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Kenneth D. Cole[a]; Björn Åkerman[b]
[a] Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, Maryland, USA [b] Chalmers University of Technology, Department of Physical Chemistry, Göteborg, Sweden

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The Influence of Agarose Concentration in Gels on the Electrophoretic Trapping of Circular DNA[#]

Kenneth D. Cole[1,*] and Björn Åkerman[2]

[1]Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, Maryland, USA
[2]Chalmers University of Technology, Department of Physical Chemistry, Göteborg, Sweden

ABSTRACT

The density and structure of the electrophoretic traps for circular DNA in gels were strongly influenced by the concentration of agarose used to form the gels. Agarose gels were cast in a range of concentrations from 0.25% to 2.5% (1% is 1 g/100 mL). The trapping behaviors of two DNA circles with sizes of 13 kilobase pairs (kbp) and 52.5 were studied. The electric field strength (E) for the onset of trapping, the apparent trap density, and release characteristics of circles from traps were studied using both direct current and field inversion gel electrophoresis (FIGE)

[#]Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedures. Such identification does not imply recommendation by NIST, nor does it imply that the materials or equipment are necessarily the best available for the purpose.

*Correspondence: Kenneth D. Cole, NIST, Mailstop 8312, 100 Bureau Drive, Gaithersburg, MD 20899; Fax: (301) 330-3447; E-mail: Kenneth.Cole@nist.gov.

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experiments. Direct current mobility indicated that the onset of trapping, indicated by a reduction in velocity and increase in band smearing, occurred at significantly lower value of E in 0.25% gels compared to 1% gels or 2% gels. The critical value of E for complete immobilization of the 13 open circular form was 16 V/cm in 0.25% gels, and 20 V/cm in 1% and 2% gels. FIGE mobility measurements, done by varying the reverse times, were used to determine the time required to free a circle from a trap. The time span of the reverse pulse time (from first release to complete release) was increased in the 0.25% gels compared to the time span for the 1% gels or 2.5% gels. These results indicated that the electrophoretic traps were more heterogeneous in the lower concentration gels. FIGE experiments, in which the forward pulse times were varied, were done to determine an average distance that a circle traveled before trapping occurred. The average distances before the 13 kbp open circles were trapped ($E = 22\,V/cm$) were approximately 80 μm in 0.25% gels, 180 μm in 1% gels, and greater than 500 μm in 2.5% gels. The higher trap density and longer trap length in low concentration gels explains the experimental observations that, under some conditions, DNA circles migrate faster in higher concentration gels compared to their migration in lower concentration gels.

INTRODUCTION

The migration of circular DNA can be completely arrested in gels when the electric field strength (E) reaches a critical value. The factors that determine the formation of the electrophoretic traps in gels are not well understood. A better understanding of these factors would allow control of their formation for the design of better gels for the selective separation of circular DNA. By studying the process of trapping using circular DNA, we can infer characteristics of the trap structure. An important feature of electrophoretic trapping is that it is reversible. Trapped circles can be released from traps by either by turning the field off or reversing the direction of the field.[1,2] This implies that the traps are open ended (dangling) and they will have an effective length that threads the DNA circle. The traps can be regarded as an energy barrier that the circles must overcome to migrate through the gel under the influence of the electric field. Measuring the E required to immobilize a circle (the critical E) provides information about the size of the energy barrier.[3] Measuring the time and velocity it takes for a DNA circle to escape a trap can yield information about the effective length of a trap.[4]

The phenomenon of electrophoretic trapping of circular DNA was first described in agarose gels[5] and later in polyacrylamide gels.[4] Mickel et al

observed that the size of open circular DNA trapped in agarose gels could be decreased by increasing the value of E.[5] They first proposed that the DNA circles could become looped over the traps and immobilized by E.

Electrophoretic trapping of circular DNA is the result of the interaction of the DNA and the gel driven together by E. The physical properties of the DNA circle and the gel traps both determine the conditions under which electrophoretic trapping will occur. The critical E for a given DNA circle is determined by the trap structure and the interactions of the circle with the trap. We measured the critical E for several plasmids in 1% (mass percent) agarose gels.[6] The critical E required to trap the supercoiled forms was significantly higher than that of the open circular forms. Adding starch polymer to agarose, gels reduced the critical E for the open circular form of a 13-kbp plasmid to one-third of the control value (1% agarose gels), but the critical E for the supercoiled form was not significantly changed.[7] These results suggest that the traps formed by starch in the gels are active for open circular DNA but not for supercoiled DNA.

