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Short communication

A single molecule detection method for understanding mechanisms of electric field-mediated interstitial transport of genes

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Abstract

The interstitial space is a rate limiting physiological barrier to non-viral gene delivery. External pulsed electric fields have been proposed to increase DNA transport in the interstitium, thereby improving non-viral gene delivery. In order to characterize and improve the interstitial transport, we developed a reproducible single molecule detection method to observe the electromobility of DNA in a range of pulsed, high field strength electric fields typically used during electric field-mediated gene delivery. Using agarose gel as an interstitium phantom, we investigated the dependence of DNA electromobility on field magnitude, pulse duration, pulse interval, and pore size in the interstitial space. We observed that the characteristic electromobility behavior, exhibited under most pulsing conditions, consisted of three distinct phases: stretching, reptation, and relaxation. Electromobility depended strongly on the field magnitude, pulse duration, and pulse interval of the applied pulse sequences, as well as the pore size of the fibrous matrix through which the DNA migrated. Our data also suggest the existence of a minimum pulse amplitude required to initiate electrophoretic transport. These results are useful for understanding the mechanisms of DNA electromobility and improving interstitial transport of genes during electric field-mediated gene delivery.

Keywords: Single molecule detection; DNA electromobility; Interstitial transport; Electric field-mediated gene delivery; Non-viral gene delivery

1. Introduction

The efficacy of non-viral gene therapy in the treatment of solid tumor is limited by poor gene delivery from the extracellular domain into the nucleus of cells since the delivery has to overcome various physiological barriers, including interstitial structures, cell membranes, cytoskeleton, and nuclear envelope, which are tissue- and cell-dependent [1–3]. DNA diffusion in tumor interstitium is negligible as indicated by the small diffusion coefficient ($<10^{-9}$ cm^2 s^{-1}) [2,4]. Convective transport, due to a uniformly elevated interstitial pressure, is also negligible everywhere in solid tumors except at the tumor periphery [1]. Furthermore, the plasma membrane and nuclear envelope are impermeable to naked DNA during passive diffusion. Therefore, non-viral gene transfer is inefficient without development of novel strategies [5–9].

Local application of pulsed electric fields is one the strategies that has been shown to improve delivery of exogenous genes into cells both in vitro [10–12] and in vivo [7–9,13–19]. The improvement occurs through two potential mechanisms: electroporation and electrophoresis [20,21]. These mechanisms, especially electroporation, have been studied extensively in vitro [22–28], in which electric pulses create transient pores in the plasma membrane that allow transport of traditionally non-permeant molecules into cells via both diffusion and electrophoresis [29,30]. However, DNA administered into tissues can be successfully delivered into cells only if these molecules are located within a critical distance from the plasma membrane during electroporation. Beyond this distance, the DNA molecules cannot reach the plasma membrane before the pores created by electroporation are closed. This critical distance depends on the rate of interstitial and transmembrane

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transport. Therefore, any improvement in the transport may significantly increase the extracellular domain within which DNA molecules are eligible for cellular uptake during electroporation.

Both interstitial and transmembrane transport can be improved through electrophoresis [2,31]. A recent report by Satkauskas et al. has demonstrated that following a prerequisite cell permeabilizing pulse, electrophoretic pulses play an important role in determining the efficiency of in vivo gene transfer [32]. The ability of an applied electric field to enhance interstitial transport of DNA has also been investigated in excised tumor tissue [2] and agarose gel acting as a tissue phantom [31]. These studies have uncovered surprising relationships between applied pulsing parameters and resulting gene transport behaviors, and led to questions concerning the underlying mechanisms of transport.

The objective of this study was to develop a reproducible single molecule method for understanding mechanisms of electric field-mediated extracellular transport of DNA. The rationale of the study was that DNA–DNA interactions were negligible during interstitial transport since DNA concentration in tissues was low in most gene delivery studies. As a result, the experimental results were determined mainly by transport behaviors of single DNA molecules. Using this method, we investigated the dependence of DNA transport on pulse magnitude, pulse duration, pulse interval, and pore size in agarose gels exposed to pulsed electric fields.

