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Is pulsed electric field still effective for RNA separation in capillary electrophoresis?

Zhenqing Li[a,b,c], Xiaoming Dou[a,b,c,*], Yi Ni[a], Qinmiao Chen[c], Shuyi Cheng[a], Yoshinori Yamaguchi[d,b,a]

[a] Institute of Optical-Electrical Engineering, University of Shanghai for Science and Technology, 516 Jungong Rd., Yangpu, Shanghai 200093, China
[b] Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care (ASMeW), Waseda University, 513 Wasedatsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan
[c] Department of Physics, Optical Engineering, Shanghai Jiao Tong University, 800 Dongchuan Rd., Minhang, Shanghai 200240, China
[d] Photonics Advanced Research Center, Graduate School of Engineering, Osaka University, Yamadaoka Suita-city, Osaka 565-0871, Japan

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ABSTRACT

Pulsed field capillary electrophoresis (PFCE) is a predominant technique to cope with difficulties in resolving large DNA strands, yet it is still unclear whether pulsed electric field is effective for the separation of higher mass RNA. In this paper we focused on the role of pulsed electric field in large RNA fragments analysis by comparing RNA separation performance in PFCE with that in constant field CE. Separation performance in terms of migration mobility, plate numbers, resolution, and selectivity has been tested for the analysis of RNA from 0.1 to 10.0 kilo nucleotide (knt) under different electrophoretic conditions. Denaturation, important to obtain uniform and identifiable peaks, was accomplished by heating the sample in 4.0 M urea prior to analysis and the presence of 4.0 M urea in the electrophoresis buffer. Results demonstrate that unlike DNA in PFCE, the pulsed electric field mainly affects the separation performance of RNA between 0.4 and 2.0 knt. The migration mobility of long RNA fragments is not a strong function of modulation depth and pulsed frequency. Moreover, the logarithm of RNA mobility is almost inversely proportional to the logarithm of molecule size up to 6.0 knt with correlation coefficient higher than 0.99 in all the polymer concentrations measured here. Resonance frequency of RNA in PFCE was also observed. While these initial experiments show no distinct advantages of using PFCE for RNA separation, they do take further step toward characterizing the migration behavior of RNA under pulsed field conditions.

1. Introduction

For many years, slab gel electrophoresis has been the customary method in the characterization of nucleic acids [1–5]. With the advent of capillary electrophoresis, it started to lose its dominance in DNA analysis. However, this trend has not been followed in RNA field, let alone in the application of pulsed field capillary electrophoresis (PFCE) to RNA analysis.

Several groups have reported the capillary electrophoresis (CE) separation of low molecular mass RNA (transfer RNA and 5S ribosomal RNA, 70–135 bases) from bacterial [6,7], RNA molecules from 100 to 2000 bases with formamide [8], and RNA fragments up to 2.6 kilo nucleotide (knt) [9,10]. In order to resolve the high molecular mass in reasonable amount of time by CE, traditional method is to reduce the polymer concentration in background electrolyte (BGE) and/or increase the separation voltage. However, previous researches show that in CE, lower concentrations of polymer are useful for fast screenings at poor resolution, whereas with the increase of polymer concentration, separation is improved in the medium size range but at the cost of lower mobility and, therefore, increased run times [11], and even the rate of deterioration of the capillary increases with increasing gel concentration [12]. Furthermore, at large molecular weight and/or strong electric fields, nucleic acids become roughly oriented along the field direction and move at a velocity which is approximately size-independent [13–15], thus the user must reach a compromise between longer read lengths (lower fields) and fast separations (higher fields).

Because molecular orientation can be influenced by external field modulation [16–19], an effective method to solve this problem is the introduction of pulsed electric field into CE. Experiments have demonstrated that PFCE is an ideal method for the separation of long DNA fragments. Besides reducing the analysis time for DNA, PFCE can also improve the separation performance for large DNA molecules [20,21]. The success of this technique lies in the forced reorientation of DNA chains in a new direction of the external field. As the reorientation time is size-dependent, separation of different fragments becomes possible.

