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Capillary electrophoresis of RNA in hydroxyethylcellulose polymer with various molecular weights

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ABSTRACT

Recent research demonstrates that large numbers of long noncoding RNAs (lncRNAs) in mammals exhibit indices of functionality, and thus analysis of longer RNAs is of great significance. In the present work, we investigated the effect of molecular weight on the separation performance of long RNA by capillary electrophoresis (CE). Results demonstrate that (1) low molecular weight of hydroxyethylcellulose (HEC) (90k) favors the separation of short RNA (<1000 nt). The resolution for short RNA was improved and the migration time was linearly extended with the increase of polymer concentration. (2) In the longer chain HEC (250k, 720k and 1300k), the resolution for the small RNA fragment (<1000 nt) became better as the polymer concentration increased, whereas the resolution for the large ones (>3000 nt) deteriorated. (3) Based on logarithmic plot, there exist two migration regimes for RNA in short chain HEC (90k), three regimes in moderate chain HEC (250k and 720k), and four regimes in the long chain HEC (1300k). Such a systematic investigation of long RNAs may be useful for research on lncRNAs in the length range of 100–10,000 nt.

1. Introduction

Capillary electrophoresis (CE) was widely employed in the analysis of DNA because of its numerous advantages, e.g., high resolution, fast speed, and excellent reproducibility. However, this trend has not been followed in the RNA field during the past few years [1], because the RNA was assumed to have been largely relegated to an intermediate between gene and protein, encapsulated in the central dogma 'DNA makes RNA makes protein' [2]. Recently, increasing research demonstrates that lncRNAs in mammals exhibit indices of functionality [3,4] and have been shown to be dynamically expressed during cellular differentiation [5], including in the brain [6,7], and thus analysis of longer RNA is of great significance [8].

So far, there were only a few studies about RNA or lncRNAs analyzed by CE. For example, Katsivela's group first demonstrated the separation of low molecular weight RNA (transfer RNA and 5S ribosomal RNA, 70–135 bases) from single bacterial strains [9,10]. Todorov et al. performed comparison of RNA, single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) behavior in semidilute neutral polymer solutions by CE [11,12]. Zhong et al. reported an excellent method for high-throughput RNA expression analysis based on laser induced fluorescence (LIF) CE [13]. Zhong's lab combined rolling circle amplification with LIF-CE for small RNA detection and the detection limit of this approach was down to 35 amol [14]. Li et al. reported a pioneering work that used CE to separate the miRNA and its methylation product. It showed that capillary zone electrophoresis with UV absorption detection and a reduced running temperature achieved good separation of miR173/miR173* and miR173-m/miR173*-m with a detection limit of around 1 μM [15]. Saevels et al. described how to measure the catalytic rate of a synthetic hammerhead ribozyme in cleaving its substrate [16]. Moreover, various non-crosslinked matrixes were employed as sieving polymers for small [17], medium size [10] and large RNAs [18], including polyacrylamide [10], cellulose derivatives [19], and poly(*N*-vinyl pyrrolidone) [20]. Our group also developed the online analysis of RNA by CE [21,22].

Moreover, the separation mechanism of the probes in the polymer solutions has been extensively investigated in the past few

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years. Ogston sieving [23], reptation [24], and biased reptation model [25] were employed for the description of nucleic acids migration in polymer solutions. For Ogston sieving, the probes are deemed to be migrated through the gel much like an undeformed ball of radius R_g, when R_g is smaller than the gel pore size; while for R_g is larger than the gel pore size, the probe must deform in order to migrate through the gel, and we call it reptation regime [26]. Prof. Muthukumar successively investigated the electrophoretic mobility of a polyelectrolyte in semidilute solutions of neutral polymers [27]. They derived a formula for the electrophoretic mobility of the polyelectrolyte in the polymers in terms of molecular weight, concentration of neutral polymer, Debye length and solvent quality. They also analyzed the mechanism of the poly(styrenesulfonate) in dilute solution of neutral polymers, and measured the dependence of mobility of the probe on the molecular weight and concentration of host chains by experiment [28]. Cottet et al. discussed the analytical performances and migration mechanisms of polystyrenesulfonates in entangled polymer solutions. They mainly investigated the influence of the mesh size and of the lifetime of the obstacles of the separating network [29]. Todorov et al. researched the migration behavior of RNA in semidilute polymer solutions [11]. So far, research on the relationship between the separation performance of long sized RNA and molecular weight of polymer is still insufficient by now.