Additional insight into trap formation was given by forming gels with agarose that had reduced chain length (higher concentration of agarose ends). The critical E and trapping characteristics for a series of plasmids was measured in control gels and gels formed using the reduced molecular weight agarose.[6] The results showed that the critical E was not changed for the plasmids, but the density of traps significantly increased in gels formed with the reduced molecular weight agarose.[6] These results indicate that it is possible to modify the density of the traps in agarose gels.

Field inversion gel electrophoresis (FIGE) was developed to improve the resolution of large linear DNA in agarose gels,[8] but it has also proven to be a valuable tool for studying the electrophoretic trapping of circular DNA.[4] The effect of agarose gel concentration on the trapping of large open circular DNA was investigated using FIGE and rotating gel electrophoresis to relieve the trapping of large open circles (48.5 kbp and 97 kbp).[9] They observed that when the agarose gel concentration was decreased, the mobility of large open circles decreased. This is in contrast to linear DNA, where a decrease in agarose gel concentration always resulted in increased mobilities. To explain the anomalous mobility results (termed atypical sieving) the researchers hypothesized that the increased trapping (reduced mobility) was due to a change (s) in one or more of the following characteristics of the traps: number, length, shape, and stiffness.

We confirmed the importance of agarose gel concentration on trapping of a 13-kbp plasmid and presented some preliminary results in a review.[3] In this article, our aim is to provide a more complete study of the effect of agarose concentration on trapping. We used two DNA circles with sizes of 13 kbp and

52.5 kbp, the small open circle requiring a high value of E (20 V/cm) and the large open circle being trapped at a low value of E (3 V/cm). The trapping behavior of the open circles was compared to their supercoiled counterparts (not trapped at these value of E) and linear markers using agarose gel concentrations that were above and below the concentration of the 1% gels, we characterized previously.

MATERIALS AND METHODS

Materials

DNA standards and restriction enzymes were obtained from Invitrogen Life Technologies (Carlsbad, CA, US). Agarose (SeaKem LE) was from BioWhittaker Molecular Applications (Rockland, ME, US). The circular DNA used a plasmid of size 13.1 kbp (pYA101) and a cosmid clone of size of 52.5 kbp (American Type Culture Collection, ATCC #59882, Manassas, VA). The DNA was prepared using commercial ion-exchange column kits (Marligen, MD). The DNA concentrations were determined by fluorescent dye (Hoechst 33258) binding (BioRad, Hercules, CA, US) using supercoiled standards.

Electrophoretic Mobility and Field Inversion Gel Electrophoresis

Agarose gels were prepared by boiling the agarose in buffer and pouring it into slabs containing either two or three sections with different agarose concentrations. The gels were run in a submarine mode with buffer recirculation and maintained at 20°C. The buffer used for mobility measurements was 45-mM tris (hydroxymethyl)aminomethane, 45-mM boric acid, and 1-mM ethylenediaminetetraacetic acid pH 8.3 (TBE). The value of E was determined by touching a set of electrodes (connected to a volt meter) to the surface of the gel during the electrophoresis run. A short electrophoresis step (E = 3 V/cm for 25 min) was done to run the DNA into the gel. This was done to avoid the effect of the gel edge on trapping. The gels were stained in ethidium bromide (1 μg/mL) for 1 h and destained by performing several changes of water. The velocity was calculated from the distance migrated relative to the starting position (which was determined on an identical but separately stained gel) and the running time. The distance migrated was measured to the center of mass of the band, determined visually,

assisted by adjusting contrast and brightness using image processing software. Velocities were calculated using the average of two or three lanes on the gel.

