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Separation and quantification of viral double-stranded RNA fragments by capillary electrophoresis in hydroxyethylcellulose polymer solutions

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Abstract

Capillary electrophoresis (CE) is an analytical technique widely utilized to resolve complex mixtures of nucleic acids. CE uses a variety of polymers in solution that act as a molecular sieve to separate nucleic acid fragments according to size. It has been shown previously that purified dsDNA can be resolved efficiently by solutions of hydroxyethylcellulose (HEC) polymer, providing a rapid and high resolution method of separation. We have applied this separation technique to viral double-stranded (ds) RNA segments derived from rotavirus process samples. HEC polymers of various molecular masses and concentrations were identified and compared for their ability to separate dsRNA based on the extent of expected polymer network formation. The HEC polymer exhibiting the most desirable separation characteristics was then used for subsequent optimization of various method parameters, such as, injection time, electric field strength, dye concentration and capillary equilibration. The optimized method was then applied to the quantification of genome concentration based on a representative segment of the rotavirus genome. This study demonstrated that purified viral dsRNA material of known concentration could be used to generate an external standard curve relating concentration to peak area. This standard curve was used to determine the concentration of unknown samples by interpolation. This novel RNA quantification assay is likely to be applicable to other types of virus, including those containing dsDNA.

Keywords: Viruses; RNA; Hydroxyethylcellulose

1. Introduction

Rotaviruses are dsRNA viruses classified as a genus within the family *Reoviridae*. Structurally, they are characterized as triple layered icosahedral particles consisting of an outer (VP7/VP4), intermediate (VP6) and an inner protein capsid (VP2), which contains the 11 genome segments ranging in size from 667 to 3302 base pairs (bp) [1]. Rotaviruses have been identified as the major cause of severe gastroenteritis in human infants and young children [2]. For this reason a vaccine against rotavirus continues to be of major importance for public health, especially in developing countries. Typically, viral vaccines are produced by virus propagation in cell substrates, followed by a series of filtration/purification steps to generate a virus suspension which will comprise the final vaccine. The development of a successful viral vaccine requires monitoring of the virus concentration at each of the processing steps to ensure acceptable step yields and consistent process performance. In most cases, this is achieved by measuring the viral content by biological potency or physical mass assays. Potency measurements are typically based on plaque assays, tissue culture infectious doses that will infect 50% of the cells ($TCID_{50}$) or quantitative polymerase chain reaction (qPCR) methods and are inherently more variable than physical determination assays. In addition, potency assays are usually laborious, costly and have a low sample throughput. Hence a quick, precise and accurate particle/genome quantification assay is highly desirable, especially during the process development phase of a new candidate vaccine.

Over the course of the development of a rotavirus (RV) quantification assay, initial experiments have indicated that several rotavirus dsRNA genome fragments can be quantified using a simple slab-gel electrophoresis assay in the presence of an intercalating dye, such as ethidium bromide. The

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fluorescent intensity of resolved bands was found to be proportional to the amount of viral genome present. However, the assay was laborious, susceptible to loading inconsistencies and inherently variable due to limited resolution and sensitivity. It was therefore deemed inappropriate for the development of a reliable and precise quantification assay. Nevertheless slab-gel electrophoresis clearly demonstrated the potential of resolving and quantifying individual genomic segments as a measurement of total genomic concentration.

The principle of slab-gel electrophoresis separation is analogous to that used by capillary electrophoresis (CE). Specifically, the CE method relies on a separation technique in which charged molecules move through a narrow-bore fused-silica capillary containing a sieving solution in the presence of an electric field. Molecules associate with an intercalating dye in solution and are detected during separation through an aperature in the capillary. Compared to slab-gel electrophoresis, CE technology represents a potentially more precise and more sensitive analytical tool with which to detect and measure viral genomes/particles. Over the last decade many advances have been made in the separation of nucleic acids using CE in the DNA sequencing field. CE can offer faster and more efficient separations than slab-gel electrophoresis due to the use of higher electric fields that can be applied to capillaries [3,4]. Separation of nucleic acid molecules is achieved by injecting samples into a coated capillary that has been precharged with a running buffer composed of polymers and a background electrolyte. The polymers act as a molecular sieve in solution to separate nucleic acid fragments based on size. This mechanism of separation is related to classical Ogston sieving described for agarose slab-gel electrophoresis [5]. Sample detection is accomplished by the use of an intercalating dye (YOPRO-1) and laser-induced fluorescence (LIF). The limit of detection for nucleic acids has been reported to be as low as 10^{-12} M with the use of LIF detection [6]. Thus, based on the improved control over parameters affecting separation and detection, CE separation of nucleic acid fragments is faster, more precise, and potentially more efficient and reproducible than traditional slab-gel methods [7].

To develop a CE method, the sieving properties of the stationary phase need to be optimized to afford efficient separation. When using uncross-linked polymers, the formation and geometry of the network is dependent on the extent of interaction between individual polymers, and hence is a function of their size distribution, molecular mass and concentration. The point where polymers begin to interact and form a network in solution is referred to as the entanglement threshold [3]. The entanglement threshold is dependent on the molecular mass, hydrophilicity and polydispersity and can be determined experimentally [8]. Large polymers are able to interact with one another at lower concentrations and therefore have entanglement thresholds at lower concentrations compared to small polymers. An entanglement of high-molecular-mass polymers will create a network with large pores appropriate for separating larger fragments of RNA/DNA. Small molecules will move through such a network without much resistance and therefore will not be effectively separated, whereas, larger fragments can become entangled with polymers and must change their conformation in order to move through the dynamic pores in solution, resulting in size-dependent separation. These two separation regimes have been described as Ogston type sieving and reptation, respectively. Low-molecular-mass polymers, on the other hand, have been shown to induce separation of small molecules but only when used in high concentrations, hence, optimal separation is usually achieved when the contour length of the polymer is comparable to the contour length of the nucleic acid [9].