In this work we investigated the separation performance of RNA ranged in size from 100 to 10000 nucleotide (nt) by CE coupled with fluorescence detection methods. They were resolved hydroxyethylcellulose (HEC) polymer with different molecular weight: 90k, 150k, 720k, and 1300k. We believe that such a study will shed light on the research of long noncoding RNAs.

2. Material and methods

2.1. Chemicals

10× TBE buffer was purchased from Bio-Rad (Hercules, CA, USA). HEC with viscosity average molecular weight (Mv) of 90k, 250k, 720k, and 1300k was from Sigma (St Louis, MO, USA). SYBR Green II was bought from Invitrogen (Carlsbad, CA, USA). Urea was purchased from Wako Pure Chemical Industries (Osaka, Japan). RNA Marker RL 1000 and RNA Marker RL 10000 were from Takara (Shiga, Japan). Pure water was from ultra-pure water machine (Purescience, Shanghai, China). RNA was denatured by heating in a denaturing solution (4.0 M urea) at 65 °C for 5.0 min, and then was cooled on ice for 3.0 min. The HEC solutions consisted of 0.5× TBE, 1× SYBR Green II and 4.0 M urea. The viscosity of the separation buffer was measured by SNB-1 (SunnY Hengping Scientific Instrument Co., Ltd, Shanghai, China). The entanglement threshold of the HEC solution was determined by evaluating the dependence of specific viscosity on polymer concentration [11,30]. Results showed that the entanglement threshold for HEC solution with 4.0 M urea was about 0.02%, 0.06%, 0.32% and 0.45%, which correspond to the molecular weight of 1300k, 720k, and 250k, 90k, respectively.

2.2. Capillary electrophoresis

The experimental setup was described before [31,32]. High-voltage power supply (Matsusada Precision, Shiga, Japan) was used to drive electrophoresis. The excitation wavelength from a mercury lamp was filtered (U-MWIB-3, Olympus, Japan) to be 460–495 nm to detect the RNA fluorescence signal, which was about 512 nm. The fluorescence emission was collected by a 60× objective (PlanApo/IR, Olympus, Japan), 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). Each time, fused-silica capillary (ID/OD = 75 μm/365 μm) (Polymicro Tehchnologies, Phoenix, AZ, USA) was covalently coated with polyacrylamide (Sinopharm Chemical Reagent Co., Ltd.(SCRC), Shanghai, China) [33,34]. All experiments were run at 900 V. The total capillary length was 9 cm, with an entrance to detector length of 6 cm. The entire detection system was enclosed in a dark box. RNA sample was electrokinetically introduced into the capillary at 100 V/cm for 1.0 sec. After each run, the capillary was flushed for 1.0 min with sterilized water by pump. All separations were performed at 26 °C and performed 5 times to check the reproducibility.

