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Formation of a resistive region at the anode end in DNA capillary electrophoresis

We have studied the formation of a resistive region in the capillary during DNA separation. This effect is caused by an unequal change in the mobilities of cations and anions at the interface between the running buffer solution and the capillary. We studied the motion of the resistive region boundary by sequential removal of portions of the affected capillary end. We found that in the process of developing the resistive region the distribution of the electric fields in the capillary changes from uniform to extremely nonuniform, with a very high field (above 1 MV/cm) in the resistive region and a reduced field (80 V/cm) in the rest of the capillary. Though theoretically a resistive region may appear either at the anode (detection) or the cathode (injection) end of the capillary, all previous publications report the formation of the resistive region at the cathode side. In our experiments, however, the anomalous region is formed at the anode. Thus, the separated DNA peaks move towards the slowly progressing resistive region. Our results indicate that the DNA is stopped at the boundary and does not enter the region. When the resistive region is clipped off the peak motion resumes. This suggests that there exists a potential barrier at the resistive layer boundary that prevents the drift of the peaks towards the anode. The formation of the resistive region interferes with a normal separation process causing a gradual decrease of the capillary current and the deceleration and eventual quenching of the peak motion. For the ABI chemistry, we experimented with adding polymers to the electrode buffer to equate the transference numbers for anions and cations, and found the conditions at which this effect is completely eliminated.

Keywords: Anode resistive region / Anomalous conductivity / Capillary electrophoresis EL 5289

1 Introduction

Under certain conditions in capillary electrophoresis a resistive region forms in the capillary adjacent to one of the electrodes and interferes with a normal separation process. This undesired phenomenon is largely eliminated in commercial instruments whose sequencing protocol implicitly includes an effective antidote. Groups who design their own equipment and sequencing protocols often fall victims to this problem. Our group at SUNY Stony Brook developed a sensitive single-photon-counting miniature fluorescence detector that was incorporated in a compact "personal" size single-lane DNA sequencing instrument [1]. To test this instrument, we designed our own procedures for the DNA separation processes using commercially available chemistries. In our first sequencing experiments we used the Beckman-Coulter chemistry that employs coated capillaries in conjunction with the Beckman-Coulter polymer. The Beckman-Coulter protocol produced high-quality electropherograms with read lengths of approximately 600 bp. Figure 1a is an electropherogram of a four-color BigDye-labeled sample sequence obtained on our instrument using our standard Beckman-Coulter protocol with coated capillary. Since the actual shape and magnitude of the signals are important for this discussion, here and elsewhere in this paper we present as-received software-unshaped electropherograms. The amplitudes of the peaks gradually decrease, due to the injection bias [2], as the separation progresses. At the very end the electropherogram exhibits a large massive peak produced by long ($>$ 600 bp) unseparated fragments. With the Beckman-Coulter protocol, the current through the capillary is stable during the entire run. This protocol sustains several continuous sequencing runs with the same polymer in the capillary.

Later on we switched to a more popular ABI chemistry that employs uncoated capillaries in conjunction with self-coating less viscous polymers (POP-4 and POP-5) and ABI running buffer with EDTA recommended for Prism 310 single-capillary sequencing instrument in the anode and cathode vials. However, using this ABI recommended protocol we observed inferior results. Our typical electro-

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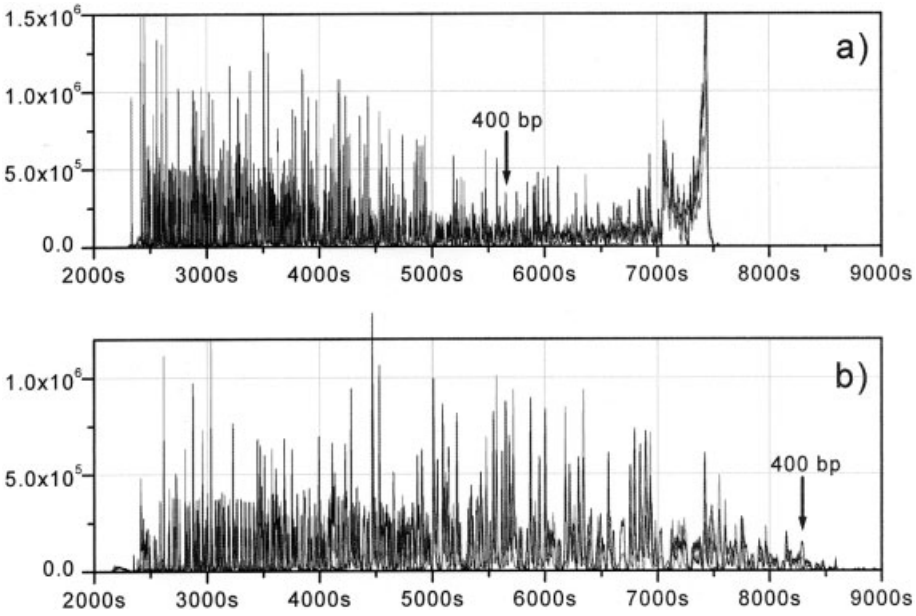