Separation conditions that utilize an entangled mesh of polymers to separate fragments are said to be operating under semi-dilute solution conditions. However, it has also been shown that nucleic acids can be separated in dilute and ultra-dilute solutions [10]. These solutions refer to concentrations below the entanglement threshold. Separation of this type does not use Ogston or reptation type sieving because a network has not been formed. Barron et al. [10] proposed that separations of this type were due to transient entanglements whereby polymers in solution interact with DNA fragments transiently to create a drag force. Larger fragments encounter more polymers and therefore migrate more slowly than smaller fragments. Due to the wide range of fragment sizes in the RV genome (650–3300 bp) the optimal separation technique or polymer size may not be the same for all segments. A screen of various polymer sizes and concentrations is therefore likely to be necessary in order to identify separation conditions for all segments of the RV genome.

This study focused on developing a CE method for separating and quantifying RV genome segments from samples of various process intermediates to guide development efforts. This investigation specifically examined HEC polymers due to their persistence length and extended conformation in solution when compared to other polymers, such as, polyethylene oxide or linear polyacrylamide. HEC polymers were screened to determine the optimal polymer size and concentration resulting in a robust and reproducible fragment resolution. While most of the HEC polymers separated RV fragments to some extent, results of this study show that one specific HEC polymer (HEC250) provided better control over the separation and peak resolution for the RV dsRNA genome. The polymer screen was followed by an optimization of various parameters impacting the separation, such as, injection time, electric field strength, dye concentration and capillary equilibration. The study indicates that these parameters are directly related to the efficiency and resolution of the RV fragments. By manipulating these conditions, the optimal resolution and separation efficiency was achieved for the RV genome. The optimized method was then used to quantitate genomic segments through the use of an external standard of known concentration.

2. Experimental

2.1. Instrumentation

All CE experiments were carried out using a P/ACE MDQ Molecular Characterization System (Beckman-Coulter, Fullerton, CA, USA) coupled to a LIF detector (Beckman). The capillaries employed were coated with polyacrylamide (Beckman) measuring 50 cm total in length (40 cm effective length) and had an internal diameter of 100 μm. To separate the negatively charged dsRNA fragments samples were run in reverse polarity and injected at the cathode using hydrodynamic injection at 0.5 psi (1 psi = 6894.76 Pa). All separations were performed at 25 °C and samples were stored at 25 °C between separations. Raw data were analyzed using the integration system of the 24 Karat software (Beckman) and then exported to Microsoft excel for further data analysis. Excitation and emission wavelengths were 488 and 508 nm, respectively.

2.2. Buffers and polymers

The following hydroxyethylcellulose (HEC) polymers were used in this study: number-average molecular mass (M_n) ≈ 90 000–105 000 and M_n ≈ 140 000–160 000 (Polysciences, Warrington, PA, USA), weight-average molecular mass (M_w) ≈ 250 000 (Sigma–Aldrich, St. Louis, MO, USA), M_v ≈ 300 000, M_v ≈ 720 000, and M_v ≈1 000 000 (donated by Aqualon, Wilmington, DE, USA). From this point forward the polymers will be referred to as HEC105, HEC160, HEC250, HEC300, HEC720 and HEC1000, respectively. All polymers were dissolved in a 1× TBE buffer (89 mM Tris, pH 8.3, 89 mM boric acid, and 2 mM EDTA) (Bio-Rad, Hercules, CA, USA) at specific percent w/v concentrations. Polymer-buffer solutions were mixed on a stir plate for 24 h, filtered through a 0.45 μm cellulose acetate disposable filter (Corning, Corning, NY, USA), and sonicated for 10 min. Just prior to CE analysis YOPRO-1 dye (Molecular Probes, Eugene, OR, USA) was added to the polymer–buffer solution at various concentration.

2.3. Viscosity measurements

Several concentrations of the HEC250 polymer were analyzed for their viscosity. All measurements were obtained at 25 °C using a Rheometric Scientific ARES LS instrument which measured steady shear viscosity at shear rates ranging from 50 to 500 s^{-1}. Measurements were used to generate a double log plot of polymer concentration versus viscosity to determine the entanglement threshold for the polymer of interest.

2.4. Sample preparation

Samples were treated with RNase-free, DNase I (Roche, Indianapolis, IN) to digest cellular DNA and then heat-treated at 70 °C for 15 min to inactivate the DNase, denature the protein capsid and release the 11-segment viral genome. A mass standard ladder (Bio-Rad) was used as a control in experimental testing of polymers. All DNA and RNA samples were diluted in molecular grade biology water within a range of 1–45 ng/μL.