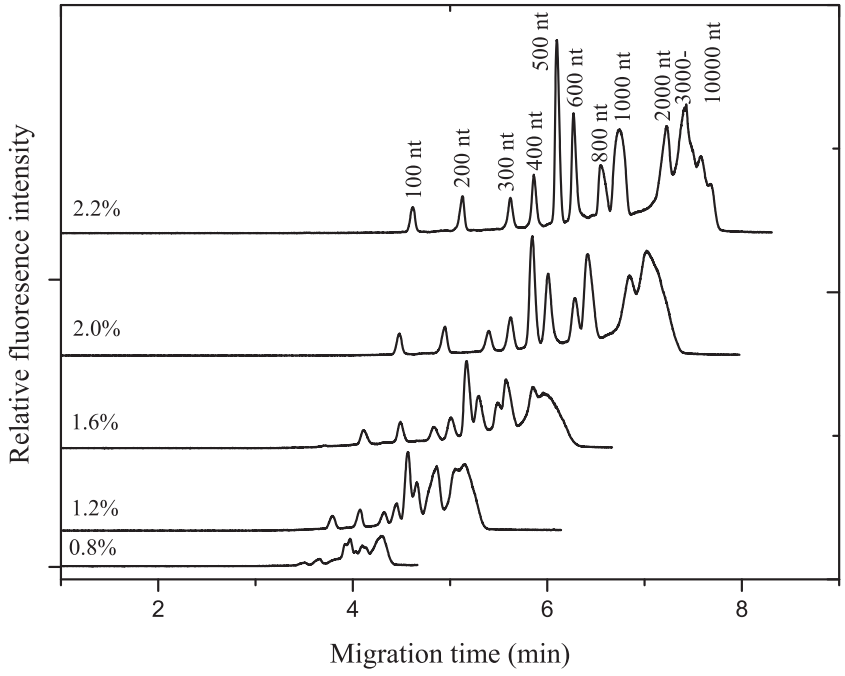

Fig. 1. Electrophoresis of RNA sized from 100 to 10,000 nt in HEC (Mv~90k) with concentration varied from 0.8% to 2.2%. CE was performed at 100 V/cm. The total length and the effective length of the capillary are 9.0 cm and 6.0 cm. Sample Loadings: 100 V/cm (1.0 sec).

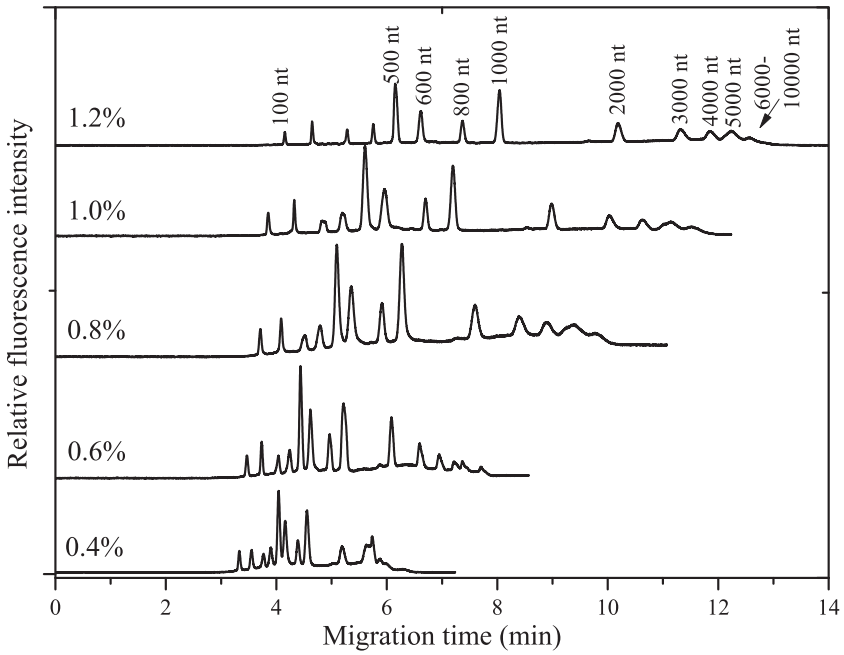

Fig. 2. Electrophoresis of RNA in HEC (250k) with concentration varied from 0.4% to 1.2%. Other electrophoretic conditions were the same as those in Fig. 1.

3. Results and discussion

3.1. Electrophoresis of RNA in HEC 90k

In order to investigate the effect of molecular weight on the separation performance of RNA, we first separated RNA ranging in size from 100 to 10,000 nt in HEC polymer (Mv~90k). Fig. 1 shows the electropherogram of RNA in HEC (Mv~90k) with concentration varied from 0.8% (wt%) to 2.2% (wt%) at 100 V/cm of electric field strength. As shown in Fig. 1, HEC (Mv~90k) with concentration at 2.2% (wt%) and the more diluted one offered limited resolution for larger RNA (>2000 nt). According to Eq. (5) and Eq. (8) in Ref [29], this was possibly because the for HEC with lower molecular mass, the radius of gyration of the swollen coil, R_g, is relatively small, and the entanglement threshold of the polymer corresponds to the concentration C^* was relatively high, and thus short-chain HEC failed to form effective sieving matrix for the large RNA fragment. When the concentration of HEC increased from 0.8% (wt%) to 1.6% (wt%), an improvement in small-RNA (<500 nt) separation was seen, but to a lesser extent. We also resolved the sample in background electrolyte containing no HEC. It showed that in 0% HEC solution, only one peak was observed in the electropherogram, indicating that no separation happened. This was because no sieving matrix was formed for the RNA fragment. Through Fig. 1, we can find that the separation of large RNA(>1000 nt) was extremely weak, and peak broadened when the concentration of the polymer was lower than 1.2%. This was possibly because the polymer concentration was so low that HEC chains behaved independently of each other, "collisions" between RNA molecule and HEC chains seldom happened. Therefore, we can conclude that in HEC (Mv~90k), the separation performance of RNA was improved with the increase of polymer concentration. Furthermore, it is interesting to notice that when RNA was smaller than 1000 nt, its migration time was linearly increased with the growth of polymer concentration and the correlation coefficient (R) was above 0.99 for the fitting line. We also calculated the resolution between the neighboring RNA fragments. The resolution (R) was calculated based on the following equation.