* Corresponding author at: Institute of Optical-Electrical Engineering, University of Shanghai for Science and Technology, 516 Jungong Rd., Yangpu, Shanghai 200093, China. Tel.: +86 21 55276023; fax: +86 21 55276023.
E-mail addresses: zhenqingli@163.com (Z. Li), xm.dou@yahoo.com.cn (X. Dou).

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However, to the best of our knowledge, reports on the application of PFCE to RNA are extremely scarce. In order to separate the RNA fragments with a wide range, we conducted an initial capillary electrophoretic experiment of RNA in the pulsed field condition [22], and realized denaturing and separating RNA simultaneously in capillary within 15 min. While this work showed acceptable separations, the denaturing process which is frequently used for RNA separations was omitted. Thus, one of the focuses of this work is to further explore the role of pulsed field in the performance of RNA by CE. Under the partial denaturing conditions used in this work, we systematically compared the separation performance of RNA in PFCE with that in CE under a series of square-wave pulsed fields. Operation parameters, such as polymer concentration, modulation depth, and pulsed frequency were varied in experiments. RNA marker sized from 0.1 to 10.0 knt was denatured in 4.0 M urea prior to analysis, and then electrokinetically injected into the capillary with HEC polymer containing 4.0 M urea for separation.

2. Materials and methods

2.1. Chemicals

Urea for RNA denaturation was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 10× TBE buffer was from BIORAD (Hercules, CA, USA) and was diluted in sterilized water to 0.5× TBE buffer as the running buffer. HEC with a molecular size of 250 000 was obtained from Sigma (St Louis, MO, USA). SYBR Green II was from Invitrogen (Carlsbad, CA, USA). The sieving buffer was consisted of 0.5× TBE, 1× SYBR Green II and 4.0 M urea. Perfect RNA™ Markers (Novagen, USA) were 0.1–1.0 knt (1000 μg/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). These two RNA markers were mixed with the same volume and then the mixture was stirred using a magnetic stirrer at least 1.0 min to make the sample concentration 500 μg/ml, then the RNA samples were diluted in 4.0 M urea and 0.5× TBE to the final concentration of 100 μg/ml, and then it was stored at −80 °C refrigerator before use. Thus a combined marker with molecular mass distributed from 0.1 to 10 knt was obtained. Prior to analysis, the RNA sample was denatured by heating in a denaturing solution (4.0 M urea) at 65 °C for 5.0 min, and then was fast cooled on ice for 3.0 min. 0.1 and 1.0 kilo base pairs (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 10.0 kbp. As supplied, the 0.1, 0.5, 1.0 and 5.0 kbp fragments are brighter than the other fragments and serve as the visible reference indicator.

2.2. Pulsed field capillary electrophoresis

The experimental setup is similar to that described before [20,21]. High-voltage power supply obtained from TREK Inc. (MODEL 610E, Medina, NY, USA) was employed to drive electrophoresis. Total capillary length (l_t) was 15 cm (8 cm effective length (l_e)) (ID/OD = 75 μm/365 μm; Polymicro Technologies, Phoenix, AZ, USA). The capillaries were coated with polyacrylamide to suppress the electroosmotic flow [23,24]. 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 waveform for pulsed field electrophoresis was generated from A/D converter controlled by Labview software. The generated voltage from A/D converter was amplified by TREK voltage amplifier. The pulsed-field electrophoresis was performed at frequencies of: 10, 12.5, 16.7, 25, and 50 Hz with and average voltage 100 V/cm in various modulation depths of a square waveform. Modulation depth was calculated by the following equation:

$$\text{Modulation depth}\ \ (\%) = 100 \times \frac{V_f - V_{DC}}{V_{DC}}$$

where $V_{DC} = (V_f t_f + V_b t_b)/(t_f + t_b)$, V_f refers to the forward separation voltage, V_b is the backward, t_f is the forward time duration and t_b is the backward. The entire detection system was enclosed in a dark box. RNA sample was electrokinetically injected into the capillary at 100 V/cm for 2.7 s. After each run, the capillary was flushed with sterilized water by pump for 1.0 min. All separations were performed at 26 °C in the clean room controlled by air-conditioner.

