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Capillary Electrophoresis of Supercoiled DNA Molecules: Parameters Governing the Resolution of Topoisomers and Their Separation from Open Forms

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We describe the separation of covalently closed and open circular DNA forms with capillary electrophoresis. This technique is expected to be applied in the research of novel anticancer molecules targeting the activity of topoisomerase I. The separation of a plasmid mixture containing fully supercoiled molecules, single topoisomers, and their relaxed and open circular forms was tested in an electric field of 200 V/cm using Tris/borate buffer with the addition of magnesium ions at low concentrations and various sieving polymers. The resulting separation is quite simple to achieve and is clearly comparable to that obtained in agarose gels run at low voltage, but with an improved resolution, a higher quantitativity, and a higher speed of analysis. We identified three main parameters that influence the separation: (I) Low concentrations of $MgCl_2$ in the separation buffer are required for a good resolution of topoisomers. (II) Cellulose derivatives can be used as sieving polymers; in our hands, HPMC and HEC worked best. (III) High molecular mass forms of sieving polymers allow the best separations.

The covalently closed circular forms of plasmids have special structural features, of which the most common is superhelicity. These features have been traditionally studied by slab gel electrophoresis.[1] The common limit of these methods, even today, is the need of separating the supercoiled forms in very low electric fields, so as not to alter the conformation of topoisomers, with loss of resolution. Electric fields from 1 to 3 V/cm imply time-consuming analytical runs and make the massive screening of DNA topoisomers impracticable.

The study of the enzymes that control topological properties of DNA molecules such as superhelicity has led to the discovery of new therapeutic strategies and new drugs in cancer research.[2] Among the recently discovered molecules with promising applications as anticancer drugs are camptothecin and its derivatives, inhibitors of DNA topoisomerase I (topo I).[3] Camptothecins work as "poisons" for topo I by blocking the catalytic cycle at the stage of the DNA−topo I covalent intermediate (cleavable complex). In its catalytic cycle, topo I facilitates the transfer of a phosphate ester bond from the DNA backbone to the hydroxyl group of a tyrosine in the active site of the enzyme, followed by swiveling of the DNA duplex along the other strand (that is left intact) and transfer of the phosphate ester bond back to the DNA backbone.[4,5]

A typical assay for in vitro topo I activity involves the relaxation of a supercoiled plasmid by topo I, in the absence or in the presence of inhibitors. All the topological forms generated in the assay (residual fully supercoiled forms, single topoisomers, and their relaxed and open circular (OC) forms) are then separated and quantitated by agarose gel electrophoresis.[6]

Considering the limits of agarose gel electrophoresis, we evaluated the possibility of separating DNA topoisomers by capillary electrophoresis (CE). In principle, this technique should show a higher power of resolution, be faster, and be suitable for complete automation. CE is an analysis tool capable of separation at high speeds and with high resolution; it requires high electric fields for the separations[7] and consequently allows shorter working times. It also allows easier automated sample processing and quantitative data analysis. A wide range of CE applications in DNA analysis have been described, the most recent development being highly automated DNA-sequencing protocols.[8,9]

We have studied several physicochemical parameters important for the separation of topoisomers in the high electric fields required for CE: the effect of different sieving polymers, all derived from cellulose; the effect of the average length of the polymer molecule; and the effect of magnesium ions in the electrophoretic buffers.

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We describe here the results of the separation of DNA topoisomers in CE and compare them with those obtained by classical slab gel electrophoresis.

EXPERIMENTAL SECTION

Materials. Ethidium bromide (EtBr) was purchased from Sigma (St. Louis, MO). Supercoiled plasmids used in this work were pUC 18 and pHOT1, a modified version of pUC 12 in which the sequence