$$R = \frac{2(t_B - t_A)}{W_B + W_A} \quad (1)$$

where t_B is the time corresponding the electrophoretic peak, and W_B is the time width of the electrophoretic peak. The resolution for the RNA in HEC (90k) was tabulated in Table 1.

3.2. Electrophoresis of RNA in HEC 250k

To study further the separation properties of HEC solutions, we have deeply resolved RNA (100–10,000 nt) in HEC (Mv~250k) with concentration varied from 0.4% (wt%) to 1.2% (wt%) at 100 V/cm, and the results were depicted in Fig. 2. A comparison of electropherogram of Figs. 1 and 2 clearly revealed that substantial improvement in RNA separations was achieved in longer-chain HEC solutions. When the concentration of HEC (Mv~250k) was increased from 0.4% (wt%) to 1.2% (wt%), there was no great change of the migration time for RNA smaller than 500 nt. In contrast, the migration time for 10,000 nt RNA was extended from 6.0 min (0.4% (wt%) HEC) to 12.0 min (1.2% (wt%) HEC). Furthermore, the resolution between neighboring RNA fragments (Fig. 3) improved greatly with the increase of polymer concentration. Data in Fig. 3 show that a significant increase in resolution for short RNA fragments (<3000 nt) was achieved as the concentration of HEC (Mv~250k) was increased, while for large ones (4000 and 5000 nt), the resolution reached the maximum at 0.6% (wt%) HEC (Mv~250k), and then decreased with the increase of polymer concentration. Detailed information of the resolution was tabulated in Table 2.

Table 1
The resolution for the neighboring RNA fragment in HEC (90k) with various concentrations. The electrophoretic conditions were the same as those in Fig. 2.

RNA (nt)	1.20%	1.60%	2.00%	2.20%
100–200	1.993	2.573	3.013	3.139
200–300	1.775	2.097	2.697	2.624
300–400	0.920	1.057	1.386	1.535
400–500	1.008	1.162	1.219	1.729
500–600	0.828	1.000	0.945	1.071
600–800	1.005	1.390	1.535	2.081
800–1000	0.000	0.708	0.885	1.283
1000–2000	0.723	1.203	1.795	1.884
2000–3000	0.490	0.443	0.666	0.654

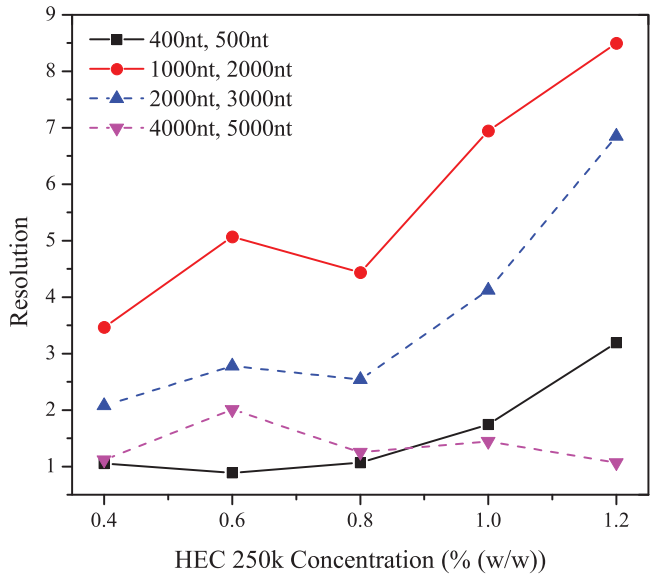

Fig. 3. The resolution of neighboring RNA fragment in HEC 250k. The electrophoretic conditions were the same as those in Fig. 2.