Field inversion gel electrophoresis (FIGE) was performed using commercial pulsing instruments. Before beginning the pulsing step, the DNA samples were run into the gel (approximately 1 mm) using a direct current field (3 V/cm for 25 min). The velocities for the FIGE experiments were calculated by using an effective time of electrophoresis.[4] The effective time was calculated by multiplying the actual running time by a correction factor to compensate for the time spent in the reverse direction. The correction factor was calculated using the following formula:

$$(t_f - t_r)/(t_f + t_r)$$

where t_f is the forward pulse time and t_r is the reverse pulse time.

RESULTS

Direct Current Mobility Experiments

Gels were run at different values of E to determine the effect of E on mobility of the 13 kbp circles. One set of gels (1% and 0.25%) was run for 1450 min·V/cm (product of time and E) (Fig. 1A and B). A second set of gels (2% and 1%) was run for 4100 min ·V/cm, to measure the low mobilities in the 2% gels (Fig. 1C, D). The gels were run at a constant product of E and time to facilitate comparisons, but the actual migration distances increased with higher values of E giving, as expected, higher mobilities, except when trapping reduced the mobilities of the open circles. Since the amount of DNA loaded can influence the mobility and the band sharpness, each series of gels were measured using a consistent set of DNA samples. By comparing the results of the same DNA samples, it was possible to compare the effect of E on mobility and band smearing among the different gels. Duplicate gels gave values of the mobilities that agreed to within ±5%. To obtain data on the degree of smearing of the bands (reduced sharpness), the length of the band in the direction of migration was measured and was referred to as the band length.

The decrease in mobility of the 13 kbp open circles with increased E was coincident with an increase in band length of the DNA (see Fig. 1). The increase in band length reached a maximum and then decreased as trapping increased. Figure 1 shows that the onset of trapping (indicated by the decrease in mobility) occurred at a lower E in the 0.25% gels compared to the 1% and 2% gels. The maximal band length occurred at approximately 7 to 9 V/cm in

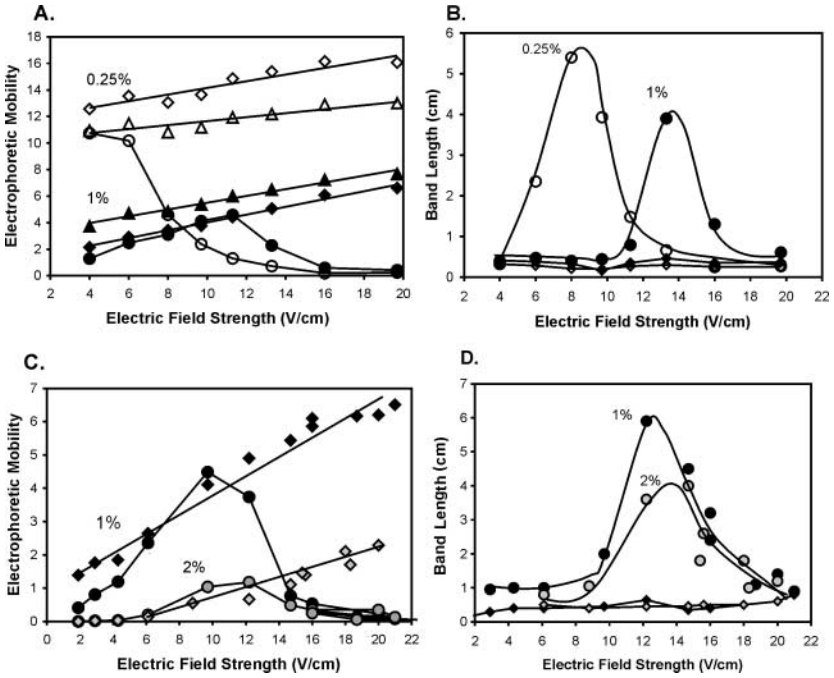


Figure 1. Direct current mobilities and band lengths of the 13-kbp plasmid and the linear marker. Electrophoretic mobilities and band lengths of the 12.2 kbp linear marker (triangles), the open circular form (circles), and the supercoiled form (diamonds) of the 13-kbp plasmid in 0.25% gels (open symbols), 1% gels (black symbols) and 2% gels (gray symbols). The gels in A and B were run for a total of 1450 min·V/cm (product of time and electric field strength). The gels in C and D were run for a total of 4100 min·V/cm. The electrophoretic mobility equals 10^{-5} $cm^2/(V \cdot s)$. The mobilities and band lengths were measured as described in the text.