3. Results and discussion

3.1. Separation of RNA by CE and PFCE

Fig. 1 plots the nucleic acids separation with direct current (DC) and alternating current (AC) electric field in CE. The data for DNA were obtained in 0.5× TBE buffer containing 0.8% HEC, 1× SYBR Green II. As Fig. 1A demonstrates that for 100 V/cm, the apparent efficiency, such as resolution, and theoretical plate number of the first ten peaks, is greater compared to Fig. 1B, where full separation of DNA molecules up to 1.0 kbp was attained. In Fig. 1B, an upper limit of 8.0 kbp was achieved at 100 V/cm DC with 50 Hz of pulsed frequency, 167% of modulation depth. The electropherograms show that PFCE yielded rapid migration and better resolution than CE for DNA molecules larger than about 1.5 kbp, albeit at the expense of deteriorated resolving power for DNA fragments between 0.5 and 1.0 kbp. Denaturation, essential to obtain uniform and identifiable peaks, was accomplished by denaturing RNA sample in 4.0 M urea prior to electrophoresis and the presence of 4.0 M urea in BGE for electrophoresis. Fig. 1C and D plots the separation of the same size of RNA sample in 0.5× TBE buffer with 0.8% HEC, 4.0 M urea, and 1× SYBR Green II by CE and PFCE. It reveals that RNA fragments can be well resolved up to 6.0 knt under both electric field conditions, besides that there is a little difference for RNA between 1.5 and 6.0 knt in size. As clearly shown in Fig. 1C and D, for small RNA fragments (<1.0 knt), they nearly migrate at the same speed in PFCE and CE, while for RNA fragments longer than 1.0 knt, they migrate a little slower in pulsed electric field condition than in constant field CE, although the average electric field strength of PFCE is the same as CE.

Through Fig. 1, we have noticed there is dramatic migration difference between DNA and RNA in CE and PFCE. This difference may be caused by two reasons: (i) the persistence length of RNA is 1.5–1.9 fold longer than that of DNA [25,26], thus the flexibility of RNA and DNA in the sieving matrix is different; (ii) the presence of urea increases the viscosity of HEC solutions [27], consequently RNA fragments move slower than DNA and thus the band broadens. The phenomenon observed above indicates the necessity of investigating the influence of pulsed electric field on the migration behavior of RNA molecule in CE. Moreover, we also noticed that in PFCE, RNA fragment moves slower in certain HEC polymer containing 2.0 M acetic acid [22] than containing 4.0 M urea. This maybe because the stronger ability of 2.0 M acetic acid to enhance the viscosity of HEC than 4.0 M urea. Meanwhile, the acidity of 2.0 M acetic acid in BGE (pH < 4.0) was stronger than 4.0 M urea acid in BGE in BGE (pH 7.0–8.0). Consequently, the fluorescent intensity of the SYBR Green II binding to RNA was weekend.