Table 2
The resolution for the neighboring RNA fragment in HEC (250k) with various concentrations. The electrophoretic conditions were the same as those in Fig. 2.

RNA (nt)	0.40%	0.60%	0.80%	1.00%	1.20%
100–200	1.923	2.246	3.452	6.351	4.219
200–300	1.643	2.488	2.185	4.303	4.897
300–400	1.143	1.127	1.144	2.220	2.731
400–500	1.055	0.889	1.069	1.746	3.191
500–600	0.959	1.055	1.330	1.210	2.766
600–800	1.703	2.132	2.064	3.161	4.702
800–1000	1.000	1.572	1.194	2.179	2.709
1000–2000	3.465	5.070	4.439	6.945	8.498
2000–3000	2.077	2.781	2.539	4.121	6.851
3000–4000	0.722	2.222	1.628	2.305	2.565
4000–5000	1.116	2.007	1.253	1.446	1.068
5000–6000	0.740	1.254	1.159	0.995	1.353

Table 3
The resolution for the neighboring RNA fragment in HEC (720k) with various concentrations. The electrophoretic conditions were the same as those in Fig. 4.

RNA (nt)	0.40%	0.60%	0.80%	1.00%	1.20%
100–200	1.198	1.444	2.132	2.535	3.959
200–300	0.868	1.479	2.253	2.004	4.222
300–400	0.774	1.086	1.374	0.917	3.446
400–500	0.731	0.933	1.344	1.175	1.754
500–600	1.067	1.126	0.920	0.718	1.267
600–800	1.248	0.833	1.983	2.074	2.531
800–1000	0.823	1.128	1.744	1.169	1.830
1000–2000	2.356	2.739	3.573	3.531	6.328
2000–3000	1.687	1.461	1.727	1.789	3.180
3000–4000	1.552	1.553	1.727	1.516	1.590
4000–5000	1.317	1.392	1.613	1.144	1.135
5000–6000	0.928	1.036	1.033	0.911	0.000
6000–1000	2.723	2.605	1.841	1.483	0.000

3.3. Electrophoresis of RNA in HEC 720k

Next, we have carried out RNA separation in HEC (Mv~720k) with concentration ranging from 0.4% (wt%) to 1.8% (wt%) at 100 V/cm. Data in Fig. 4 demonstrated that nearly each of the RNA fragment sized from 100 nt to 10,000 nt could be baseline resolved, which was consistent with the resolution tabulated in Table 3. However, it should be noted that the migration time of 10,000 nt RNA in 0.4% (wt%) HEC was about 6.0 min. By contrast, it was more than 30.0 min in 1.8% (wt%) HEC, indicating that the migration time was prolonged dramatically if the polymer concentration was increased. This is because the viscosity was increased with increasing polymer concentration [35], and thus reduced the velocity of RNA fragment in polymer, and thus the movement of RNA fragment was retarded by the concentrated network of the polymer. The radius of gyration of the swollen coil, R_g was estimated by the following equations [29].

$$R_g = \left(\frac{K}{6^{3/2}\Phi}\right)^{1/3} M_W^{(a+1)/3} \quad (2)$$

where M_W is the average weight molecular mass, K is the Mark–Houwink coefficient, and Φ is a constant related to Flory viscosity constant. Eq. (1) predicts that the radius of gyration R_g will be enlarged if the molecular mass of polymer increases. Therefore, the sieving ability for the relatively large RNA fragment was improved. Moreover, the extension in migration time may cause RNA diffusion in polymer solution, and finally widen the peak in the electropherogram. Those two hypotheses were also proved by the electrophoretic result of RNA in 1.6% (wt%) and 1.8% (wt%) HEC (Mv~720k). The peaks corresponding to RNA fragment larger than 3000 nt nearly cannot be recognized because the peak broadens. Consequently, longer RNA was resolved with higher resolution in the more diluted concentration of HEC (Mv~720k), which is shown in the insert of Fig. 4.

Table 4
The resolution for the neighboring RNA fragment in HEC (1300k) with various concentrations. The electrophoretic conditions were the same as those in Fig. 5.