2. Materials and methods

A small-scale electrophoresis chamber was constructed on a glass microslide (VWR, West Chester, PA, USA) to allow continuous observation of single fluorescently labeled DNA molecules undergoing electrophoretic transport in agarose gel (Fig. 1). Platinum wire electrodes (A-M Systems, Inc., Carlsborg, WA, USA), inlaid along opposite walls of the electrophoresis chamber, were used to deliver the electric field. The electrodes were of sufficient diameter (0.127 mm) to span nearly the entire depth of the chamber.

All experiments were performed using bacteriophage T2 DNA (Sigma, St. Louis, MO, USA) stained with YOYO-1 intercalating dye (Molecular Probes, Eugene, OR, USA) at a 1:10 dye to base pair ratio. Solutions of 1.0, 2.0, 3.0, or 4.0% w/v low gelling temperature agarose (Sigma) were prepared in the slightly heated 1× Tris–Acetate–EDTA (TAE) buffer, allowed to cool for 2 min, then mixed with an equal volume of YOYO-1 labeled T2 DNA in 1× TAE containing 6% v/v β-mercaptoethanol. Approximately 50 μl of the mixture was pipetted into the electrophoresis chamber mentioned above. The chamber was then sealed with a glass microslide and the solution formed the gel in 15 min. The final sample medium contained 0.5, 1.0, 1.5, or 2.0% w/v agarose, 3% v/v β-mercaptoethanol, and approximately 3×10^{-6} μg/μl YOYO-1 labeled T2 DNA.

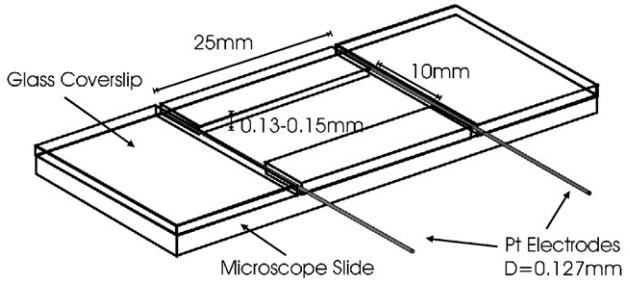

Fig. 1. Schematic of the small-scale electrophoresis device developed for the observation of single DNA molecules undergoing electrophoretic transport under pulsed electric fields.

Images of YOYO-1 labeled DNA during pulsed electric field application were acquired using a 100× oil immersion objective on an inverted microscope (Axiovert 100 TV, Zeiss, Thornwood, NY, USA), and captured with an intensified CCD camera (DAGE-MTI, Inc., Michigan City, IN, USA) connected to a videocassette recorder. The electric field was supplied by an ECM 830 electro square porator (BTX, San Diego, CA, USA). The resulting videos were digitized and analyzed using image analysis software (Image-Pro Plus®, Media Cybernetics, Inc., Silver Spring, MD, USA). Agarose gel deformation due to pulse delivery was negligible as determined by preliminary experiments using 1.0-μm-diameter yellow-green latex microspheres (Polysciences, Inc., Warrington, PA, USA) as a gel marker (data not shown).

DNA movement was quantified and reported in terms of the net DNA displacement per pulse, and the DNA electromobility. Total DNA displacement, d, was defined as the distance between the location of the midpoint of a DNA molecule before application of a 10-pulse train and the location of the midpoint of the same molecule 10 s following the completion of the 10-pulse train. The DNA displacement per pulse, d_p, was calculated by,

$$d_p = \frac{d}{N} \tag{1}$$

where N is the number of pulses in the applied pulse train. The DNA electromobility, μ, is given by,

$$\mu = \frac{v}{E} \tag{2}$$

where v is the magnitude of the velocity vector of the observed molecule, and E is the magnitude of the applied electric field. In this report, v was obtained by,

$$v = \frac{d_p}{t_p} \tag{3}$$

where t_p is the duration of the pulse.