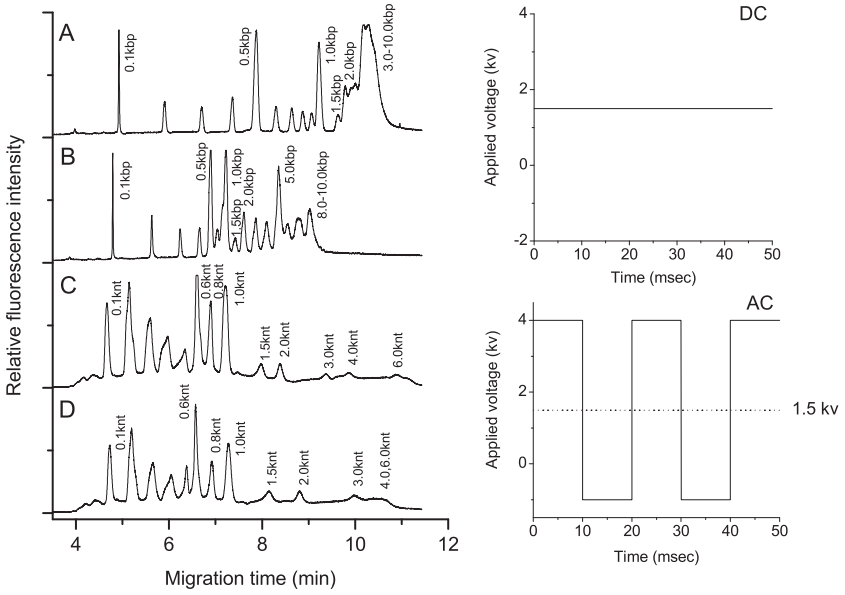

Fig. 1. Electropherograms of (A) DNA in CE, (B) DNA in PFCE, (C) RNA in CE, (D) RNA in PFCE. The BGE for DNA separation: 0.5× TBE buffer containing 0.8% HEC and 1× SYBR Green II, while BGE for RNA: 0.5× TBE buffer containing 0.8% HEC, 1× SYBR Green II, and 4.0 M urea. PFCE was performed at 100 V/cm DC with 167% of modulation depth and 50 Hz of pulsed frequency. CE was carried out at 100 V/cm DC. The capillary: l_t/l_e = 15 cm/8.0 cm. Sample injection: 100 V/cm (6.0 s).

3.2. The effect of polymer concentration on RNA separation

The network of polymer is related to the concentration of polymer solution. Here, we investigated the migration behavior of RNA (0.1–10.0 knt) in 0.5× TBE buffer containing 4.0 M urea, 1× SYBR Green II, and HEC (250 K) with concentrations from 0.1% to 1.2%. Separations were performed at 100 V/cm DC with 167% of modulation depth and 50 Hz of pulsed frequency. We find that when HEC concentration is lower than 0.3%, the components in RNA molecular mass marker migrated together, and when HEC concentration is between 0.3% and 0.5%, the baseline of the electrophoreogram is easy to elevate. Fig. 2A demonstrates an example of the separation of RNA with 0.5%, 0.8%, and 1.2% HEC solutions. It shows that under pulsed electric field conditions, long RNA fragments (>1.0 knt) could always be resolved, while decreasing the polymer concentration impairs the resolution of short RNA fragments (<1.0 knt). Furthermore, with increasing HEC concentration, sub-peak appears (0.6 knt RNA molecule in 1.2% HEC) and the bandwidth increases.

Double logarithmic mobility vs. DNA size plot is usually employed as a measure for the selectivity and indicator of DNA movement mechanism in matrix [28]. The mechanism of DNA separation in uncross-linked polymer has been described as Ogston, Reptation, and Biased Reptation with fluctuations model [10,29,30].