RNA (nt)	0.20%	0.40%	0.60%
100–200	0.946	1.614	2.272
200–300	0.745	2.195	2.241
300–400	0.672	1.406	1.693
400–500	0.875	1.084	1.911
500–600	0.780	1.069	1.118
600–800	1.536	1.821	3.372
800–1000	0.806	1.047	2.077
1000–2000	4.216	10.656	10.984
2000–3000	3.014	5.624	8.836
3000–4000	2.882	1.605	2.806
4000–5000	2.155	0.782	0.882
5000–6000	0.887	1.184	1.456
6000–10,000	1.100	0.853	0.731

3.4. Electrophoresis of RNA in HEC 1300k

Finally, we performed CE of RNA in HEC (Mv~1300k) with concentration varied from 0.2% (wt%) to 0.6% (wt%), and the results were plotted in Fig. 5. Data in Fig. 5 showed that the separation of short (<1000 nt) and large RNA (>1000 nt) fragment was quite different in the long-chain HEC solutions. The resolution for the neighboring RNA fragments are tabulated in Table 4. A detailed description is that the resolution of short RNA fragments dramatically improved with the increase of concentration of HEC (Mv~1300k), whereas the longer ones, especially RNA fragment larger than 3000 nt was deteriorated with the increase of polymer concentration. Comparably, 0.4% (wt%) HEC offered the best resolution for both short and long RNA fragments, indicating that large HEC polymers contribute significantly to the separation of both small and large RNA fragment, which is quite different from the result of DNA in HEC polymer [36]. Another interesting phenomenon was that very low concentration of HEC 1300k provided both high resolution and short migration time for RNA, indicating that low concentration of longer chain HEC polymer may offer effective sieving matrix for the RNA molecule. This is most possi-

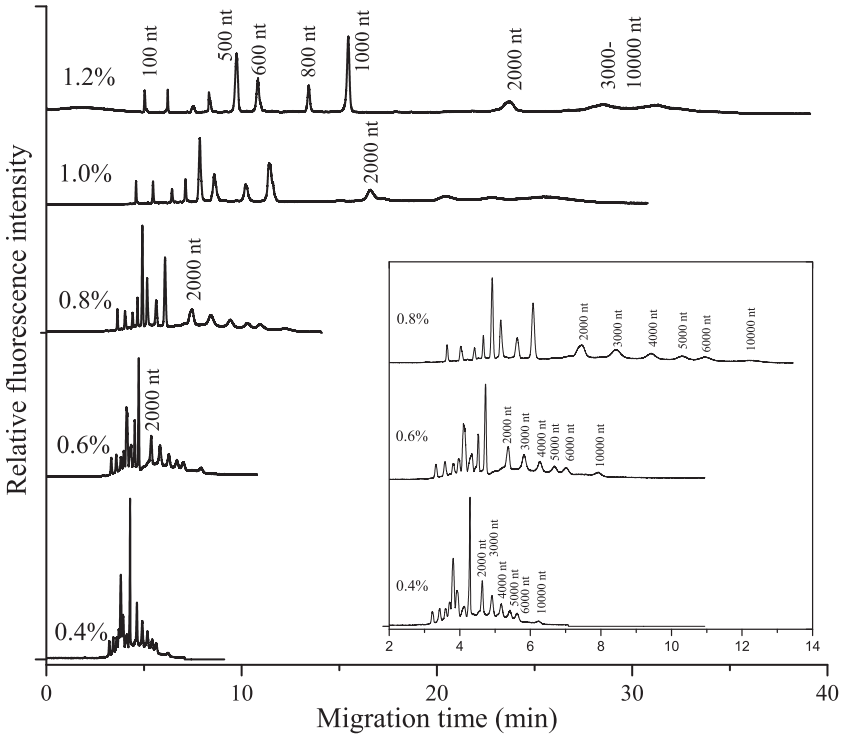

Fig. 4. Electrophoresis of RNA in HEC (720k) with concentration varied from 0.4% to 1.2%. Other electrophoretic conditions were the same as those in Fig. 1.

bly because the radius of gyration of the polymer is related to the following equations [11].

$$R_g \approx \left(\frac{[\eta] M_r}{(6.2 N_A)}\right)^{1/3} \quad (3)$$

$$[\eta] = KM^a{}_r \quad (4)$$

where N_A is Avogadro's number. Through Eq. (2) and Eq. (3), we can conclude that R_g is related to the molecular weight M_r. Therefore, high molecular weight polymer may easy offer concentrated sieving matrix at lower concentration.