TCTAGAGGATTTCGAAGACTTAGAGAAATTTCGAAGATCCCCGGGCGAGCTC

was inserted into the XbaI-EcoRI site of the polylinker.[10] Camptothecin (CPT; TopoGen, Columbus, OH) was prepared as a 10 mM stock solution in DMSO (Sigma-Aldrich, Milano, Italy). The 10X reaction buffer consisted of 100 mM Tris HCl, 10 mM EDTA, 1.5 M NaCl, 1% BSA, 1 mM spermidine, and 50% glycerol; pH 7.9. Topo I was supplied by TopoGen. Hydroxypropyl methyl cellulose (HPMC) of 86, 90, and 120 kDa average molecular masses was from Aldrich; hydroxyethyl cellulose (HEC) of 90−105 and 140−160 kDa average molecular masses was from Polysciences (Warrington, PA). Fused-silica capillaries (μSIL-FC, 50 μm i.d. × 80 cm) were from J&W Scientific (Folsom, CA).

Relaxation Assay. 500 ng quantity of supercoiled plasmid was reacted with 0. 1 U of topo I in 20 μL quantity of reaction buffer for 45 min at 37 °C; when indicated, 1 mM CPT solution in 10% DMSO was included in the reaction mixture up to a 100 μM final concentration. EtBr was added to the reaction mixture as indicated in the figure captions. The reaction was stopped by adding SDS to a final concentration of 0.2%; proteinase K was added, and the incubation at 37 °C was continued for a further 45 min. SDS was then precipitated by adding 2 μL of 2.5 M KCl, and each sample was kept on ice for about 10 min. The precipitated material was centrifuged at about 6000*g* for 5 min, and the supernate was collected and placed on a 50 kDa cutoff ultrafiltration cartridge (Microcon 50, Amicon). After a wash with 0.8 mL of Milli-Q water, the sample was collected in a final volume of about 10 μL and used in CE or in agarose gels.

Agarose Gel Electrophoresis. Agarose gels (1.5%) were prepared in 90 mM standard Tris/borate buffer either with or without 5 mM $MgCl_2$ and run at 2 V/cm for 16 h. After electrophoresis, the gel containing $MgCl_2$ was washed thoroughly in Tris/borate buffer and both were stained with 1 μg/mL EtBr; visualization and photography were performed under UV transillumination.

Capillary Electrophoresis. Capillary electrophoresis buffers were prepared by dissolving 0.4% stock polymer solutions to a final concentration of 0.1% containing 90 mM Tris/borate buffer and $MgCl_2$ at 0, 1, 2, and 5 mM final concentrations. For 140−160 kDa HEC, a 0.05% final concentration was used. Coated capillaries were used at a length of 30 cm (23 cm of effective separation distance) with purging of buffer between runs. A separation potential of 6 kV (200 V/cm electric field) and a hydrostatic loading mode were employed. The separation temperature was between 18 and 22 °C, and on-line detection was performed at 254 nm.

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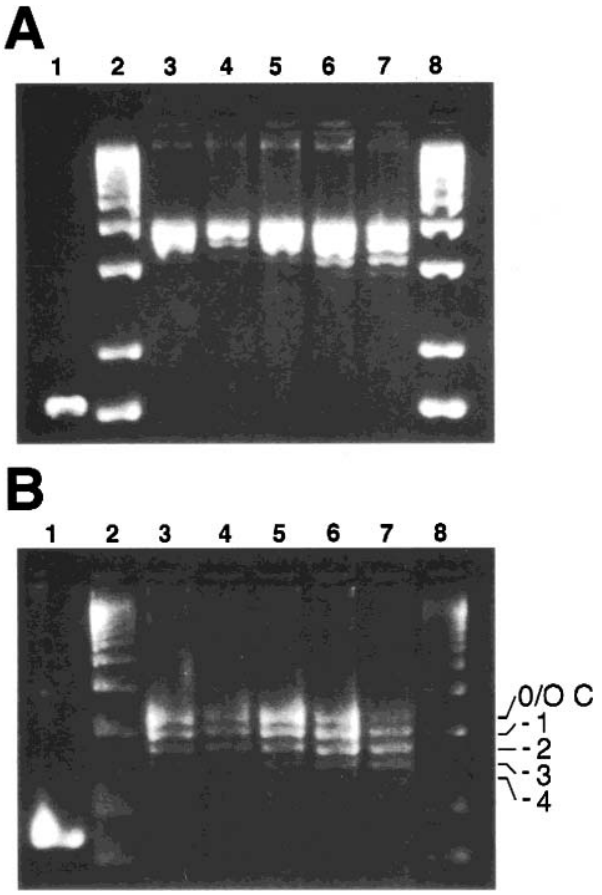

