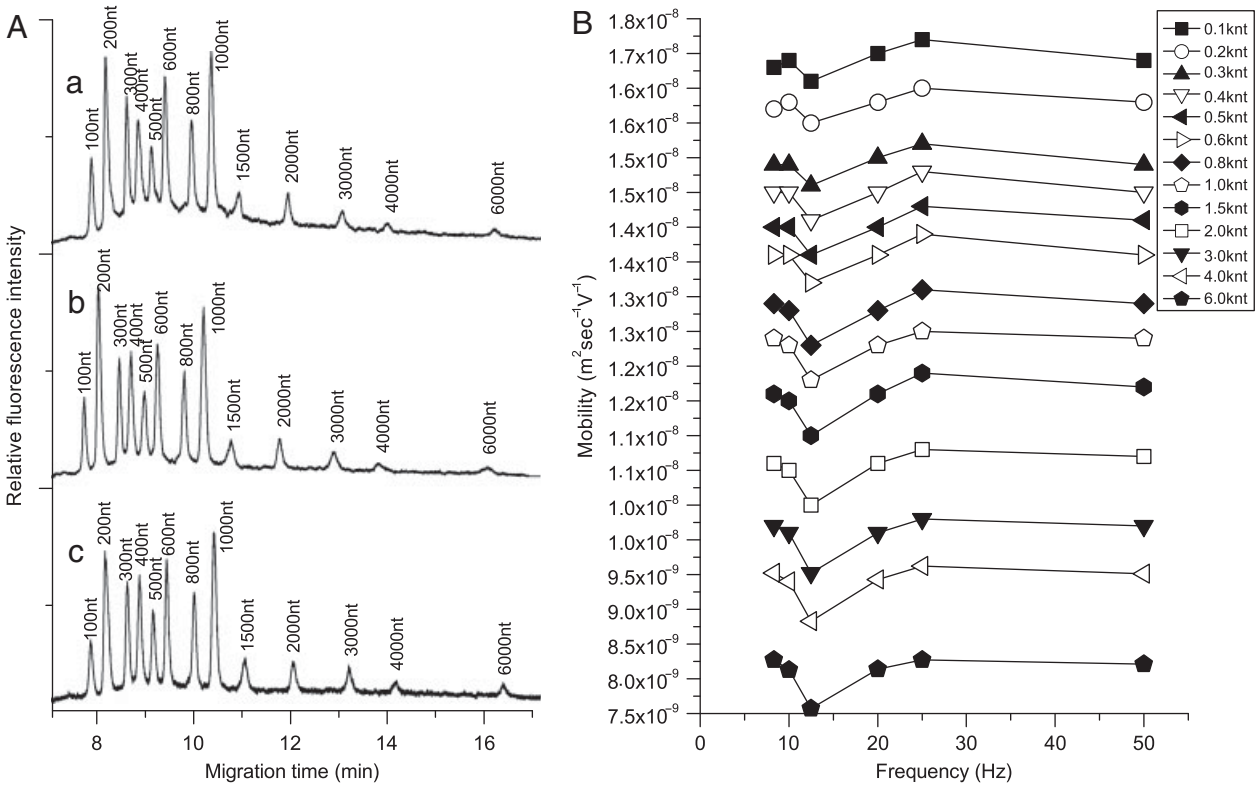

Figure 4. The effect of polymer concentration on RNA separation by PFCE. (A) Electrophoretic separation of RNA molecules in 0.8%HEC at various pulse frequencies by PFCE: (a) 10 Hz, (b) 25 Hz, (c) 50 Hz. Other conditions are the same as those in Fig. 1A. (B) Migration mobility of RNA/DNA *versus* pulse frequency under pulsed field conditions corresponding to (A).

conditions with pulse frequency from 8.3 to 50 Hz. Figure 4A plots the electropherogram of RNA separation in 0.8% HEC/0.5 × TBE at various frequencies with 100 V/cm DC with 167% modulation depth. Data in Fig. 4A demonstrate that nearly all RNA fragments were well resolved. Meanwhile, RNA fragment seems to migrate in a stable speed. Detailed information can be observed in Fig. 4B, when pulse frequency is lower than 12.5 Hz, the mobility of RNA fragment declines with the growth of pulse frequency, while the mobility increases with the increase in pulse frequency when it is greater than 12.5 Hz, and when the pulse frequency is larger than 25 Hz, RNA migration mobility will not change any more. This phenomenon implies that 12.5 Hz at which the mobility is the lowest may be the resonance frequency for RNA reorientation time and applied frequency. And it is lower than the 31.3 Hz resonance frequency of DNA in our previous report [34], which may be caused by the persistence difference between RNA and DNA. The persistence length is an important parameter that characterizes the flexibility of linear macromolecules [35]. Tinland has pointed out that the persistence length of dsDNA, ssDNA and RNA is about 50, 4 and 1 nm respectively [36], indicating that an RNA molecule is more flexible than DNA molecule, thus it will take long reorientation time for RNA molecule to reach a steady state in polymer under pulsed field conditions. Consequently, the resonance frequency is lower than DNA resonance frequency by PFCE.

4 Concluding remarks

This paper presents the investigation of large RNA molecular separation by in-capillary denaturing PFCE for the first time. We also investigated the parameters that may influence the separation performance, which included polymer concentration, pulse frequency and modulation depth. The results show that: (i) high concentration of HEC is required for the separation of short RNA fragments, for low concentration of HEC is liable for elevating the baseline of the electropherogram, meanwhile RNA fragment is inclined to overlap in low concentration of polymer; (ii) modulation depth linearly affects the migration speed for RNA in the pulsed field conditions; (iii) there exists resonance pulse frequency for RNA reorientation time and applied frequency, and RNA mobility is the lowest at the resonance pulse frequency. In addition, in order to obtain a better understanding of the migration mechanism of RNA in HEC polymer at pulsed field conditions, a more detailed study of visualization of RNA and DNA molecules *via* fluorescence video microscopy during electrophoresis is underway in our lab.

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The authors have declared no conflict of interest.

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