3.5. Comparison of RNA mobility in different molecular weight of HEC

Finally, we compared the migration patterns of RNA in HEC solution with different molecular weights (90k, 250k, 720k, and 1300k) by double logarithmic plot, and the results are described

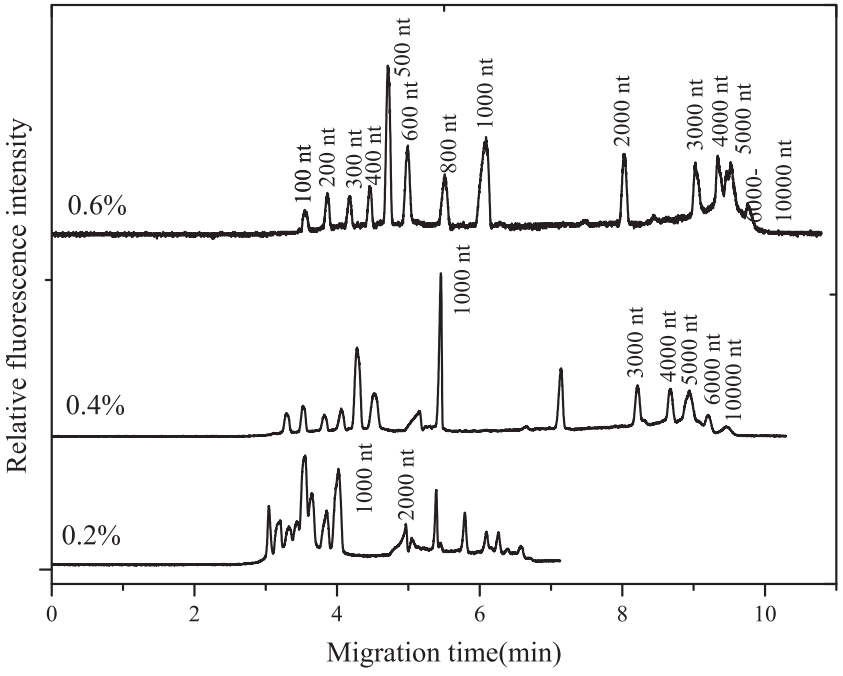

Fig. 5. Electrophoresis of RNA in HEC (1300k) with concentration varied from 0.2% to 0.6%. Other electrophoretic conditions were the same as those in Fig. 1.