Figure 1. Two electropherograms obtained using (a) Beckman-Coulter, and (b) ABI chemistries (pure buffer in the anode vials).

pherogram with the BigDye four-color sample using the ABI protocol for POP-5 polymer is shown in Fig. 1b. Somewhere after 350 bp the peak height deteriorates, the separation slows down (note the difference in the position of 400 bp marker) and eventually stops. The capillary current gradually decreases and, when the separation is practically halted, the current stabilizes at about 30% of its original value. Attempts at second injection in the degraded capillary produce no peaks. Refilling the capillary with fresh polymer usually restores the original quality.

The well-known unwelcome phenomenon is due to the formation of anomalous conductivity zones in the capillary during the separation process. Spencer [3, 4] developed a model that explained the formation of the anomalous conductivity by an unequal change in the mobilities of cations and anions at the interface between the running buffer solution and the capillary gel. According to the nomenclature introduced by Spencer, the buffer solutions are divided into "normal" and "abnormal". In normal buffers, the low-concentration zone always moves into the retarding medium (polymer). This leads to the formation of a low-ion-concentration (high-resistivity) region at the buffer/gel interface that will migrate into the retarding medium with the rate proportional to the capillary current. Depending on the sign of the derivative dT/dC (where T and C are the transference number and the buffer concentration, respectively), the low-concentration resistive region may appear either at anode (dT/dC positive) or cathode (dT/dC negative) end of the capillary. For the "abnormal" buffers, the interface will move out of the capillary into the buffer reservoir and thus rendered harmless. All common buffer systems follow the "normal" pattern.

In modern commercial sequencing equipment the formation of the resistive region is eliminated by judicious selection of the polymer/buffer solution in the anode and cathode vials. We encountered the resistive region phenomenon because we developed a sequencing protocol for a home-built sequencer using commercial chemistry. We still do not know why the Beckman-Coulter buffer works and the ABI buffer does not. Since the composition of the running buffer is proprietary information inaccessible for the consumer we can only speculate that the former contains some additives necessary to equalize the transference number, and the latter does not.

Publications about the resistive region appeared periodically in the scientific discussion in 1980s and 1990s as new research groups were entering the field of capillary electrophoresis and investigated various aspects of this effect ([3–12] and references therein). Early observations of the anomalous behavior during the capillary DNA separation were reported in cross-linked gels. The effect was often described as a decrease in the capillary current, "loss of gel stability" and "gel breakdown" [5–7]. Since the cross-linked gels were intended for multiple use, the authors resorted to clipping the cathode end of the capillary to restore the sequencing ability of the capillary [5, 8]. Swerdlow *et al.* [5] suggested that one solution to this problem would be to use some additives to the running buffer to match the transference number in the cap-

illary. With the cross-linked polymer in the capillary, they added monomers to the running buffer, in the amount to match the conductivities of the two media (the conductivity of the 6% gel-buffer solution matched that of 4.2% of monomer-buffer solution). This group was also first to report the degrading influence of the presence of a large concentration of template in the sample. They attributed this influence to the plugging of the polymer pores that leads to even larger disparity of the transference number at the buffer/polymer interface.

With the advent of linear polymers, the effect of the resistive zone formation during the separation process was also observed. Most relevant to our experience is the series of works from The University of Alberta [9–12] that investigated processes in linear polyacrylamide during the electrophoresis in precoated capillaries. The resistive zone in their capillaries was localized in the first few centimeters at the injection end. They found that the extent of the effect depend on the age of the polymer. There was an optimum period in the life of polymer when the resistive zone formed less readily. They also reported a negative effect of the template presence [9–11], explored the possibility of multiple use of capillary polymer [11], and studied the effect of buffer composition [12].