2.5. Standard curve preparation

Rotavirus containing process material was purified by cesium chloride gradient method [11]. Samples were centrifuged with a 25% sucrose cushion in a Beckman Coulter Optima XL-100 K Ultracentrifuge using SW28 rotors at 4 °C and 122 000 × *g* centrifugal force. The supernatant was discarded, pellets were resuspended in TNC buffer [10 mM Tris, pH 7.4 (Life Technologies, Rockville, MD, USA), 14 mM NaCl, 10 mM $CaCl_2$ (Sigma–Aldrich)]. Cesium chloride (Sigma–Aldrich) was added at a concentration of 50% (w/v) and the solution was mixed. This solution was centrifuged, using ultra-clear centrifuge tubes, in SW60Ti rotors for 20 h at 4 °C and 156 000 × *g* centrifugal force. Tubes were carefully removed from the ultracentrifuge rotors so as not to disturb the viral particle layers. Tubes were punctured in the bottom and fractions of decreased density were collected by gravity flow in sterile microtubes. Double and triple layer particles (DLPs and TLPs, respectively) from the rotavirus samples were collected separately. This is achieved on the basis of the difference in density, 1.36 g/cm^3 for TLPs and 1.38 g/cm^3 for DLPs [12]. Viral particles were resuspended in TNC and added to SW28 ultra-clear ultracentrifuge tubes with 25% sucrose to act as a cushion. They were sedimented for approximately 2 h at 4 °C and 122 000 × *g* centrifugal force. The pellets were resuspended in TNC and were stored at 4 °C. To measure the viral genome concentration, the genomic dsRNA was extracted by phenol:chloroform extraction. Briefly, virus was digested using proteinase K (Invitrogen Life Technologies, Grand Island, NY, USA) in a solution of 10% sodium dodecyl sulfate (SDS), 0.5 M EDTA, and 10 mM Tris (pH 7.4) and then extracted in a 25:24:1 (w/v) mixture of phenol chloroform and isoamyl alcohol (Invitrogen Life Technologies). The aqueous phase was precipitated with ethanol (100%) (AAPER, Shelbyville, KY, USA) in the presence of 3 M sodium acetate, pH 5.2 (Sigma–Aldrich) and the genomic material was resuspended in Tris–EDTA (TE), pH 8.0, and its concentration measured by absorbance at 260 nm. The number of copies of genomes was calculated by using the spectrophotometric conversion for ds-DNA (50 μg/mL) and the total number of bases in rotavirus genomes.

2.6. Data analysis

All sample data were analyzed by comparing peak spacing (*X*), peak width at half height (w_h), limit of bp resolution (*S*) and overall resolution (*R*). Peak spacing was calculated

by taking the difference between two peak velocities and multiplying by the total separation time of the experiment ($\Delta X = \Delta vt$), where Δv is the relative velocity difference and t the total separation time of the experiment [13]. The limit of bp separation measures the minimum number of base pairs that can be resolved between any two given peaks. To determine this value, a separation factor was calculated for several resolved fragments to compare the limit of bp separation [$S = 1/2(w_1 + w_2)/(\Delta t_m/\Delta MW)$, where ΔMW is the difference in size in bp] [13,14]. Resolution is a measurement of the ratio between peak width and peak separation [$R = 2(t_2 - t_1)/(w_{h2} + w_{h1})$, where t is the migration time and w_h the peak width at half height]. When the resolution between two peaks is greater than 1, the peaks are considered to be fully resolved, with $R = 0.5$ being the limit of resolution between two adjacent peaks. Values were converted from temporal data to spatial data by multiplying by the velocity of the peak in question. Mobility (μ) values were calculated by dividing the velocity (cm/min) of a given molecule by the electric field strength (V/cm), [$\mu = (L_D/M_t)/(V/L_T)$], where L_D is the distance from injection to detector (cm), M_t the migration time (min), V the voltage (V), and L_T the total capillary length (cm) [15].

3. Results and discussion

3.1. Effect of HEC polymer size on RV fragment separation

In order to resolve the RV dsRNA fragments several types and sizes of polymers were evaluated. Some of the polymers commonly used in CE include linear polyacrylamide (LPA), polyethyl oxide (PEO), and HEC. The physical properties of these polymers have a significant impact on their ability to resolve nucleic acid fragments. PEO and LPA are both flexible polymers in solution and as such do not form the strong entanglements necessary to efficiently separate large fragments unless prepared at very high concentrations [3,16,17]. High polymer concentrations create highly viscous solutions that are more difficult to load into small diameter capillaries and are unable to resolve larger sized fragments due to small pores in the network. The stiff and extended conformation exhibited by HEC polymers in solution is believed to be important when separating larger fragments (>600 bp [8]). The ability of HEC polymers to provide better resolution of fragments greater than 600 bp was an important factor when considering polymers to separate the RV fragments which fall in the range of 667–3302 bp.

Prior to the use of HEC polymers, experiments were carried out using LPA and PEO. LPA and PEO were unable to separate the RV fragments with the same degree of resolution as the HEC polymers (data not shown) and confirmed findings of published literature reports [8,16,18,19]. The sizes of the HEC polymers tested in this study were chosen based on their contour length in relation to the contour length of the RV dsRNA fragments. It has been shown that larger polymers are better suited to entangle with and separate larger fragments while low-molecular-mass polymers can separate smaller fragments when used at a high concentration [9]. HEC-buffer concentrations targeted for evaluation were determined for each size of HEC polymer based on entanglement thresholds previously determined by Barron et al [9]. The entanglement threshold for the HEC250 polymer has not been previously reported in the literature and was determined experimentally from viscosity measurements at various concentrations. In the case of the HEC300 polymer for which the entanglement threshold was also unknown from literature review, dilutions were carried out at a wider range in an attempt to encompass the threshold concentration. In this case the entanglement threshold was not experimentally determined because initial results suggested this polymer to be suboptimal.