the 0.25% gels, 12 to 14 V/cm in the 1% gels, and 13 to 15 V/cm in the 2% gels. Complete immobilization (the critical E) of the 13 kbp open circle was at 16 V/cm in the 0.25% gels and 20 V/cm in the 1% and 2% gels. In 1% agarose gels, the 52.5-kbp open circle migrated in the gel at 2 V/cm and was trapped at 3 V/cm. The 52.5-kbp supercoiled form was partially trapped at 22 V/cm. However, the band was smeared, indicating that the supercoiled form was not fully trapped. The onset of trapping was considered to occur when the mobility decreased and band length increased. When the mobility approached zero and the band length reached a minimum, the circle was considered trapped.

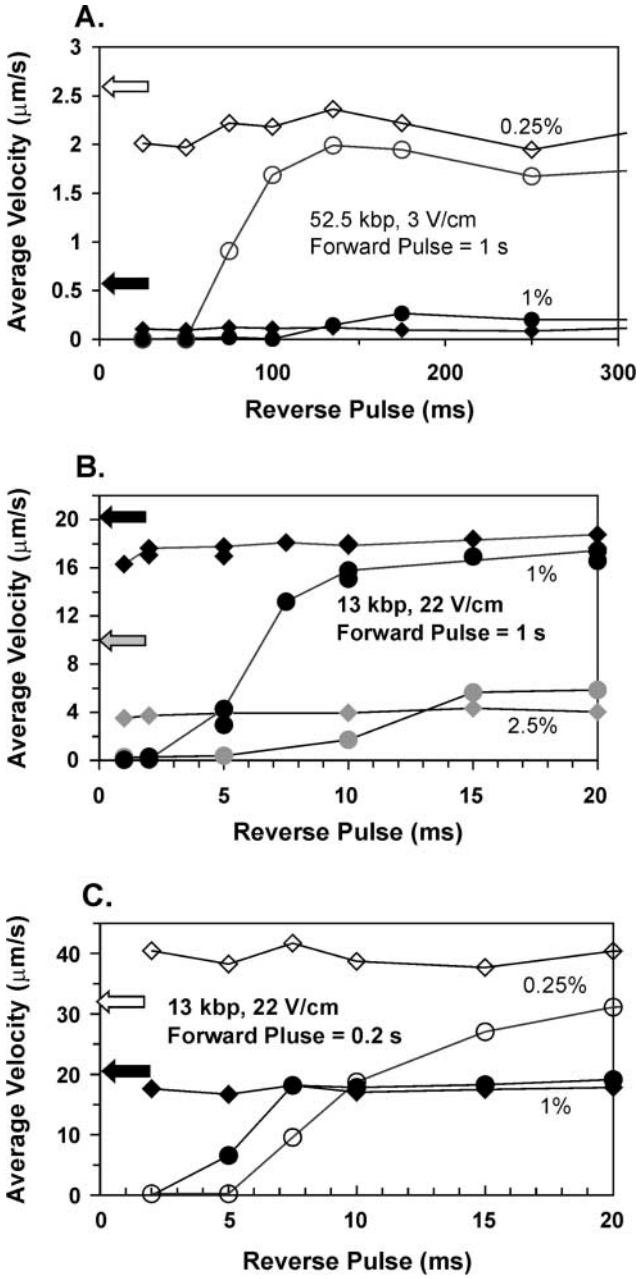
FIGE Mobility Measurements to Determine Time to Escape Traps

FIGE experiments were done by varying the duration of the reverse pulse time and keeping the forward pulse time constant. These experiments were done to determine the time (at a given velocity) required for the circle to escape the traps in a gel. The mobilities of the DNA were corrected for the time the DNA spent traveling in the reverse direction, as described in the materials and methods section. When calculated in this manner, the mobilities of the supercoiled forms and the linear markers did not change with pulse time in these experiments. An exception to this is discussed in the following section, when E was equal to 3 V/cm and short forward pulse times were used.