Figure 1. Agarose gel electrophoresis of plasmid DNA in 90 mM Tris/borate buffer (A) and in 90 mM Tris/borate buffer plus 5 mM $MgCl_2$ (B). pUC 18 was loaded in the supercoiled form (lane 1) or after relaxation in the presence of 0 (lane 3), 20 ng/mL (lane 4), 50 ng/mL (lane 5), 100 ng/mL (lane 6), and 200 ng/mL EtBr (lane 7). Lanes 2 and 8 show the 1 kb linear DNA ladder (Life Technology, Milano, Italy).

RESULTS AND DISCUSSION

Comparison of Agarose Gel and CE Separations of Topoisomers. The ability of CE to separate DNA topoisomers was compared with that of electrophoresis in agarose gels at low electric fields. We evaluated the electrophoretic behavior of pUC 18 plasmid, isolated as the native form from *Escherichia coli* cells and purified by ion exchange chromatography according to Onishi et al.[11] (see Supporting Information). A pUC 18 topoisomer ladder was obtained by topo I-catalyzed relaxation at concentrations of EtBr ranging from 0 to 200 ng/mL. Analysis of these samples in agarose gels revealed a series of five topoisomers, ranging from 0 to −4 (see Figure 1) obtained from plasmid relaxation in the presence of the different EtBr concentrations.

The separation was obtained on two different gels run in parallel: the gel in Figure 1A contained a 90 mM Tris/borate buffer; the gel in Figure 1B contained 90 mM Tris/borate plus 5 mM $MgCl_2$. Although the patterns of separation were very similar, the gel in 5 mM $MgCl_2$ showed a better resolution of topoisomers. In a preliminary attempt at separation using 1% agarose gel, the pattern of topoisomers was resolved only when $MgCl_2$ was

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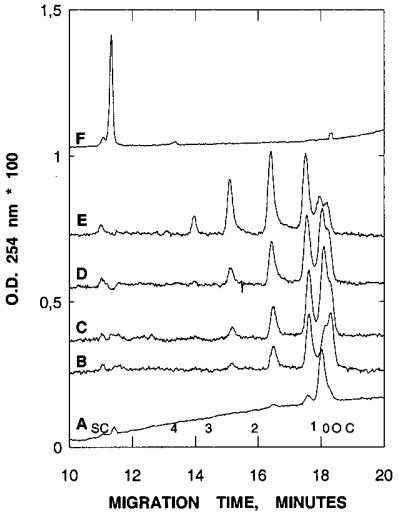

Figure 2. Capillary electrophoresis of topoisomerase-mediated relaxation of the pUC 18 plasmid in the presence of increasing EtBr concentrations. Electropherogram A is from pUC 18 relaxed in the absence of EtBr; electropherograms B−E are from plasmid pUC 18 relaxed in the presence of 20, 50, 100, and 200 ng/mL of EtBr, respectively. "SC" indicates the elution peak of the supercoiled form; "OC" indicates that of the open circular form. The electrophoresis was performed with a buffer containing 0.1% of 90 kDa HPMC (average molecular mass) and 5 mM $MgCl_2$.

included in the electrophoresis gel and buffer (see Supporting Information).

The samples shown in Figure 1 were also analyzed by CE, and the electropherograms are shown in Figure 2. In this case, 90 K HPMC was used as the sieving polymer at a concentration of 0.1% in the two alternative buffers described above for agarose gels. Figure 2 shows the electropherograms obtained in Tris/borate plus 5 mM $MgCl_2$.

Figures 1 and 2 allow a direct comparison between agarose gel and capillary electrophoreses of topoisomers. The topoisomer patterns of the samples are highly comparable between the agarose gel and the CE, when running in Tris/borate plus 5 mM $MgCl_2$. We could not obtain any CE separation of the four topoisomeric forms in running buffer without $MgCl_2$ (see Supporting Information).