The various HEC polymers were assessed at different concentrations for their ability to resolve the 11 segments of the RV genome. Most polymer solutions consistently resolved 8 of the 11 RV genome segments and in some cases could resolve 9–10 segments as the polymer concentration was increased. The resolving power of the sieving polymers was compared on the basis of peak width and spacing, limit of bp separation, and overall resolution (Fig. 1). Results indicated that peak width was marginally affected by polymer size or polymer concentration. Peak spacing measurements showed that smaller fragments are most sensitive to a change in polymer concentration; however, the only polymer that produced any significant change in peak spacing was the HEC250 polymer (Fig. 1A). HEC250 exhibited a significant increase in peak separation as the polymer concentration was increased. Concomitantly, the limit of bp separation showed a steady decreasing trend with increasing polymer concentration, indicating that fragment separation was more efficient at the higher concentrations (Fig. 1B). Overall resolution exhibited an increasing trend with the increasing HEC250 concentration up to 1.3% after which point it slightly decreases (Fig. 1C). It is noteworthy that the other HEC polymers did not show a steep increase in resolution as a function of concentration (Fig. 1C). This HEC polymer screen indicated that the HEC250 polymer has the most potential to impact the separation of the RV fragments and that it yields the highest resolution of separation at a concentration of 1.3% (w/v). Qualitative analysis of representative electropherograms indicated that this concentration yielded the most optimal and consistent separation conditions for the RV dsRNA fragment (Fig. 2). It is noteworthy that peak 5 consistently resolved with high separation from other peaks over several polymer concentrations. HEC720 and HEC1000 polymers were not pursued in the polymer screen due to poor resolution that was based on the visual inspection of the electropherograms (data not shown). Based on these results all further experiments were conducted using the HEC250 polymer at the 1.3% (w/v) concentration.

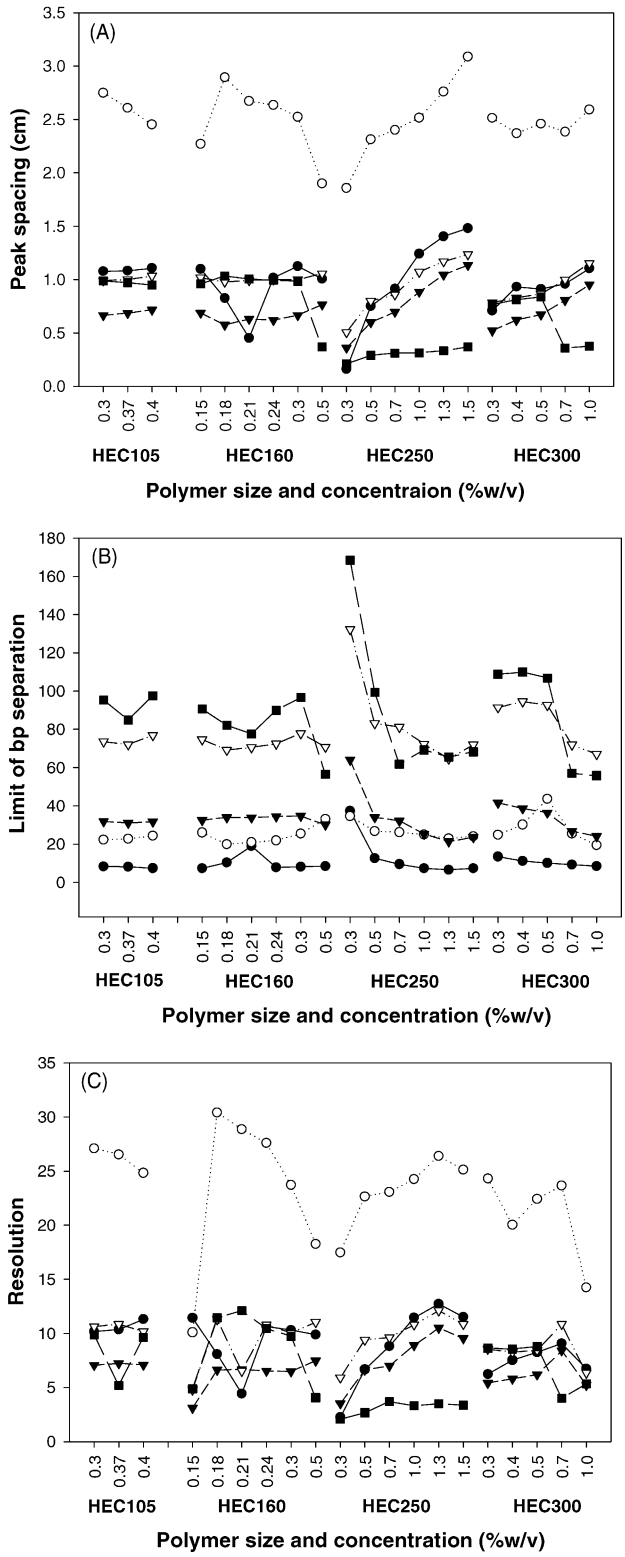

Fig. 1. Results of the HEC polymer screen on separation of RV genomic fragments. (A) Peak spacing, (B) limit of bp separation, and (C) resolution dependence on polymer molecular mass and concentration. Fragment sizes shown are (●) 667 bp, (◯) 751 bp, (▼) 1356 bp, (▽) 1581 bp, (■) 2362 bp.