The average pore size in agarose gel has been characterized previously by the radius, r_p, of the largest latex spheres that can enter the gel [33]. The value of r_p is empirically related to the concentration of agarose, ϕ, in the gel through Eq. (4),

$$r_p = 118\cdot\phi^{-0.74} \quad \text{(nm)} \tag{4}$$

in which the unit of r_p is nanometer (nm) and the unit of ϕ is percent weight by volume (% w/v) that is calculated as

100 times the mass of agarose (g) divided by the volume of gel (cm^3). Eq. (4) is valid for 0.2% w/v $\leq \phi \leq$ 4.0% w/v.

3. Results and discussion

The characteristic electromobility behavior, observed under the majority of pulsing conditions investigated in this study, included distinct temporal phases of stretching, reptation, and relaxation (Fig. 2). Relaxed T2 DNA has a radius of gyration of approximately 1.2 μm [34], which is larger than the predicted pore radius of agarose gel (71, 87, 118 and 197 nm for 2.0, 1.5, 1.0 and 0.5% w/v agarose gels, respectively) using Eq. (4). Prior to pulse application, a DNA molecule often spanned multiple pores, occupying the full volume of each pore in segments of spherical conformation, joined through inter-pore gaps by stretched, linear segments. The initial pulses in an applied pulse train acted to stretch the DNA in the direction of the applied electric field, which is consistent with previous reports showing the orientation of large DNA molecules in the direction of the applied field [35]. The stretching phase could be characterized as either single or double end elongation. Double end elongation resulted from the DNA strand hooking around an agarose fiber. The reptation phase of electromobility started once the strand had been stretched sufficiently to pass through pores in the agarose gel. In instances of double end elongation, the lagging end of the DNA strand was pulled around the hooking agarose fiber, and then followed the path of the leading end.

The first two stages of electromobility (stretching and reptation) were distinguishable by plots of the DNA displacement