All above-quoted papers describe the situation when the resistive layer is formed at the cathode end of the capillary. Our unique situation is that we consistently observe the resistive region at the anode end. The location of the resistive zone (cathode or anode) depends on the sign of d*T*/d*C* that in turn is determined by mobilities, sizes and concentrations of the ions of the buffer. Not knowing the chemical composition of the ABI buffer and other consumables, we do not attempt to explain the near-anodic location of the resistive region in our capillaries. However, the position of the resistive region at the anodic (detection end) gives us an opportunity to directly observe the behavior of the DNA peaks in this region.

2 Materials and methods

The experiments were carried out at room temperature, using our single-lane sequencing instruments [1] equipped with an Nd-YAG laser ($\lambda = 532$ nm, 10 mW; Intelite, Minden, NV, USA) as a fluorescence excitation source. Our fluorescence detection module employs a single-photon counter (Hamamatsu H6240–02). Both the excitation light and the fluorescent response are delivered to and from the detection/excitation window *via* multimode optical fibers. The uncoated capillaries employed in this study were from Polymicro Technologies, (Phoenix, AZ, USA) 60 cm long, 75 μm ID fused silica. For convenience of comparison we used commercially available ABI (Foster City, CA, USA) four-color sequencing standards labeled with BigDye (PN 4304154) and dRhodamine (PN 4303120) dye sets. The ABI consumables used in our experiments were ABI running buffer (PN 402824) and polymers POP-4 (PN 4028380) and POP-5 (PN 4313087). With coated capillaries we used Beckman-Coulter (Fullerton, CA, USA) polymer (CEQ separation gel LPAI, PN 608010) and separation buffer (PN 608053). The detection window 1 cm long was formed by removing the Kevlar coating at 5.5 cm from the anode end of the capillary. The DNA samples were processed according to the ABI protocol and introduced into the capillary by electrokinetic injection (3 kV, 40 s). After the injection the inlet end of the capillary was placed into a tube with the corresponding (BC LPAI or ABI 310) buffer. The outlet end was placed either in a pure buffer or in various mixtures of the polymers and the buffer. The separation was conducted at voltages corresponding to the electric field of 200 kV/cm. The electric current through the electrode-capillary system was monitored during the entire run.

3 Results

3.1 Distribution of electric fields in a capillary after formation of resistive region

To find the distribution of electric fields in the capillary after the resistive region had been formed, we subjected a POP-5 filled 60 cm long uncoated capillary with both ends immersed into pure buffer-filled vials to a DNA separation process and obtained an electropherogram similar to that in Fig. 1b. After completion of the run, we sequentially cut off ten 1 cm long pieces from the anodic end of the capillary and each time measured the resistance of the remaining part. The second capillary was subjected to an electric stress identical to that endured by the first capillary but without the DNA injection and separation.

In Fig. 2 we plot the measured resistance of the capillaries after each consecutive cut of a 1 cm long segment from the anodic end. The solid line in the figure represents a freshly filled capillary with a uniform distribution of its resistance along its length. The slope of this line (18 Mohm/cm) yields a differential resistance of the capillary, that is, the resistance of a 1 cm long capillary segment. Two curves in the figure represent the capillary resistances, one after the DNA separation and the other after just the electric stress. Both curves exhibit two regions with vastly different slopes. High slopes near the anodic end are indicative of a high differential resistance of this segment of the capillary. As we move away from the anode, the slope sharply decreases and approaches

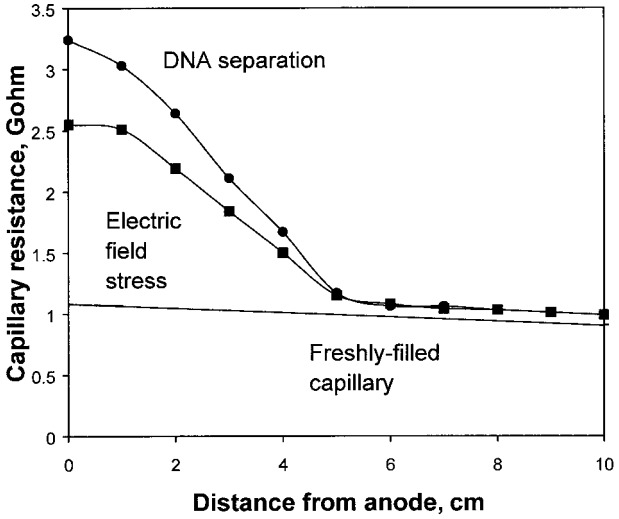

Figure 2. Specific resistance plotted as function of distance from the anodic end of the capillary measured by consecutive clipping of 1 cm pieces of the capillary from the anodic end. Experiments were carried out with pure buffer in the anode vials.