At short reverse pulse times, the open circles remain trapped until a threshold time was reached, at which time, the circles began to move (observed as a smear). Finally, with longer reverse pulse times, the circles reached a plateau velocity where they traveled as a discrete band (Fig. 2). The smearing of the circles at the intermediate release times indicates that the open circles are only partially released. The velocities were measured at the center of the smeared band and the mobilities are thus averages of the population of circles.

A low value of E equal to 3 V/cm was sufficient to trap the 52.5 kbp open circles. The low velocity of the 52.5 kbp circle in 2% agarose gels at 3V/cm precluded measurements, but we measured velocities in 0.25% and 1% gels (Fig. 2A). The results shown in Fig. 2 revealed that reverse pulse times of 0.1 s and 0.18 s were sufficient to completely free the large open circles from 0.25% and 1% agarose gels, respectively. The reverse pulse time required for the release of the 52.5 kbp circle was considerably shorter than the time required to release 115 kbp and 220 kbp open circles (full release time 3 s and 6 s, respectively) under the same conditions (3 V/cm in 1% agarose).[10] These results indicate that when the size of the open circles is increased beyond 52.5 kbp, escape from the traps becomes greatly hindered.

FIGE experiments were also done at 22 V/cm (sufficient to trap the 13 kbp open circles). In the first set of experiments, a constant forward pulse time of 1 s was used and the reverse pulse times varied. The 13 kbp open circle reached a plateau velocity in 1% and 2% gels (Fig. 2B), but not in the 0.25% gels. In the 0.25% gels, the velocity of the 13 kbp open circle was approximately one-half the velocity of the supercoiled form (results not shown). This result indicated that the rapid trapping during the 1 s forward pulse reduced the velocity in the 0.25% gels (also see results in the following section). A second set of gels was prepared using a forward pulse time of 0.2 s and the reverse pulse times varied (Fig. 2C). Under these conditions, the 13 kbp



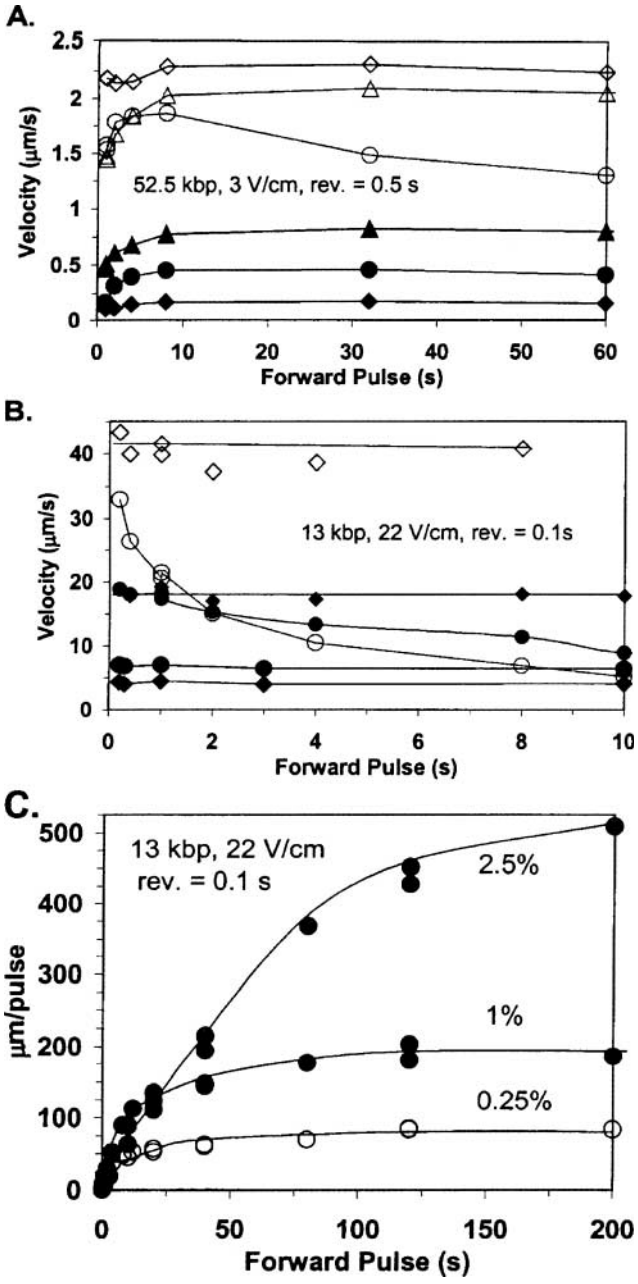
open circles reached plateau velocity in the 0.25% gels much closer to the velocity of the supercoiled form. The velocity of the 13 kbp open circle form in the 1% gels was the same with either a 1 s or a 0.2 s forward pulse (see Fig. 2B and C).