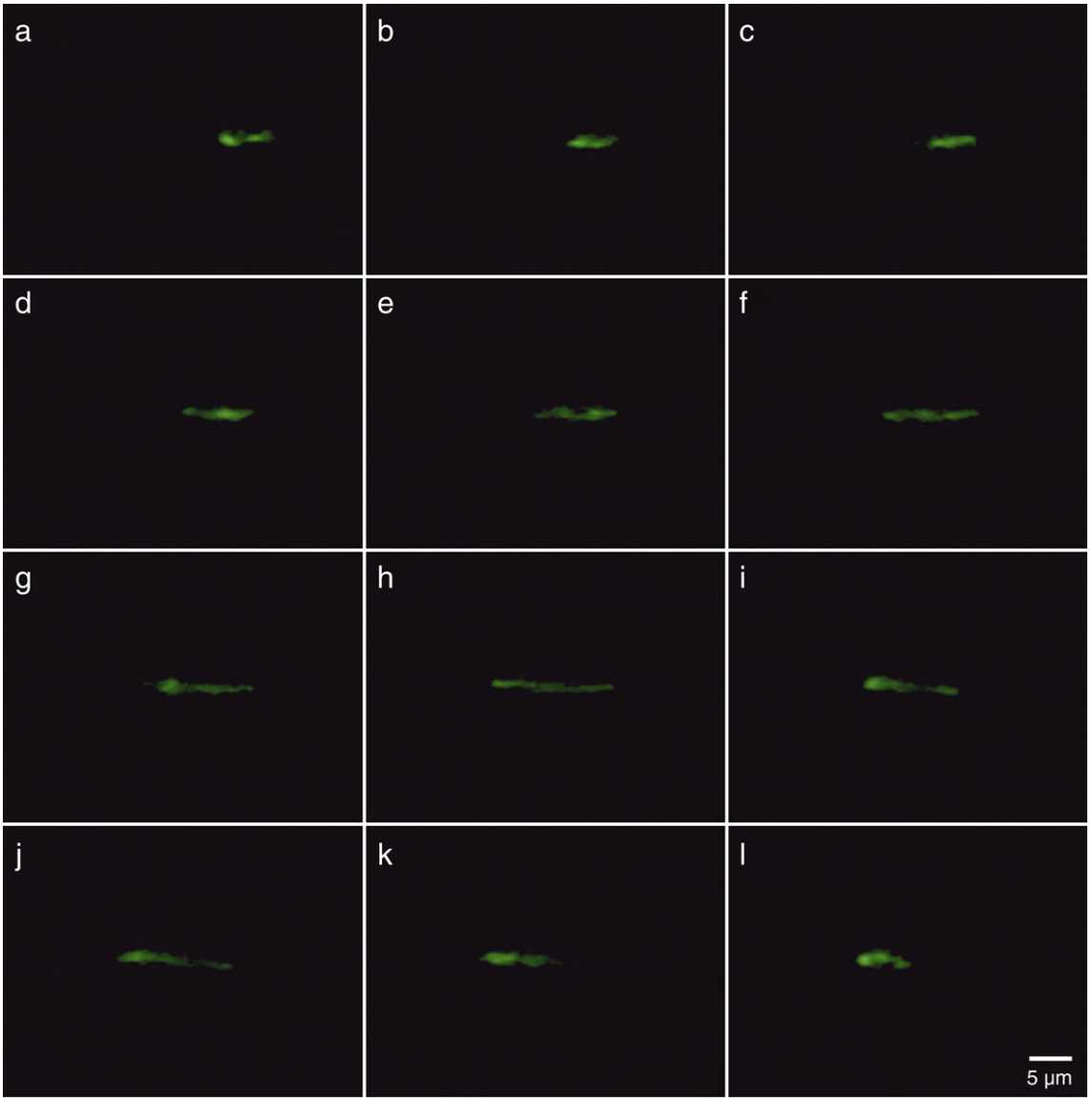

Fig. 2. Characteristic electromobility behavior of a single T2 DNA molecule in the presence of a pulsed electric field showing distinct temporal phases of stretching, reptation, and relaxation. A pulse train consisting of 10 pulses with field strength of 100 V/cm, duration of 30 ms, and pulse interval of 1 s, was applied. Still images of the resulting DNA behavior were captured prior to the application of the pulse sequence (a), immediately following each of the 10 pulses (b–k), and 10 s following the completion of the pulse sequence (l).

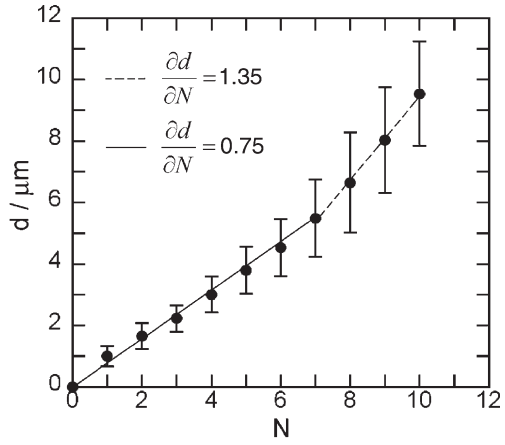

Fig. 3. DNA migration distance, d, as a function of pulse number, N, for DNA molecules in 1.0% w/v agarose gel during the application of a sequence of 10 pulses with field strength of 100 V/cm, duration of 30 ms, and pulse interval of 1 s (n=20). The step increase in slope at N=7 is indicative of the transition from the stretching to the reptation phase of mobility.

as a function of pulse number (Fig. 3). Linear regression analysis revealed a step increase in slope that occurred at the transition from the stretching to the reptation phase of mobility. The relaxation phase of mobility occurred following the end of the pulse train as the DNA strand relaxed into its globular form. Instances in which this characteristic behavior was not observed included experimental runs with a pulsing sequence of insufficient amplitude to initiate electromobility. These cases will be discussed in more detail below.