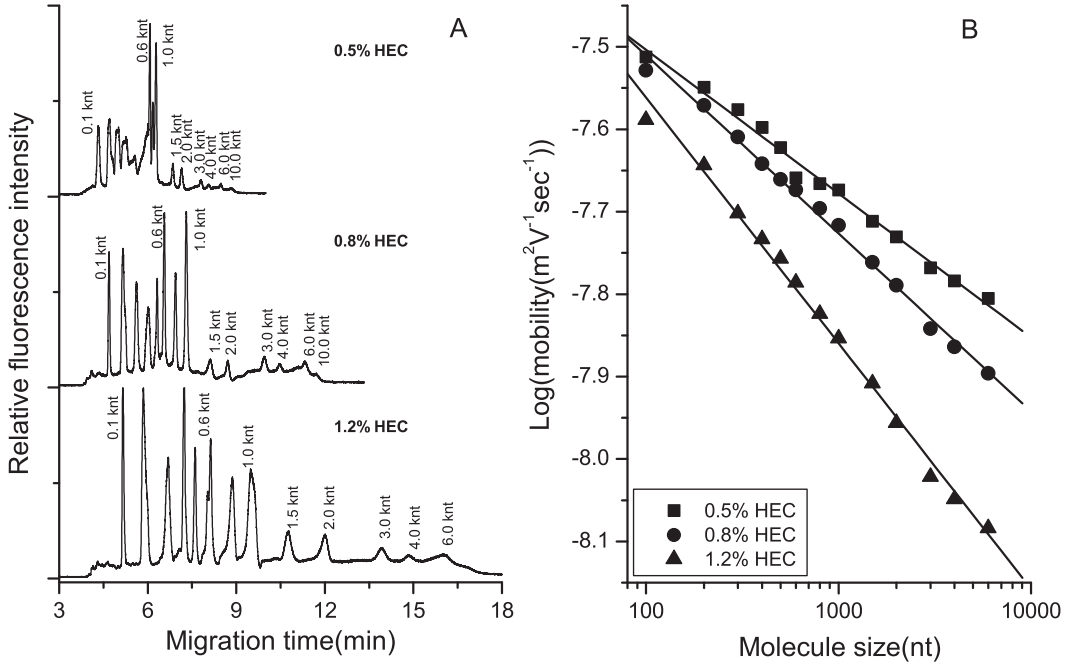

Fig. 2. The effect of polymer concentration of HEC on the separation performance by PFCE: (A) electropherograms of RNA up to 10.0 knt in various concentrations of HEC; (B) migration mobility versus RNA molecular mass at three different concentrations of HEC in BGE: 0.5%, 0.8%, and 1.2%; other electrophoretic conditions are as those in Fig. 1.

However, for RNA shorter than 10.0 knt, Fig. 2B shows that the situations are remarkably different from the corresponding size of DNA [11]. The logarithm value of mobility is almost inversely proportional to the logarithm of molecular size of RNA (the straight line in the plot is a guide to the eye only and the correlation coefficients (R) in 0.5%, 0.8% and 1.2% HEC solution are 0.995, 0.997 and 0.997, respectively), suggesting that under pulsed electric field conditions, RNA may undergo only one migration mechanism in the polymer, and the linear relationship in 4.0 M urea would allow sufficiently accurate determination of molecular weight of RNA in a manner analogous to the use of sodium dodecyl sulfate gels to determine protein molecular weight. However, this is quite different from our observation of RNA movement in HEC polymer containing 2.0 M acetic acid [22]. Fig. 3 depicts an example of the separation of RNA size marker under pulsed field conditions with 100 V/cm DC, 50 Hz of pulse frequency and 167% of modulation depth with 0.8% HEC polymer containing 4.0 M urea and 2.0 M acetic acid, respectively. It reveals that the mobility of RNA shows an approximately parabolic manner when it is denatured by 2.0 M acetic acid, inducing that the acidity may influence the structure of HEC polymer network.

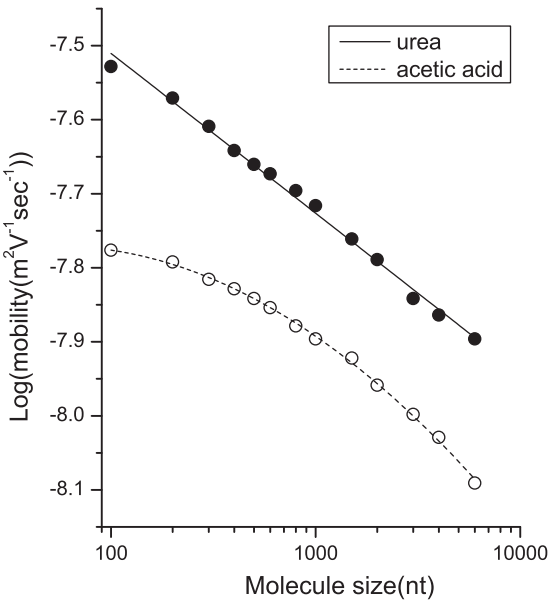

Fig. 3. RNA molecule size vs. log(mobility) with 4.0 urea and 2.0 M acetic acid as denaturant respectively by PFCE.