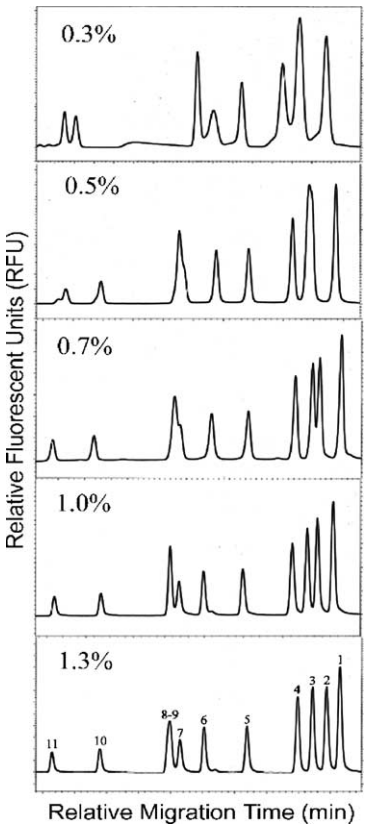

Fig. 2. Visual appearance of the electropherograms for HEC250 polymers and dependence on concentration.

3.2. Determination of the entanglement threshold and implications on dsRNA migration mechanism

In order to experimentally determine the entanglement threshold of the HEC250, its viscosity at various concentrations was determined using shear rates ranging from 50 to 500 s^{-1}. Polymer solutions behave as Newtonian fluids at low concentrations and low shear rates while non-Newtonian, shear-thinning behavior is seen at higher polymer concentrations with increased shear rates. As the polymer concentration increases a network begins to form between polymers; when the shear force is increased the polymer network breaks and viscosity is reduced [20]. By plotting the logarithm of specific viscosity (η_{sp}) against logarithm of HEC polymer concentration (%, w/v) the entanglement threshold for HEC250 can be determined. A deviation in linearity denotes the point where polymers begin to form a network in solution and is indicative of the entanglement threshold (Fig. 3) [8]. These results demonstrated that the threshold concentration of the HEC250 polymer is approximately 1.0%, and hence very close to the experimentally determined optimal HEC250 polymer concentration of 1.3% chosen for detailed analysis.

The entanglement threshold is the point where pores are formed in the mesh of polymers in solution which enables

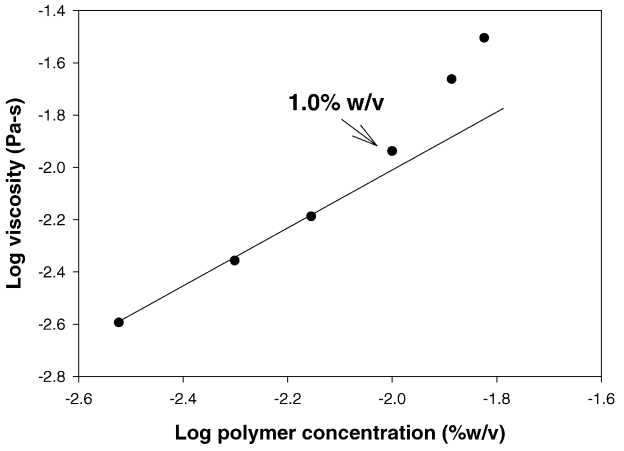

Fig. 3. Determination of entanglement threshold of HEC250 polymers. Log–log plot of viscosity and polymer concentration allows the determination of the entanglement threshold at the point of deviation in linearity (arrow).

separation of fragments to take place via a sieving mechanism. In the case of smaller fragments separation follows an Ogston type sieving regime where fragments migrate through pores in random coil formation. The pore sizes are large enough that RV fragments can pass through them without altering their shape. The reptation migrating regime causes larger fragments to orient themselves and migrate in an extended, rod-like form through dynamic pores in the network. The difference in migration regimes can be visualized by a plot of log mobility verses the fragment size in base pairs (Fig. 4). The RV fragments are compared to a wide range of DNA fragments to clearly identify sieving regimes. The steep negative slope refers to Ogston sieving and the deviation from linearity represents the change to the reptation regime. These results suggest that the smaller RV fragments (667–751 bp) migrate as random coils and that larger fragments (>2500 bp)

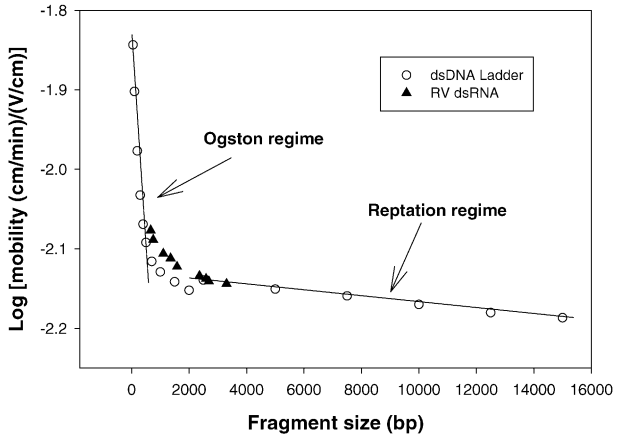

Fig. 4. Mobility behavior of RV dsRNA genome fragments. Plot of the log of mobility against the fragment size in bp shows the separation regimes existing at 1.3% (w/v) polymer solution. RV fragments are compared to an extended range of dsDNA fragments to indicate the separation mechanism at work.

follow the reptation model. Fragments >1000 and <2500 migrate in the transition region and hence are separated by a combination of these two mechanisms.

3.3. Effect of polymer concentration (viscosity) on signal

The cost of the improved resolution at 1.3% HEC250 was the loss of signal as the polymer concentration was found to be inversely proportional to the measured peak area for a representative peak (Fig. 2, peak 5). This effect was hypothesized to be a direct result of increased specific viscosity due to higher polymer concentrations in the buffer while the injection pressure and time remained constant. A calculation of injection volume ($V_i = \Delta P D^4 \pi / 128 \eta L$, where ΔP is the pressure drop, D the capillary internal diameter, η the specific viscosity and L the total length of capillary [6]) revealed that there was a decrease in hydrodynamic capillary loading due to increased viscosity (Fig. 5A). To compensate the injection time was increased by 2 s intervals until further improvements in the resolution were no longer observed. With the exception of 751 bp fragment, peak width remains constant with respect to injection time, until an 11 s injection after which peak broadening occurs (Fig. 5B). These plots show that the signal can be increased by increasing the injection time up to 11 s, although beyond this the increased injection time was found to be detrimental to the resolution because of increased sample diffusion.