FIGE Mobility Measurements to Determine Average Trap Distance

The previous FIGE experiments allowed us to pick a reverse pulse time that was sufficient to free the circles from the traps. The next series of FIGE experiments were done by varying the forward pulse time using a constant reverse escape pulse. The effect of increasing the forward pulse times was to force the circles to spend an increasingly larger portion of their time stalled in a trap, before a reverse pulse freed them.

Figure 3A shows the effect of the forward pulse time on the velocity of the 52.5 kbp open circle at 3 V/cm in 0.25% and 1% gels. The effect of the forward pulses at 3 V/cm showed complex behavior at the shorter forward pulse times (see Fig. 3A). The velocity of the linear marker (48.5 kbp) initially increased and then reached a plateau velocity that did not change at longer forward pulse times. The velocity of the open circle form initially increased, reached a maximum, and then began to decrease with longer forward pulse times. The effect was more pronounced in the 0.25% gels compared to 1% gels. The 52.5-kbp supercoiled form displayed a smaller increase in velocity to reach a plateau value when the forward pulse time was increased. The observation of a decrease in velocity for short pulse times for the linear marker is consistent with observations in pulsed field electrophoresis. A limitation of FIGE when used for linear DNA is that the mobilities go through a minimum when pulse

Figure 2. Field inversion gel electrophoresis of the 52.5 kbp and 13 kbp circles. Velocities of the open circular form (circles) and the supercoiled form (diamonds) in 0.25% gels (open symbols), 1% gels (black), and 2.5% gels (gray). A. Velocity of the 52.5-kbp DNA was measured with a forward pulse of 1 s at 3 V/cm and the indicated reverse pulse times. Arrows show velocities of the linear marker (48.5 kbp) in 1% gels (black) and 0.25% gels (open). B. Velocity of the 13-kbp DNA was measured with a forward pulse of 1 s at 22 V/cm and the indicated reverse times. Arrows show velocities of the linear marker (12.2) in 1% gels (solid) and 2.5% gels (gray). C. Velocity of the 13-kbp DNA was measured with a forward pulse of 0.2 s at 22 V/cm and the indicated reverse times. Arrows show the velocities of the linear marker (12.2 kbp) in 1% gels (solid) and 0.25% gels (open).



times are changed and the location of the minimum varies with the size of the linear DNA and the electric field strength.[11,12]

We were able to measure the effect of FIGE on the velocities of the 13 kbp open circle at 22 V/cm in 0.25%, 1%, and 2.5% gels (Fig. 3B). The velocities of the supercoiled form and linear markers did not change with the duration of the forward pulse at this value of E. The velocity of the open circle form decreased monotonously when the forward pulse times were increased again, with the greatest effect seen in the 0.25% agarose gels. Under these conditions, when the forward pulse was greater than 2 s the 13 kbp open circle will be traveling faster in 1% agarose gels compared to 0.25% gels. The decreased mobility in lower concentration gels (atypical sieving) was previously described for larger circles (48.5 kbp).[9]

When the forward pulse time was increased to a sufficiently long time, the entire population of circles would have encountered a trap by the end of the forward pulse. Over many trap and release cycles, the circles sample the entire distribution of distances between traps. This suggested to us that we could determine the average distance between traps by dividing the distance traveled by the circles in a gel by the number of trap and release cycles. Figure 3C shows the data plotted in this manner. This data indicated that the 0.25% and 1% reached a limiting distance of approximately 80 μm and 180 μm, respectively. The 13 kbp open circle had not reached a true plateau in 2.5% gels, but the distance traveled was greater than 500 μm per cycle. The data show that the low velocity of the 13 kbp open circles in 2.5% gels and the long distances between traps make it difficult to reach the limit distance where almost all of the circles are trapped by the end of the forward pulse. The low velocity at 3 V/cm prevented this type of analysis for the 52.5 kbp circles. Even at the longest forward pulse time available (1000 s), the open circles had not reached a plateau distance in the 1% or 0.25% gels (data not shown). Some of the data in Figs. 3B and 3C appeared as a preliminary report in a review