3.3. The effect of modulation depth and pulsed frequency

Modulation depth is the ratio of AC field to DC field, which is an important parameter for DNA separation by PFCE [31,32]. We have investigated the separation of RNA molecular mass marker in 0.5× TBE buffer containing 0.8% HEC, 1× SYBR Green II, 4 M urea at 100 V/cm DC field, 50 Hz of pulsed frequency and modulation depth varied from 80% to 200%. We found that under different modulation depths, the RNA fragments could be resolved up to 6.0 knt by PFCE (data not shown), and the peak for 10.0 knt RNA molecule sometimes disappeared probably due to its extremely small volume in the sample. In order to investigate in detail the influence of pulsed electric field on the separation performance, we have compared the plate number and resolution by PFCE with CE. Fig. 4 demonstrates the effect of varying the modulation depth on resolution (Fig. 4A), plate number (Fig. 4B), and selectivity (Fig. 4D) of the components in the RNA mass marker. Data shown on Fig. 4A reveal that resolution increases for RNA molecules up to 3.0 knt and subsequently

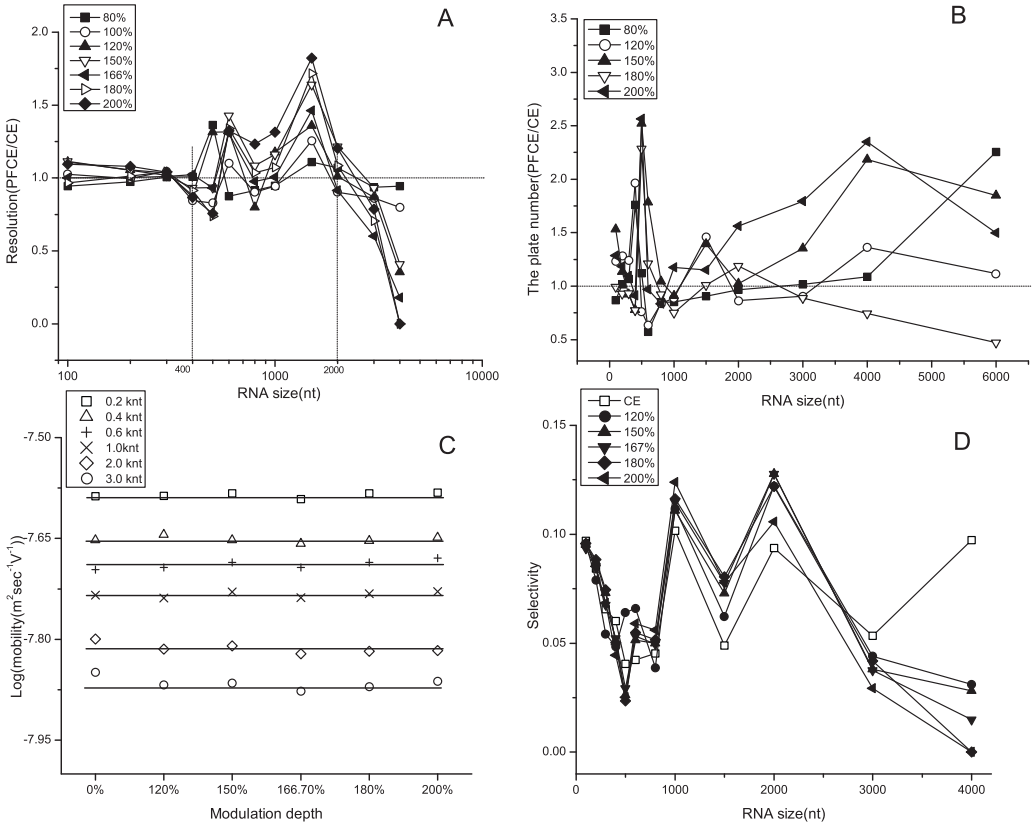

Fig. 4. The effect of modulation depth on RNA separation by PFCE: (A) the ratio (PFCE/CE) of resolution of adjacent RNA fragments; (B) the ratio (PFCE/CE) of plate numbers of RNA; (C) migration mobility of RNA; (D) the selectivity of RNA at modulation depths from 80% to 200%; other electrophoretic conditions are as those in Fig. 1.