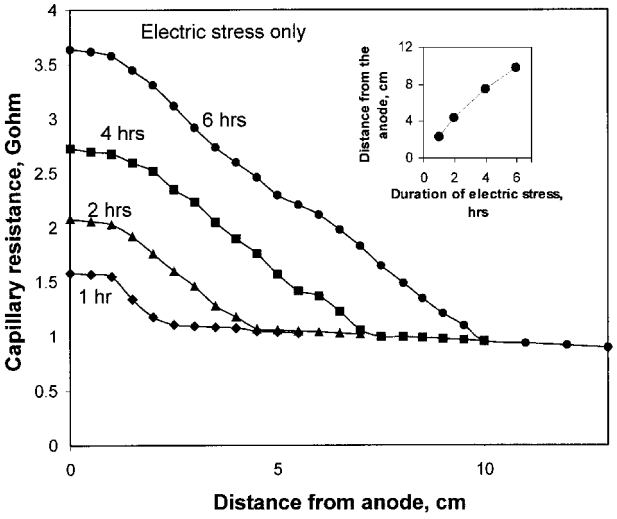

Figure 3. Dynamics of the development of the resistive region. The insert shows the rate of the expansion of the resistive region from the anode as a function of time.

that of a freshly filled capillary. As can be inferred from this figure, the highly resistive area of the capillary is only several centimeters long and is equal in length for both experiments. Noteworthy, both the slope of the curve and the magnitudes of the resistance for the capillary exposed to DNA are higher than for that exposed to the electric stress only. The current at the end of the run with DNA injection, 3.5 μA, is lower than that at the end of the "only electric field" run, 5.2 μA. This can probably be attributed to the clogging effect of DNA template [5, 11] that exacerbates the disparity in the transference numbers.

From the data in Fig. 2 we can calculate the redistribution of the electric fields in the capillary. In the first capillary subjected to the DNA separation, the resistance of the depleted segment is 2.15 Gohm. With the capillary current of 3.5 μA, the voltage drop on this segment is 7.5 kV, out of applied 12 kV. Maximum field in this region can be determined as a maximum slope of the curve equal to 1.7 kV/cm (compare with the original uniform field of 200 V/cm). In the remainder of the capillary the electric field is only 80 V/cm.

3.2 Dynamics of the development of the resistive region

We prepared four new freshly filled 60 cm long capillaries and subjected each to the separation electric stress of different durations, from 1 to 6 h. After each run, the anodic end of each capillary was removed piece by 0.5 cm piece, and the capillary resistance was measured at each step. The resulting resistances are shown in Fig. 3. The general behavior of the resistance curves is similar to that observed in the first two experiments. From the figure, we can infer the velocity of the inward migration of the resistive zone by plotting the distance of the interface (the inflection point on the curves) from the anode end (see the insert). Though the curves in Fig. 3 exhibit some roughness in the resistive region, their average slopes (average specific resistances) are very similar. The roughness may result from a scatter introduced by the uneven cuttings of 0.5 cm capillary fragments. The specific resistances in the remainder of the capillaries after removal of the depleted area also do not depend on the time of the stress (the slopes of all the curves far from anode are identical).

3.3 Fitting the transference number at the running vial/capillary interface

This experiment was designed to find the conditions at which the resistive region does not form, that is, when the ratio of the mobilities for cations and anions at the vial/capillary interface is the same. It was suggested [5, 12] that this ratio could be evened by adding some polymer to the running solution in the buffer vial (in our case, the anode vial). In Fig. 4 we show a series of electropherograms carried out with progressively increasing concentrations of POP-5 in the anode vial. The top electropherogram belongs to the experiment with