3.4. Effect of electric field strength during separation

Having optimized the separation buffer, the next step was to evaluate and optimize the operating parameters using 1.3% HEC250 polymer and an 11 s injection time. Electric field strength, dye concentration and capillary rinsing were examined to find the most optimal conditions for separation of the RV genome. Intense electric fields can be employed in capillary electrophoresis due to the efficient dispersion of heat through small diameter capillaries across the large surface area of the outer wall. Capillaries are generally air or water cooled to effectively remove heat. Higher electric field strengths have the advantage of decreasing diffusion of fragments due to shorter separation times. However, above a certain voltage, heat will not be dissipated as efficiently, resulting in peak broadening. The dispersion of heat is linked to the capillary diameter, buffer concentration and the voltage. To determine the heat dispersion performance of a system the voltage can be plotted versus the current to create an Ohm's law plot [6]. The point where the measured curve deviates from linearity indicates the voltage above which heat is no longer efficiently dissipated. The current was measured at varying voltage points to generate an Ohm's law plot for the 1.3% HEC250 polymer-buffer (Fig. 6A). The plot indicated a slight deviation from linearity after 16 kV (320 V/cm). Therefore, experiments were performed to evaluate electric fields ranging from 200 to 320 V/cm. A slight decrease in peak width was observed with increasing applied voltage

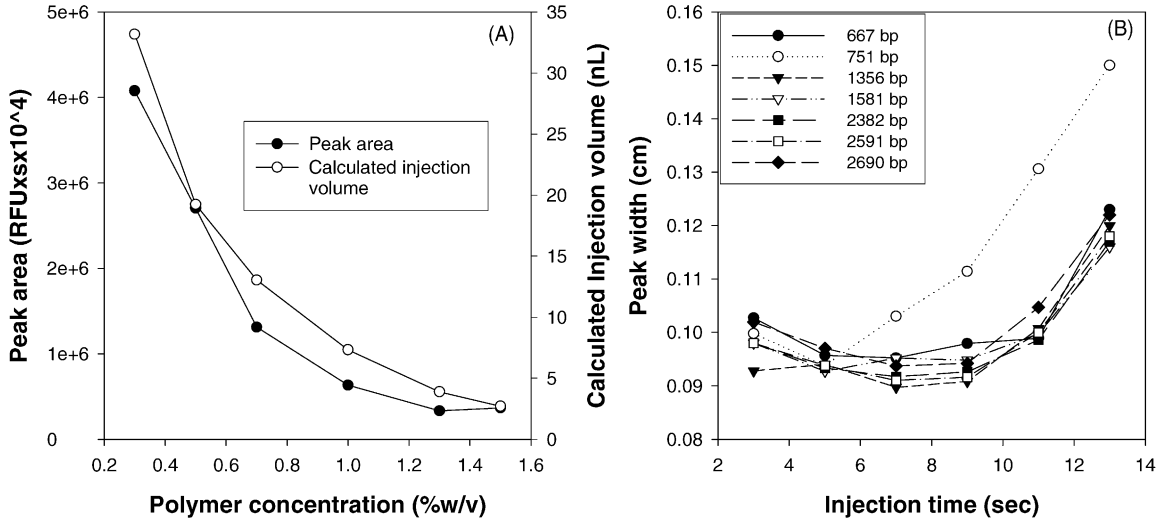

Fig. 5. Effect of polymer concentration on signal intensity. (A) A decrease in peak area is shown to correlate to the increase in viscosity for higher polymer concentrations. Injected volume was calculated using 5 s injections, 0.5 psi injection pressure, 100 μm i.d. capillary, 50 cm total capillary length and measured viscosities for each concentration of HEC250 polymer solution. (B) Peak broadening is a result of diffusion caused by the increased injection time. Diffusion is shown to have a marked increase beyond 11 s injections.

from 220 to 260 V/cm after which point most peaks began to broaden slightly (Fig. 6B). Peak spacing steadily decreased with a higher electric field as expected, but electric fields as high as 280 V/cm do not compromise resolution significantly (Fig. 6B). The optimal electric field was determined to exist over a range from 200 to 260 V/cm. However, if a shorter turn-around-time (TOT) is desired, samples can be run at 280 V/cm without significant loss in resolution while saving 33% in analysis time.