The qualitative behavior of DNA observed in this study in pulsed, high field strength electric fields was similar to that reported previously for DNA in constant low voltage electric fields [36] with one significant exception. This exception is related to the relaxation behavior of the DNA between sequential pulses in a pulse train. We investigated the dependence of DNA electromobility on pulse interval (Fig. 4). The sharp decrease in electromobility with increasing pulse interval was likely to be due to partial DNA relaxation between pulses. The longer the pulse interval, the more relaxed the DNA became, and the more energy of the following pulse required to re-stretch the DNA before it could begin to reptate again through the fibrous matrix. Decreasing the pulse interval will decrease the extent of relaxation between pulses, which would increase the effectiveness of the electric field to push the DNA through the interstitium. However, short pulse intervals in sequences of high magnitude pulses, such as those required for electroporation, may be less tolerated by the treated cells. This mechanism suggested the importance of considering this pulsing parameter when developing pulse sequences for electric field-mediated gene delivery applications.

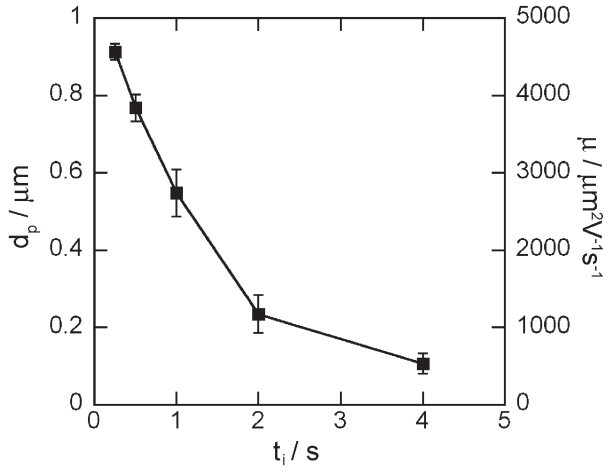

Fig. 4. DNA migration distance per pulse, d_p, and electromobility, μ, dependence on pulse interval, t_i, in 1.0% w/v agarose gel. The migration distance and electromobility decreased with increasing pulse interval. Each pulsing sequence consisted of 10 pulses with field strength of 100 V/cm and duration of 20 ms.

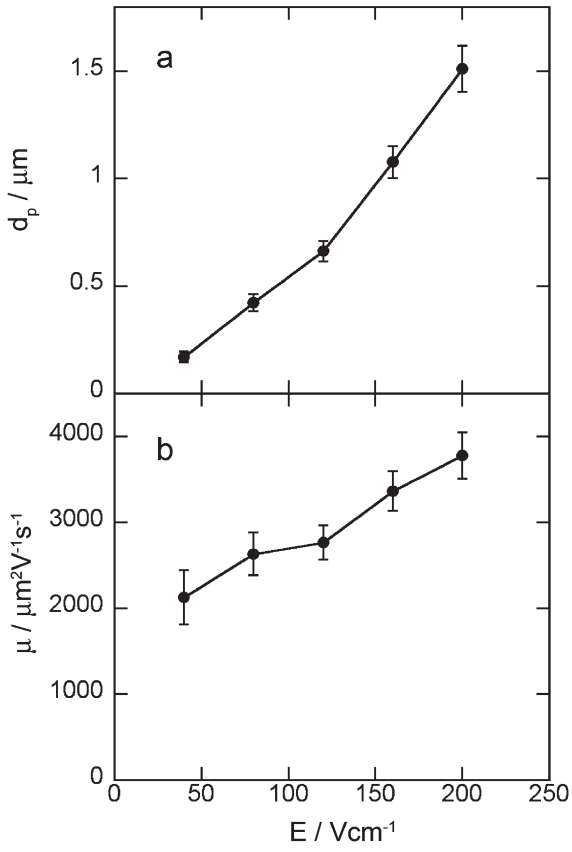

Fig. 5. DNA (a) electromigration distance per pulse, d_p, and (b) electromobility, μ, dependence on the magnitude of the applied electric field, E, in 1.0% w/v agarose gel. Both the migration distance and the electromobility increased with increasing field strength. Each pulsing sequence consisted of 10 pulses with duration of 20 ms and pulse interval of 1 s.