The effect of $MgCl_2$ on electrophoretic separation was described previously[12,13] and was attributed to the increased writhe of plasmids in solutions containing Mg^{2+} ions or high concentrations of NaCl.[13] However, we think that several additional factors are important for the separation in CE: (I) Plasmids are exposed to electric fields 2 orders of magnitude higher, and it is likely that the higher energetic stabilization of supercoiling in the presence of Mg^{2+} ions plays a relevant role in the resolution. (II) A sieving polymer different from agarose is employed for its physical state (it is a liquid compound) and its use as a solution, where this definition means that the polymer is present in solution in the form of independently moving molecules rather than as an entangled three-dimensional mesh. This involves a separation model of DNA molecules that is different from the more classical models of reptation[14,15] or Ogston.[16,17]

Some recent theoretical models quite faithfully describe the migration of linear DNA in diluted polymer solutions;[18] also, Oana et al.[19] and Hammond et al.[20] have provided good experimental descriptions of supercoiled plasmid migration in a diluted polymer. Mao et al.[21] reported a CE separation of pBR 322 in an HPMC-based buffer. Despite all this, a consistent model for the migration of DNA circular forms (supercoiled molecules, single topoisomers, and OC forms) in diluted or semidiluted polymers is not currently available. This fact raises some difficulties in providing full rational explanations for the effects on the separation that we observe here.

A comparative analysis of Figures 1 and 2 suggests that topoisomer populations can be resolved in CE without major writhing perturbations and with a resolution that is higher than that observed in agarose gels. The improvement of separation also allows a better resolution of the OC form from the lowest linking number topoisomers.

In Figure 2, the OC form appears as a product of relaxation in the absence of EtBr as a peak only partially separated from that of the fully relaxed form. Even this partial resolution is impossible to obtain in agarose gels without the use of an intercalating agent in the electrophoretic buffer.

The identification and quantitation of the OC form are important in the classification of topo I inhibitors. For instance, CPT facilitates a stabilization of the cleavable complex, which results in the accumulation of DNA nicking and, as a direct consequence, the cytotoxic effect typical of CPT. Other inhibitors induce far less nicking and have a correspondingly lower toxicity even though they are very efficient at inhibiting DNA relaxation.

The good resolution of CE also allows a better evaluation of the less abundant topoisomeric species such as the topoisomer ±2 (Figure 1 panels A and B, lane 3, and Figure 2, track B).

Quantitation by densitometry of separated sample components in agarose gels and CE (Figure 1) produces slightly different profiles of peak intensities, due to the different detection methods: fluorescence visualization after EtBr soaking for the agarose gel and UV detection at 254 nm for CE.

Since EtBr binding is affected by DNA topology, in the sense that the more negatively supercoiled the DNA is, the less efficient is the binding,[22] this fact influences the correct quantitation of DNA topoisomer abundancy in EtBr-stained agarose gels. On the other hand, in CE, DNA is detected by direct UV absorbance readings at 254 nm, giving a more accurate estimate of the relative abundance of DNA molecules. To validate this statement, we verified that the CE separation described here allows perfectly linear quantitations of the pUC 18 plasmid in a measured range from 3.9 to 390 ng/μL.

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Table 1. Electrophoretic Mobilities [(μm/min)/V] of pUC 18 Plasmid

technique−polymer	mobility in 0 mM $MgCl_2$	mobility in 5 mM $MgCl_2$
slab gel−agarose	0.51	0.65
CE−90 kDa HPMC		4.64

Table 2. Electrophoretic Mobilities [(μm)/min/V] of the OC Form of the pHOT 1 Plasmid in the Sieving Buffer Compositions Tested

	mobility			
polymer	0 mM $MgCl_2$	1 mM $MgCl_2$	2 mM $MgCl_2$	5 mM $MgCl_2$
86 kDa HPMC	9.95	5.74	5.11	4.63
90 kDa HPMC	8.20	5.34	4.76	4.32
120 kDa HPMC	7.64	5.11	4.60	3.78
MMW HEC				5.54
HMW HEC		4.44	4.44	4.00
agarose	0.51			0.65

Finally, the separation speeds of the two methods are different: while agarose gels require from 5 h to overnight, CE requires only 20−25 min. This is the result of the combined effect of the higher electrophoretic mobility and higher electric fields (Tables 1 and 2).