declines. For RNA fragments between 0.4 and 2.0 knt, the resolution shows a marked increase with the growth of modulation depth, indicating that maximum resolution of RNA for a given molecular mass range could be achieved by careful optimization of the modulation depth. Fig. 4B illustrates that the plate number was improved for RNA fragments up to 6.0 knt, which means the RNA peak width on the electrophoreogram will become narrow and its peak will be relatively high in pulsed electric field conditions. Through Fig. 4A, we also noted that when RNA molecule size is above 2.0 knt, the resolution became poor with the increase of modulation depth because of the solution temperature rise caused by Joule heating [32], which is detrimental to RNA separation. Furthermore, we have evaluated the migration mobility of RNA versus its size under different modulation depths (Fig. 4C). It shows that RNA migrated in a stable mobility even though the modulation depth was changed, and this is different from our observation of RNA migration in HEC polymer containing 2.0 M acetic acid by PFCE: the mobility of RNA is slightly increased with the increase of modulation depth. We suppose that this is because there is no great thermal variety in the HEC polymer containing 4.0 M urea induced by the change of modulation depth. Since the viscosity of HEC buffer containing 4.0 M urea is lower than containing 2.0 M acetic acid, the thermal conductivity of the former solution is higher than the one of the latter solution. Consequently, the heat is easy to emanate in the former solution. Moreover, there is a linear relationship between the mobility and the molecular mass from 0.1 to 6.0 knt, the correlation coefficient seems deteriorate with the increase of modulation depth, and 100% of modulation depth offers the highest correlation coefficient (data not shown). This is probably because at 100% of modulation depth, the existence of the space time during each pulse provides an opportunity for the Joule heat to dissipate before the next electric pulse is applied, and therefore 100% of modulation depth corresponding to the highest linear correlation coefficient may be the most suitable for quantitative analysis of RNA. Therefore, 100% of modulation depth was employed in subsequent experiments.

In addition to modulation depth, pulsed frequency also influences the molecular migration behavior in PFCE [20]. Here we resolved the RNA ladder in 0.5× TBE buffer containing 0.8% HEC, 1× SYBR Green II, 4 M urea at 100 V/cm DC field, 100% of modulation depth and pulsed frequencies altered from 10 to 50 Hz. Data on Fig. 5A and B reveal the effect of pulsed frequency on resolution and plate numbers, respectively. It shows that under pulsed electric field conditions, the plate numbers of RNA were improved not so much, and pulsed frequency mainly impact on the molecular mass RNA between 0.6 and 2.0 knt. We have also noticed that at any pulsed frequency, the migration mobility of RNA is inversely proportional to the molecular weight of RNA. Furthermore, the mobility of RNA reaches the lowest at 16.7 Hz of pulsed frequency which referred to resonance frequency, corresponding to the lowest correlative coefficient between RNA mobility (data not shown). The nature of this mobility minimum in PFCE has been explained by Duke and Viovy [33], who supposed that if duration of the forward pulse is equal to the time needed for a DNA chain to undergo transition from its random-walk form to a stretched V-shape conformation, a sufficiently large DNA molecule reaches the mobility minimum in the forward direction. In addition, the resonance frequency in urea is greater to the one (12.5 Hz) in acetic acid. This is probably due to the fact that the viscosity of HEC with 4 M urea is weaker than the polymer with 2 M acetic acid, because we have noticed that the same RNA sample moves slower in the same concentration of HEC polymer with acetic acid.