The net displacement of DNA has been shown previously in our lab to increase with the strength and duration of the applied electric field in both agarose gel [31] and excised tumor tissue [2]. We further confirmed these results here by demonstrating an increase in net DNA displacement and electromobility per pulse when the pulse strength (Fig. 5) and pulse duration (Fig. 6) were increased in 1.0% w/v gel. Furthermore, the net DNA displacement per pulse and DNA electromobility values reported here for single DNA molecules are consistent with those reported previously for DNA clouds in agarose gel [31].

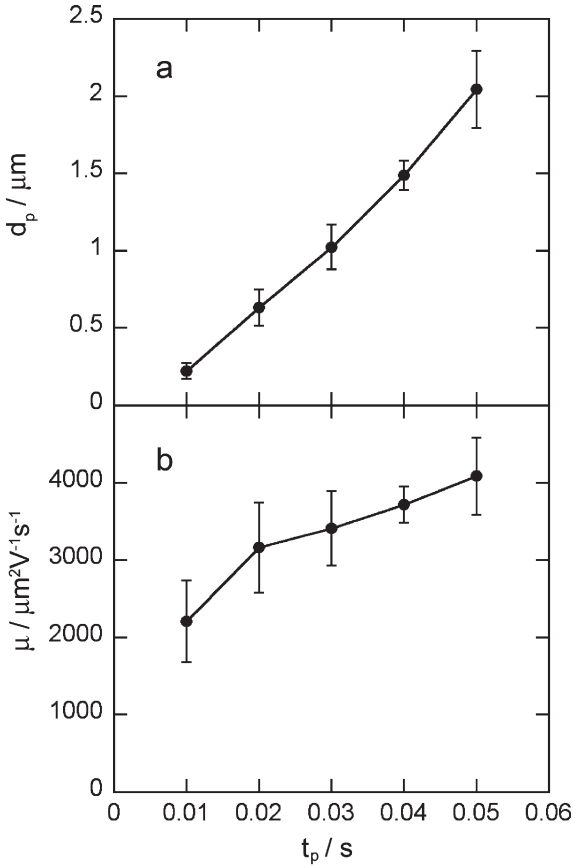

Fig. 6. DNA (a) electromigration distance per pulse, d_p, and (b) electromobility, μ, dependence on pulse duration, t_p, in 1.0% w/v agarose. The migration distance and electromobility increased with increasing pulse duration. Each pulsing sequence consisted of 10 pulses with field strength of 100 V/cm and pulse interval of 1 s.

DNA electromobility depended strongly on the pore size of the matrix through which it migrated (Fig. 7). The smaller the pore size with respect to the radius of the DNA molecule, the more the molecule had to be stretched before it was able to pass through the pore. The entropy barrier associated with this stretching had to be overcome by the applied electric field first, before energy from the applied field was available to push the DNA through the matrix. When the magnitude of the electric field was insufficient to overcome the entropy barrier, no electrophoretic transport occurred (Fig. 8). In these instances, the DNA molecule was seen to be stretched to an extent, but the transition from the stretching phase to the reptation phase of mobility never occurred. At the end of the pulse sequence, the DNA molecule relaxed back to its original position. This finding delineates the importance of recognizing and accounting for a minimum pulse amplitude when designing pulse parameters for electric field-mediated gene delivery applications. It is possible that, while keeping the pulse amplitude constant, the entropy barrier could be overcome by increasing pulse duration. This trend is likely valid until the electrophoretic force is less than the force required to deform the DNA molecule to a certain extent, at which point the applied field would no longer be able to push the DNA through the fiber matrix in tissues or gels, regardless of the duration of the pulse.