Parameters Controlling the CE Separation of Topoisomers. To optimize the capillary electrophoretic separation of plasmid DNA circular forms, ladders of topoisomers were generated by controlled relaxation of the pHOT 1 plasmid with topo I.[10] The separation of these topoisomer ladders was carried out in an electrophoretic buffer containing different sieving polymers and $MgCl_2$ concentrations ranging from 0 to 5 mM. Four different polymer compounds were used [HEC, HPMC, hydroxypropyl cellulose (HPC), and methyl cellulose (MC)], and different polymer lengths were tested. We used low polymer concentrations[22] for two main reasons: (I) An unentangled polymer solution has a lower viscosity, which allows easy purging and washing of the capillary after each run, thus avoiding sample cross-contamination. (II) A more diluted polymer solution allows shorter separation times and enhances separation speeds.

In the case of HEC, the entanglement thresholds for different polymer lengths were calculated;[23,24] the vaues were 0.37% and 0.21%, respectively, for 90−105 kDa and the 140−160 kDa compounds. On the basis of these data and the work published by Hammond et al.[20] we chose to use polymer concentrations well below these values and test whether they could also be used for topoisomer separation.

HPC and MC polymers gave poor separation, even at the highest $MgCl_2$ concentrations in the electrophoresis buffers.

HEC and HPMC polymers of different molecular masses were tested in Tris/borate buffers containing respectively 0, 1, 2, and 5 mM $MgCl_2$ (Tables 1 and 2 and Figures 3 and 4). Although a working concentration of 10 mM $MgCl_2$ was previously used,[12,13] we tried lower concentrations because maintaining a lower current intensity during CE would have been preferable. The migration time expressed as a percent ratio (topoisomer migration time/open circular form migration time) was plotted as a function of superhelicity for the various polymers at each $MgCl_2$ concentration. A population of topoisomers ranging from 0 to −5 was also prepared (see Experimental Section), and in this case, too, none of the separations performed in the absence of $MgCl_2$ allowed any resolution of topoisomers.