3.5. YOPRO-1 dye concentration optimization

The YOPRO-1 intercalating dye was chosen over other dye candidates due to appropriate spectral matching with the P/ACE MDQ instrument, high binding affinity for double-stranded nucleic acids, and low quantum yield of fluorescence in the unbound state [21]. The dye was added to the separation buffer just prior to charging the capillary for on-capillary intercalation. During electrophoresis the positively

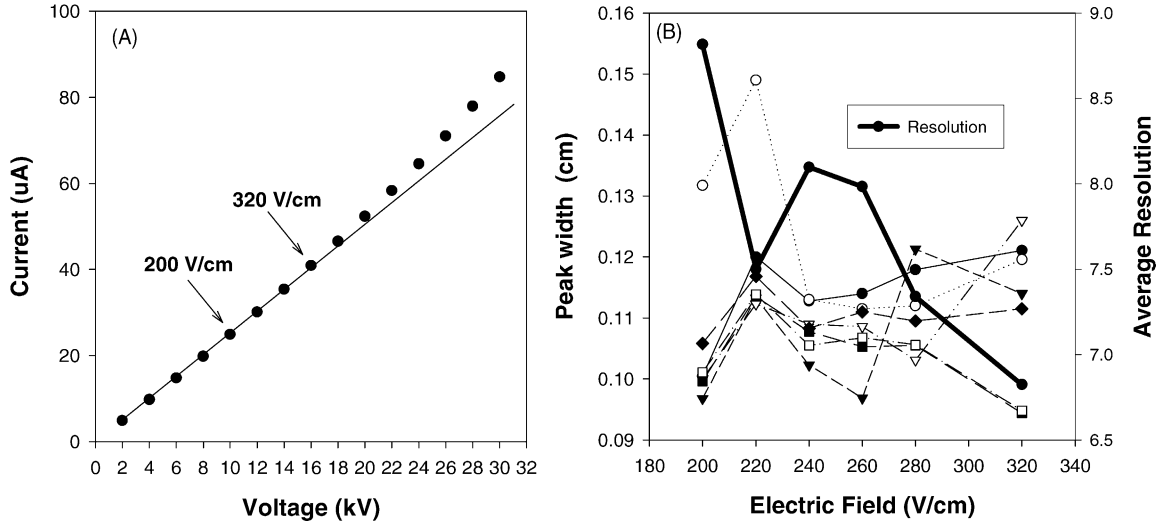

Fig. 6. Effect of electric field on RV dsRNA. (A) Plot of current as a function of the applied voltage shows linearity until the system no longer efficiently dissipates the heat generated in the capillary, resulting in a drop of resistivity. The Ohm's law plot indicates that 320 V/cm is the upper operational limit for a 1.3% HEC250 buffer solution. (B) Plot of peak width for several RV fragments as a function of voltage indicates that generated heat causes increased diffusion of the fragments above 280 V/cm. Overall resolution is highest at 200 V/cm. As the electric field is increased, the resolution decreases due in part to the loss in peak separation. In contrast, peak broadening is not observed below 280 V/cm. Fragment sizes shown are (●) 667 bp, (◯) 751 bp, (▼) 1356 bp, (▽) 1581 bp, (■) 2362 bp, (□) 2591 bp, (◊) 2690 bp.

charged dye molecules will migrate toward the cathode while the dsRNA fragments migrate toward the anode. As YOPRO-1 migrates it will bind to the dsRNA by intercalation at a constant dye/bp ratio. This results in a partial unwinding of the helix and a stiffening of the RNA molecule. The binding of positively charged dye molecules causes an overall decrease in the negative charge of the RNA and results in an increase in migration time. These alterations can change the way the fragments migrate and generally tend to improve resolution by stabilizing the double stranded fragments [22].

Several concentrations of YOPRO-1 were tested in an attempt to optimize the dye:bp ratio for the RV genome. Peak area and migration time were both found to increase with an increase in dye concentration and then progressively plateau (Fig. 7A). However, peak width began to increase beyond 500 nM and overall resolution was shown to decrease concurrently (Fig. 7B), suggesting an increase in diffusion. As such, a dye concentration of 500 nM was determined to be the optimal concentration for use with the 1.3% HEC250 polymer for optimal RV separation.

There may be a point at which too much dye in the buffer causes other dye binding interactions to take place, such as, electrostatic or groove binding [23,24]. These interactions can cause an uneven distribution of dye among fragments of the same molecular mass and result in peak broadening. Lower amounts of dye added to the separation buffer cause baseline instability and may result in premature buffer depletion of dye molecules.

3.6. Methanol rinsing and conditioning of the capillary

During experiments with dye-free buffer, it was determined that the capillary became contaminated with intercalation dye after multiple dye-containing sample runs. Various capillary rinsing methods were investigated, such as, water, buffer and methanol rinses. The results indicated that water rinsing alone was not sufficient to remove the dye from the capillary. Methanol rinsing achieved the greatest decrease in dye contamination when compared to water or buffer rinses and has been reported as an effective rinsing method by others [21]. Methanol rinsing has also been shown to be an effective capillary conditioning procedure to reduce wall effects, which can cause peak tailing and loss of resolution [25,26]. It was determined that a daily capillary rinse with methanol followed by dye-free buffer combined with inter-sample run rinses of methanol and dye-buffer could reduce the amount of excess dye in the capillary as well as increase run to run reproducibility and extend the life of the capillary.

3.7. Quantitation of RV samples

An external standard curve was prepared from purified RV material for which the dsRNA concentration had been quantified by spectrophotometry. From the previous method development optimizations it was determined that peak 5 consistently resolved as a single peak regardless of polymer concentration or parameter optimizations and is therefore likely the most robust peak to use for quantification purposes. This material was prepared in six concentrations and then resolved using the same buffer as RV samples to be quantified. Peak areas were measured and a standard curve was generated by plotting peak area against known genome concentration (Fig. 8). Measured sample peak areas were used to interpolate genome titers from the standard curve (data not shown). The reproducibility of the area of peak 5 where $n = 7$ runs gave a relative standard deviation (R.S.D.) of 13%. Preliminary results have shown that DNase-treated RV samples can be quantified without lengthy pre-treatments that can result in loss of genome.