Figure 3. Field inversion gel electrophoresis of the 52.5 kbp and 13 kpb circles. The velocities of the supercoiled forms (diamonds), open circles (circles), and linear markers (triangles) in 0.25% gels (open symbols), 1% gels (black symbols), and 2.5% gels (gray symbols). A. Velocity of the 52.5-kbp DNA measured with a reverse pulse of 0.5 s at 3 V/cm and the indicated forward times. The linear marker was 48.5 kpb. B. Velocity of the 13 kbp DNA was measured with a reverse pulse of 0.1 s at 22 V/cm and the indicated forward times. The linear marker was 12.2 kbp. C. Data for open circles shown in Figure 3B (plus additional points) plotted by dividing total distance migrated by the number of pulse cycles.

article.[3] This article confirmed the initial observations and extended the data for additional times and gel concentrations.

DISCUSSION

The electrophoretic trapping of circular DNA requires certain physical characteristics of the gel structures not probed by other techniques. The requirements for these traps are that they must have an open end (a dangling fiber), posses a shape (diameter) that will allow threading of a DNA circle, and be sufficiently rigid to counteract the forces acting upon it by the circle driven by E. The structures in gels that act as traps for circular DNA have not been directly detected, but it is logical to expect their formation based on the knowledge of the manner in which agarose forms gels. A large amount of experimental evidence supports the model that the agarose forms a double helix and aggregation of the helices into bundles (fibers) forms the strong three-dimensional gel network.[13–16] Agarose gels have found such great utility for separations because of their strength and the relatively large pores between the fibers. Characterization of the agarose fibers with electron microscopy revealed that the fibers laterally aggregate, form branches, and have an observed width of 15 nm to 30 nm.[17] Waki et al[18] studied the structure of agarose gels varying in concentration from 0.25% to 4% using electron microscopy. They determined fiber widths of 6 nm to 12 nm (corresponding to 10 to 30 double helixes per fiber) with the lower numbers found in the lower concentration gels. A study using very dilute concentrations of agarose (less than 0.05%) found that rods with diameters of 5 nm formed.[19] Agarose will fail to form gels when the concentration is below a certain critical concentration. The critical agarose concentration required to form gels and the mechanical properties of the resulting gels are dependent on the molecular weight of the agarose polymer.[20] Solutions of agarose below the critical gel-forming concentration will form fibers, but gels will not form because the fibers fail to make connections between other fibers to form a three-dimensional gel network. It is reasonable to assume that the number of dangling ends (failed connectors) would be expected to increase in lower concentration gels.

Electrophoretic traps in gels represent an energy barrier that a DNA circle must overcome to progress through the gel. Recently, we have discussed the efforts at modeling the energy barrier of a trap.[3] Changes in the length, stiffness, and shape of the traps will change the energy barrier that is present in a given gel. Changes in the energy barrier will be observed by differences in one or more of the following: (1) critical voltage, (2) electrophoretic mobility,

and (3) release characteristics from the traps for a given DNA circle. We cannot directly measure the length, stiffness, or shape of the traps, but assumptions can be made based on the experimental results.

For the interpretation of the data, it is important to consider the differences in trapping resulting from changes in the length and the stiffness of the traps. Assuming that a trap does not have a complex shape, the energy barrier it provides is due to the depth (the effective length) that a circle can thread itself into the trap (under the influence of the electric field). This effective length is determined by a number of variables that would be expected to vary in gels, including the contour length of the dangling fiber, the resistance to deformation (stiffness), and the orientation with the electric field. Flexibility in a trap would decrease the effective length of a trap if it can bend in the direction of the electric field. The direct current mobility experiments indicate that the traps in the 0.25% gels are active at lower voltages compared to the higher concentration gels. There is an approximately 40% reduction in the field strength for the onset of trapping in the 0.25% gels compared to 1% gels and 2% gels. This is compared to an approximately 20% reduction in the critical voltage to fully retain the 13 kbp open circle for the same comparison. Longer traps (active at lower voltages), but with increased flexibility, would result in retardation at lower voltages, but would result in a similar effective trap length (and critical voltage) as the traps in higher concentration gels.