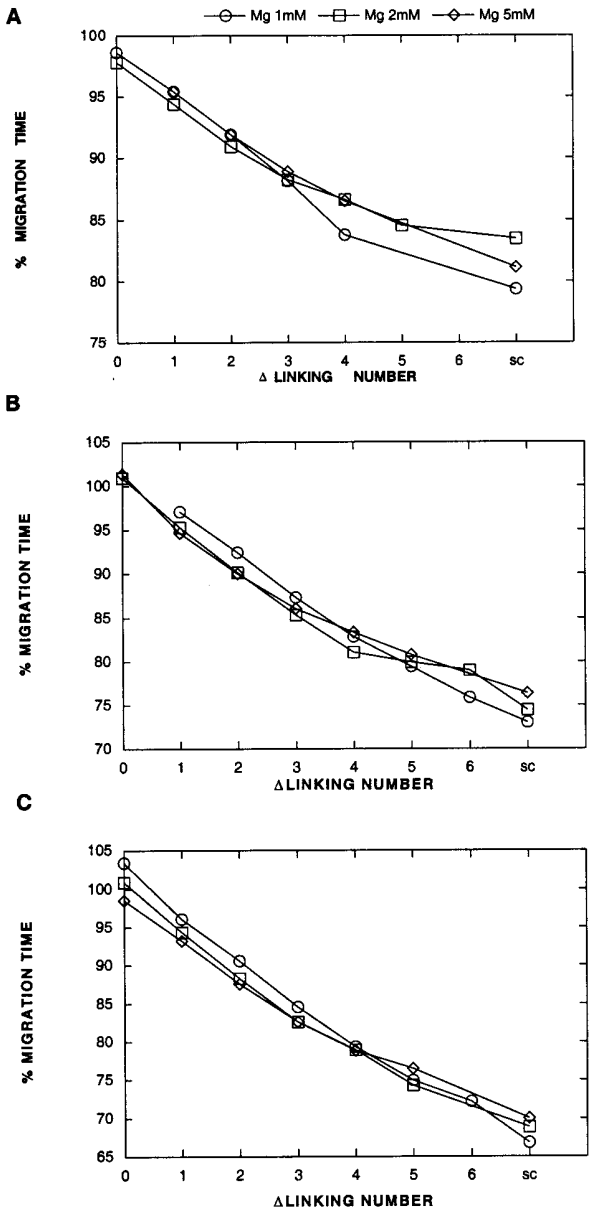

Figure 3. Diagrams of topoisomer migration times from separation in HPMC polymers of 86 kDa (A), 90 kDa (B), and 120 kDa (C) average molecular masses. The migration times of topoisomers with linking numbers from 0 to 6 were divided by the migration time of the OC form, which was taken as the reference peak with 100% retention time. The values obtained were then plotted for $MgCl_2$ concentrations of 1 (circles), 2 (squares), and 5 mM (rhombuses). The label SC on the *x* axis indicates the migration time of the supercoiled form.

Influence of Different Sieving Polymers on the CE Separation. Figure 3A shows the plot of retention times versus linking numbers of topoisomers obtained with 86 kDa HPMC as a sieving

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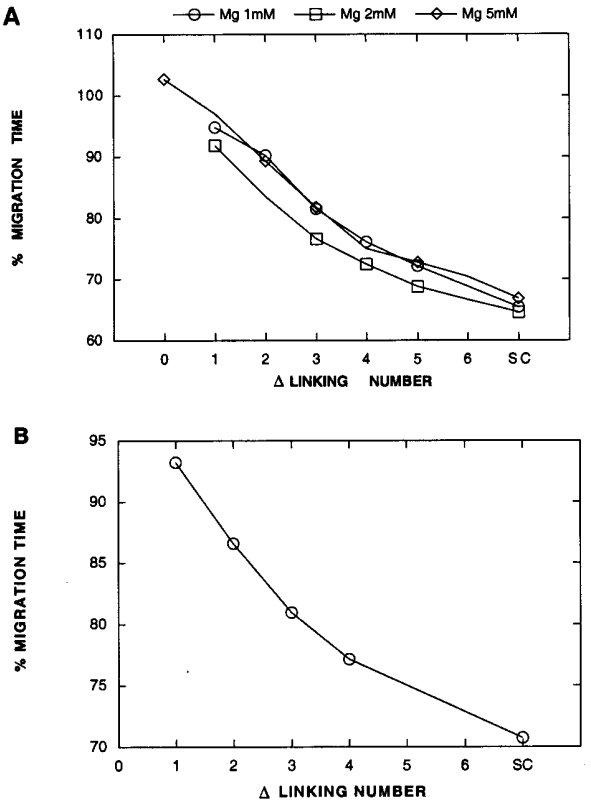

Figure 4. Diagrams of topoisomer migration times from separation in HEC polymers of 90−105 kDa (A) and 140−160 kDa (B) average molecular masses. The migration times of topoisomers with linking numbers from 1 to 5 were divided by the migration time of the OC form, which was taken as the reference peak with 100% retention time. The values obtained were plotted for $MgCl_2$ concentrations of 1 (circles), 2 (squares), and 5 mM (rhombuses). The label SC on the *x* axis indicates the migration time of the supercoiled form.

polymer; in the absence of $MgCl_2$, no separation of topoisomers was obtained. Topoisomers with the same linking number difference (ΔLk) show very similar migration times at all $MgCl_2$ concentrations tested (expressed as the percent of the migration time of the OC form).