Selectivity is another factor that characterizes the separation performance in CE. It is defined as the ratio of the mobility difference between two adjacent DNA fragments to their average mobility [12]. Higher selectivity implicates better ability to resolve the molecules. Figs. 4D and 5C depict the selectivity of RNA with

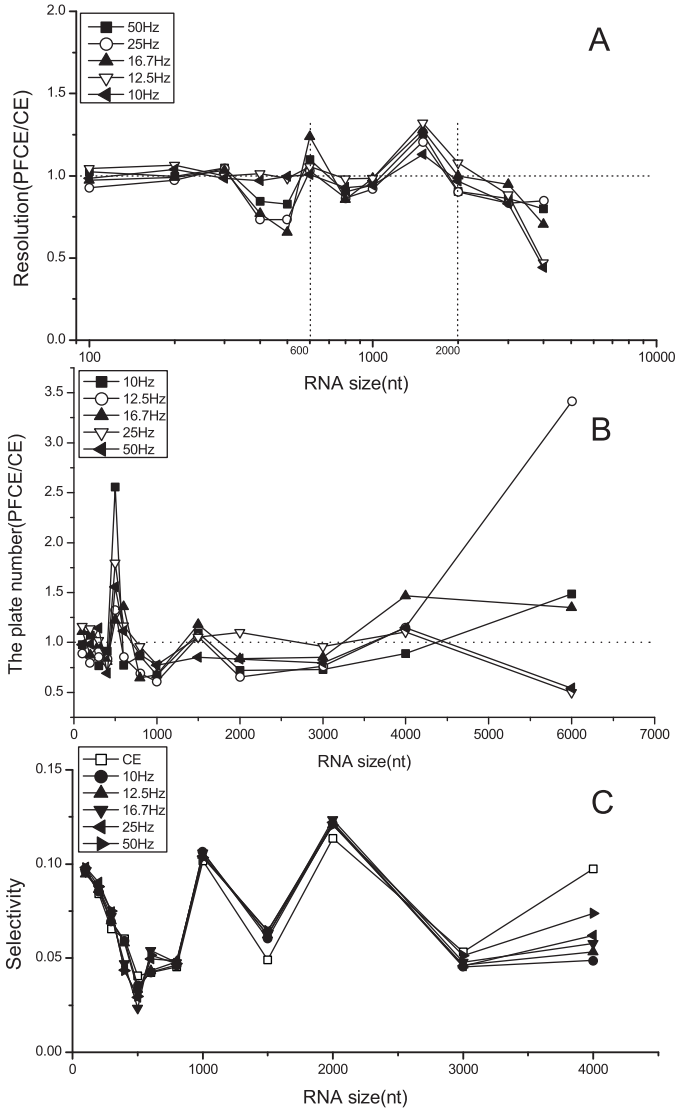

Fig. 5. The effect of pulsed frequency on RNA separation by PFCE: (A) the ratio (PFCE/CE) of resolution of adjacent RNA fragments; (B) the ratio (PFCE/CE) of plate numbers of RNA; (C) the selectivity of RNA at pulsed frequencies from 10 to 50 Hz; other electrophoretic conditions are as those in Fig. 1.

various modulation depths and pulsed frequencies, respectively. It was observed that under pulsed electric field conditions, the separation ability for RNA fragments between 1.0 and 3.0 knt was enhanced. Moreover, we noted that under certain conditions, the selectivity was almost identical for all the pulsed frequencies here. Because of the high tolerance for pulsed frequencies, a broad range of pulsed frequencies is suitable for RNA separation.

4. Conclusion

This paper systematically presents the influence of pulsed electric field on the separation performance of RNA in CE. Parameters including polymer concentration, modulation depth, and pulsed frequency were investigated for their effects on the migration mobility, plate number, resolution and selectivity. Result shows that efficient separation of RNA up to 6.0 knt could be achieved within short time. The relationship between RNA mobility and its molecular size is relevant to the denaturants employed for separation. The logarithm of RNA mobility is inversely proportional to the logarithm of RNA molecule size if it is denatured by 4.0 M urea,

which is different from the parabolic manner when it is denatured by 2.0 M acetic acid. Although there are structural similarities of RNA and DNA, the pulsed electric field mainly affects the separation of RNA between 0.4 and 2.0 knt, which is quite different from the role of pulsed electric field in separation of long DNA fragments. Though the migration mobility is not sensitive to pulsed frequency and modulation depth in the range tested, the phenomenon of resonance frequency also appears in the situation of RNA separated in PFCE.

Conflicts of interest

The authors have declared no conflict of interest.

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