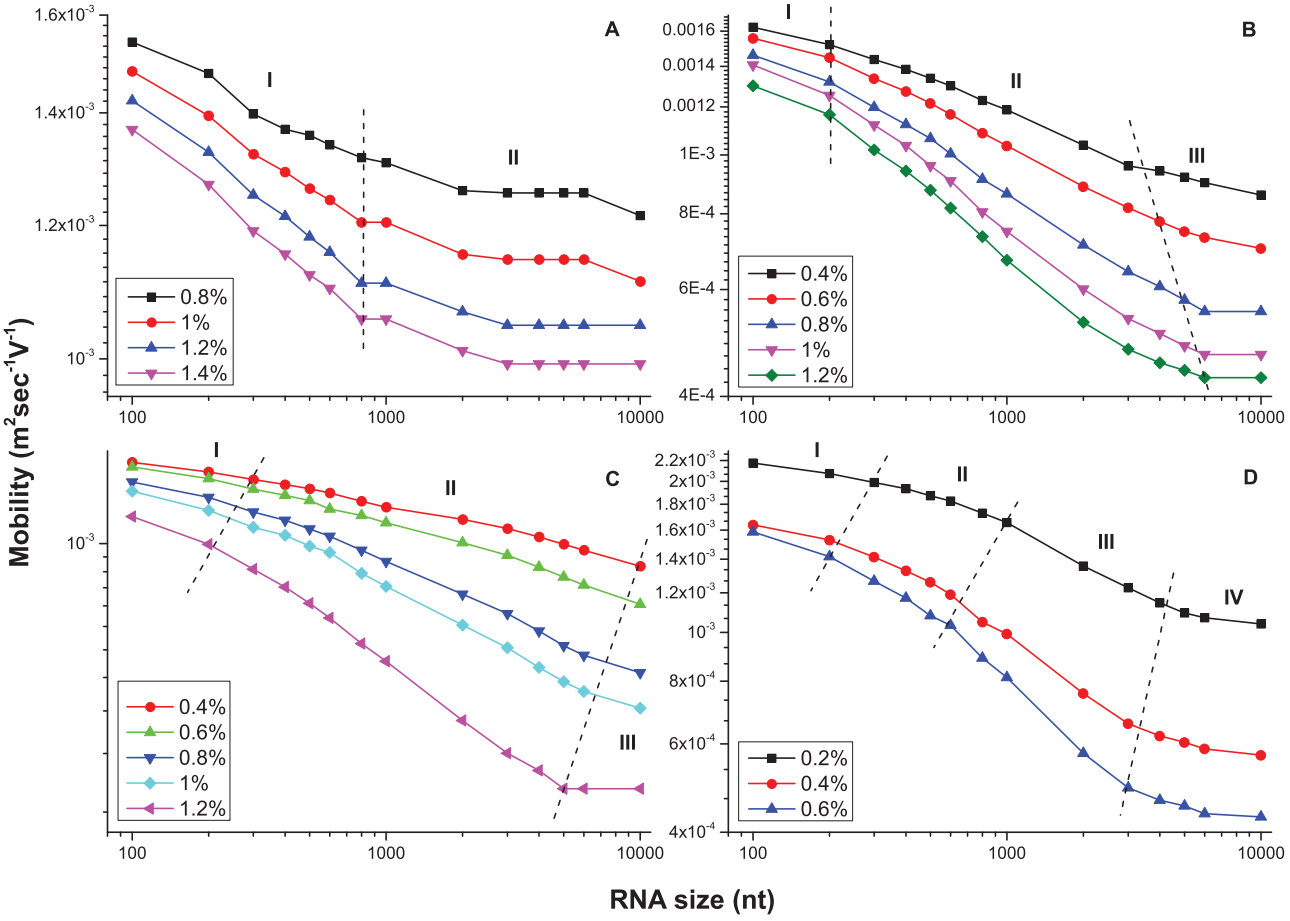

Fig. 6. The mobility of RNA in different molecular weight of HEC: (A) 90k, (B) 250k, (C) 720k, and (D) 1300k. Other electrophoretic conditions were the same as those in Fig. 1.

in Fig. 6. It shows that the migration patterns were quite different for RNA in different molecular weight HEC. As depicted in Fig. 6A, RNA fragments smaller than 800 nt migrated in Regime I, in which log(mobility) was linearly decreased with the growth of log(size). For the large ones in Regime II, especially the one above 2000 nt, there were no mobility differences for the neighboring RNA mobility, and thus they cannot be effectively resolved in HEC (Mv∼90k). Three regimes were observed in Fig. 6B. The log(mobility) seems linearly related to the log(mobility) for RNA smaller than 6000 nt, but the slope for RNA between 100 nt and 200 nt and slope for RNA sized from 200 nt to 6000 nt were different. Consequently, RNA separation performance was improved because of the dramatic mobility difference of RNA. The same situation happened in HEC (Mv∼720k), which was depicted in Fig. 6C. RNA fragments moved in four regimes when they were resolved in HEC (Mv∼1300k). In each regime, there was a linear relationship for log(mobility) and log(size), and the slop for each fitting line was different. The correlation coefficient (R) for the linear relationship in each regime was above 0.99. Through Fig. 6, we can conclude that short chain HEC with high concentration may contribute to the separation of small RNA, while relatively long chain HEC with low concentration can offer high resolution for the large RNA fragments.

4. Conclusions

We performed a systematic research on the separation of wide-range sized RNA in HEC with different molecular weight. RNA was denatured by 4.0 M urea prior to CE, and then they were resolved in HEC solution containing 4.0 M urea. The effect of polymer concentration and molecular weight of HEC on the migration time, resolution between neighboring RNA fragments, and log(mobility) was investigated, and thus the migration behavior of RNA in different molecular weight HEC was summarized. Results demonstrated that it was possible to resolve the RNA fragments in the entangled HEC solutions. Especially the result that RNA sized from 100 nt to 10,000 nt was effectively resolved in 1.2% (wt%) HEC (Mv∼250k) within 12.0 min may offer an optimal choice for wide range RNA separation. Such a study may motivate CE as an effective tool for long RNA analysis.

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