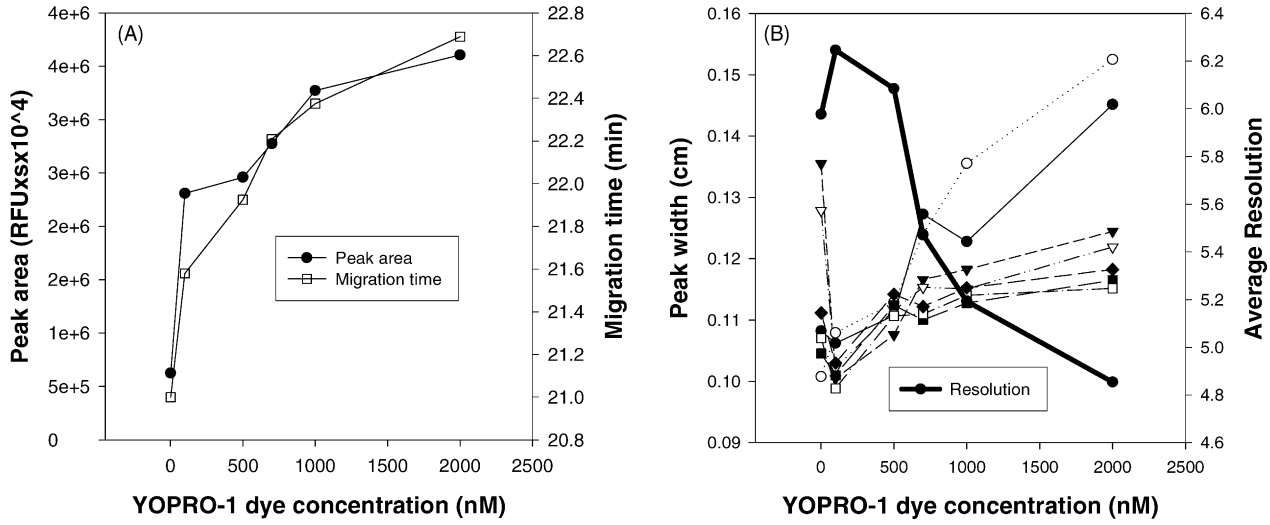

Fig. 7. YOPRO-1 dye concentration optimization. Dependence of (A) peak area, migration time and (B) peak width and average resolution on concentration of YOPRO-1 dye. Fragment sizes shown are (●) 667 bp, (◯) 751 bp, (▼) 1356 bp, (▽) 1581 bp, (■) 2362 bp, (□) 2591 bp, (◊) 2690 bp.

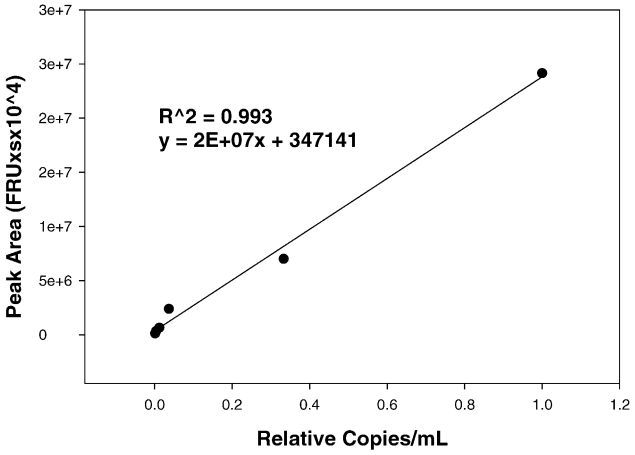

Fig. 8. A six-point standard curve constructed from the peak area (peak 5) of purified dsRNA RV genome material of known concentration.

4. Conclusions

This study has demonstrated that the dsRNA of rotavirus samples can be analyzed and quantified using capillary gel electrophoresis in the presence of HEC polymers with minimal sample preparation. Based on a comprehensive literature search, this appears to be the first report of the development of a successful capillary gel electrophoresis-based separation technique for double stranded nucleic acid without significant sample purification which holds promise for quantitative measurement.

The HEC polymers were chosen over other alternatives based on their persistence length, extended conformation and extensive use in the separation of nucleic acids. The most optimal sized polymer was found to be HEC250, as it offered the greatest control over the resolution of the RV dsRNA fragments when compared with other HEC polymers. Classical method parameters such as the concentration of polymers, the injection time, the electric field strength and the concentration of intercalating dye have subsequently been optimized with the 1.3% HEC250 polymer solution identified as optimal in this study, and have resulted in a separation method that successfully resolves 10 out of the 11 RV genomic dsRNA fragments reproducibly.

During the development of this method, the entanglement threshold for HEC250 was experimentally determined and found to be in close agreement with the polymer concentration at which separation was found to be optimal. This finding provides support to the model of polymer entanglement and pore formation as being the basis for size separation of nucleic acid molecules. In this context, the electrophoretic behavior of RV segments, with smaller fragments being separated solely by sieving and larger fragments by reptation, suggests that the separation conditions are close to optimal considering the wide range of molecular sizes encompassed by the RV genome.

As reported by McCord et al. [26] previously, we have observed a tendency for the capillary to become contaminated with dye and have implemented a comparable methanol rinsing strategy in order to obtain sample to sample reproducibility and avoid dye carry-over.

This separation procedure was applied to RV samples using an external standard curve which showed good linearity down to the picomolar range and holds promise as a fast and efficient quantification method for RV genomes in non-purified samples. This CE method offers a rapid and convenient means to qualitatively and quantitatively detect RV genomes in complex samples. The fact that no lengthy sample preparation is required and that the method is amenable to automation is a clear advantage over other mass assays involving quantitative PCR, HPLC or spectrophotometric procedures.

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