Parts B and C of Figure 3 show the separation parameters of topoisomers resulting from the use of sieving polymers containing HPMC of 90 and 120 kDa, respectively. Also in these two cases, in the absence of $MgCl_2$, there was no resolution of topoisomers. It can be noticed that, in both cases, the slopes of the curves are higher than in the case of the curves of Figure 3A. The highest slope is obtained for the 120 kDa polymer. This indicates that the higher the molecular weight of the sieving polymer used, the better the separation of the topoisomers; this also correlates very well with the fact that the time windows between the OC and the supercoiled forms for the 86, 90, and 120 kDa polymers are respectively of 18, 25, and 30% of the total retention time.

The use of a 12 kDa form of HPMC as a sieving polymer showed total inability to resolve the supercoiled form and the OC form of the plasmid DNA and was not investigated further.

In the case of HEC, three different sizes of polymer were tested in the sieving buffer, each in the presence of $MgCl_2$ concentrations ranging from 0 to 5 mM. As in the case of HPMC, the average molecular mass had an effect on the DNA separation; the lowest molecular mass did not even allow resolution of the OC from the supercoiled form.

The 90−105 kDa HEC allowed separation only at 5 mM $MgCl_2$ (Figure 4A); the 140−160 kDa HEC allowed separation to occur with only 1 mM $MgCl_2$ (Figure 4B); in the absence of $MgCl_2$, no separation was obtained.

It should be noted that the separations using the high MW HEC (MW = molecular mass in this paper) were done at a polymer concentration of 0.05% instead of 0.1%; in fact, the 0.1% concentration produced a sieving buffer too viscous to allow efficient purging and cleaning of the capillary between runs.

The $MgCl_2$ concentration also had an effect on the electrophoretic mobilities of DNA topoisomers and their OC forms. Table 1 reports the mobilities of an OC form at all $MgCl_2$ concentrations and the types of polymers tested: it can be seen that the highest effect is between 0 and 1 mM $MgCl_2$, while the differences in mobility among 1, 2, and 5 mM $MgCl_2$ are less dramatic. We are uncertain about the mechanistic basis of this phenomenon, and its detailed investigation goes beyond the scope of this article. However, we noticed that the change from 0 to 1 mM $MgCl_2$ has an important effect on both the resolution of topoisomers and the increase of their retention time; we speculate that this may correlate with a structural transition induced by the interaction of $MgCl_2$ with the plasmid and that such an interaction is already appreciable at low $MgCl_2$ concentrations.

Finally, we are unable to explain why HPMC allows a wider range of separation conditions than HEC (the HPMC molecular mass was less critical than that of HEC for achieving DNA separation and required lower starting concentrations of Mg^{2+}; compare Figures 3 and 4). This question may find an answer in the interactions of DNA with the polymers,[22] phenomena that still need detailed evaluation.

CONCLUSIONS

The transferral of plasmid separation from the agarose gel system to the CE system showed that it is possible to separate plasmid topoisomers at electric fields far higher than those allowed by a separation in agarose gels. This separation principle can then fulfill the initial requirement for a biochemical assay of topo I activity and inhibition. The scheme for separation of DNA forms (supercoiled molecules, single topoisomers, and OC forms) reported in this paper allows direct quantitation (that is, not mediated by DNA-bound EtBr fluorescence) of the amount of DNA that has been relaxed by topoisomerase I. The ability to directly distinguish the amount of relaxation from the amount of enzyme-induced nicking is of key importance in the classification of topoisomerase I inhibitors.[25,26]

We think that the significance of this report lies in the description of topoisomer separations that require only a few minutes and the establishment of passage from agarose gel to CE separations. These are probably the most important aspects in terms of technological development and high analysis numbers. In fact, once the experimental parameters required for the separation are identified, the working principle of CE can be very

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easily transferred to the capillary array system and, even more importantly to microfabricated devices.[27,28] This development of CE will completely change the data productivity of the technique.

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SUPPORTING INFORMATION AVAILABLE

Figures illustrating chromatographic purification of the open circular and supercoiled plasmid forms, CE of these isolated forms, gel electrophoresis of topoisomers in low percentages of agarose, and CE electropherograms of topoisomers in the absence of $MgCl_2$. This material is available free of charge via the Internet at http://pubs.acs.org.

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