The range of reverse times required to release the circles from the traps also reflects the heterogeneity of the traps, as indicated by smearing at the intermediate times (results not shown). The shortest time for release would correspond to circles escaping from traps with shortest trap lengths and the time for complete release would correspond to circles escaping from traps with the longest trap lengths. The 0.25% gels with a lower critical voltage (approximately 20%) should have longer traps and require longer times for complete release. To compare the release curves, it is necessary to normalize for the large differences in velocity in the gels. One way to do this is to calculate an "apparent" length of the trap by multiplying the release time by the escape velocity of the circle. We do not know the microscopic escape velocity of a circle from a trap, but the plateau velocity of the open circles would the most reasonable one to use to make comparisons.

The 13 kbp open circle in 0.25% gels was first released at 5 ms and completely released at 20 ms and using a velocity of 30 μm/sec. The calculation yields apparent lengths of 150 nm to 600 nm. The 13 open circle in 1% gels was first released at 2 ms and completely released at 10 ms (using a velocity of 18 μm/sec). The apparent lengths are 36 nm to 180 nm. The 13 kbp open circle in 2.5% gels was first released at approximately 5 ms and completely released at 15 ms (using a velocity of 6 μm/sec). This gives

the apparent lengths of 30 nm to 90 nm. These apparent lengths are calculated to compare the release characteristics of the gels, and we do not suggest that these are the actual lengths of the traps in the gels. The minimal trap lengths should be the same in all gels since the gels were run at the same voltage (acting on the same DNA). The long apparent trap lengths in the 0.25% gels are not reasonable given that the reduction in critical voltage was only approximately 20%. One important consideration is that the critical voltage is a function of the effective trap length in the forward direction once a circle is trapped. The reverse pulse time FIGE experiments measured an apparent length when the circles were being released. When released from the field, the traps are able to relax, but the DNA circles still have to escape from the trap. Long flexible traps that give a modest increase in effective trap length could present a significant barrier to escape by a circle.

The results of this study are consistent with an agarose gel structure that contains higher concentrations of dangling ends when the agarose concentration is lowered. In addition, the lengths of the dangling ends appear longer and more flexible when compared to those in higher concentration gels. As a result of this, the open circles are rapidly trapped and their motion hindered at lower voltages in low concentration gels. There are several examples of flexibility in the agarose matrix during electrophoresis. During electrophoresis, the zone of DNA in agarose gels induces an electroosmotic flow, resulting in compression and expansion of the gel in different parts of the DNA zone as detected by linear dichroism.[21] Electric birefringence was used to probe gel structure and it was found that when low pulsing fields were used, agarose gel fibers can be oriented with the field and relax.[22] Large electric fields can permanently change the gel structure when probed with electric birefringence.[23,24]

These results have practical implications for exploiting the specific mechanism of electrophoretic trapping of circular DNA. Low concentration agarose gels have enhanced properties for trapping circular DNA, in terms of higher capacity and slightly reduced critical electric field strengths needed for immobilization. In addition, the mobility of the linear DNA is higher in the lower concentration gels, allowing more rapid separations when the goal is to trap circular DNA and remove linear DNA. A consequence of enhanced trapping in lower concentration gels is that atypical sieving of open circular DNA can occur. This can be viewed as advantageous when the goal is to achieve a better separation between the open circular and supercoiled forms. Selecting a voltage where the open circular form is partially trapped (and slowed) allows a clearer separation of mixtures. The lower concentration gels permit such discrimination at lower voltages. These results also indicate that the active traps formed at lower concentration gels are more heterogeneous

than those formed with higher concentration gels. The higher effective concentration of longer flexible traps in the lower concentration gels explains the atypical sieving seen in the FIGE experiments. The lower heterogeneity and increased stiffness the traps formed in higher concentration gels should make them more favorable for measurements to support modeling efforts.

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