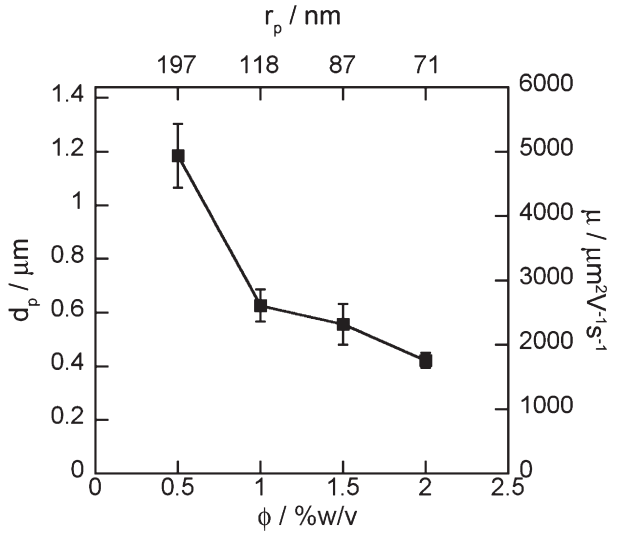

Fig. 7. Migration distance per pulse, d_p, and electromobility, μ, of DNA as a function of agarose concentration, ϕ, in the gels. All pulse sequences consisted of 10 pulses with field strength of 120 V/cm, duration of 20 ms, and pulse interval of 1 s. The observed decrease in electromobility with increasing agarose concentration correlated strongly with the decreasing pore radius, r_p, in agarose gels predicted by Eq. (4).

If the pulsing parameters are not of sufficient amplitude or duration, or the interval between pulses is too large, no electrophoretic transport through the interstitial space will occur.

It is unlikely that a uniform pulse sequence can be tailored around both electroporating and electrophoretic contributions of the applied electric field. Therefore, the approach of Bureau et al. [20] and Satkauskas et al. [21], where each contribution is dealt with individually with a combination of electroporating

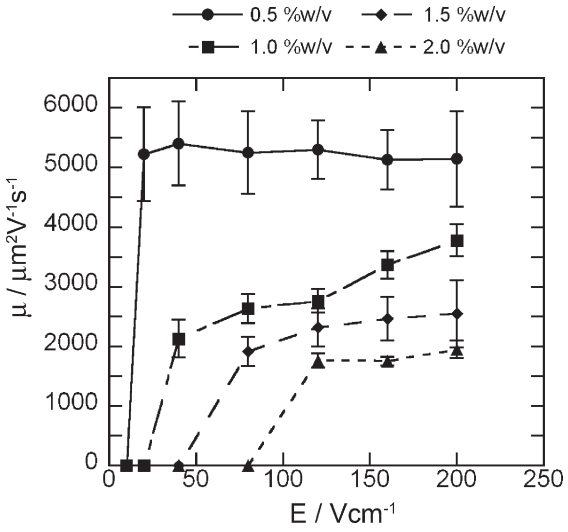

Fig. 8. DNA electromobility, μ, in 0.5, 1.0, 1.5, and 2.0% w/v agarose gels as a function of the magnitude of the applied electric field, E. There existed a minimum value of E required to initiate electrophoretic transport of DNA in fibrous matrices. No electrophoretic transport occurred in agarose gels until E was greater than a threshold level, which increased with increasing the agarose concentration.

and electrophoretic specific pulses, may be most suitable for electric field-mediated gene transfer in vivo.

In summary, we developed a reproducible method to observe the electromobility behavior of single DNA molecules in the presence of pulsed electric fields that have been used in electric field-mediated gene delivery. Using this method, we investigated the dependencies of DNA electromobility on several pulsing parameters and demonstrated that they were consistent with the electromobility behavior of a group of plasmid DNA molecules observed in pervious studies [2,31]. In addition, our study revealed the pulse interval dependence of DNA electromobility and the existence of a threshold field strength below which DNA electromobility failed to occur. These findings provide important insight into the mechanisms of DNA electromobility in tissues, which are useful for improving electric field-mediated gene delivery in tumors.

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