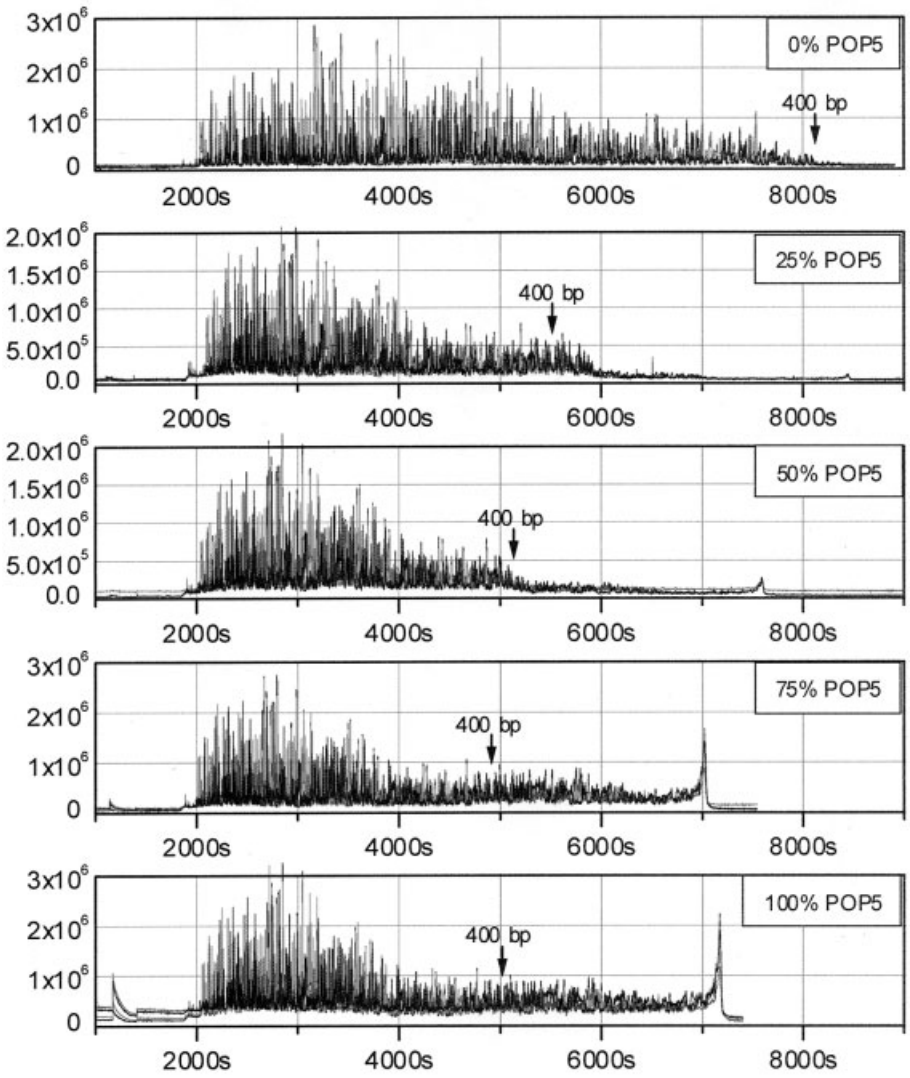

Figure 4. Electropherograms obtained with various polymer contents in the output vial.

pure buffer in the vial and the bottom corresponds to 100% polymer. The quality of the electropherograms gradually improves with increasing the polymer concentration in the output vial. The electropherogram with 75% POP-5 in the output vial is the best with 600 distinguishable peaks. The 100% electropherogram is very similar to that with 75%, and 50% electropherogram is of an intermediate quality. Adding the polymer to the cathode vial had no effect. For POP-4 polymer, the best result is achieved at 50%. Exceeding this optimum concentration led to a degradation of the separation quality.

At the optimal concentrations of POP-4 and POP-5 polymers in the anode vial the capillary can be used several times without refilling with fresh polymer. We carried out up to ten separations without noticeable deterioration of the separation quality.

3.4 DNA peaks are detained at the resistive zone

This two-stage experiment was designed to observe the behavior of the separated peaks when they approach and enter the expanding resistive region. We removed the Kevlar cover to form two detection windows, one at the standard distance 5.5 cm from the anode end and another at 12.5 cm, and carried out the injection and separation with pure buffer in the anode vial. At some point the peaks disappeared entirely. We then cut off the anode end of the capillary whose length was calculated to be about 1cm longer than the resistive region (the run took ~2.5 h; from Fig. 3, the extension of the resistive region was about 6 cm, we removed 7 cm), substituted the pure buffer in the output vial with the 75% polymer mixture, and continued the run while detecting the fluo-

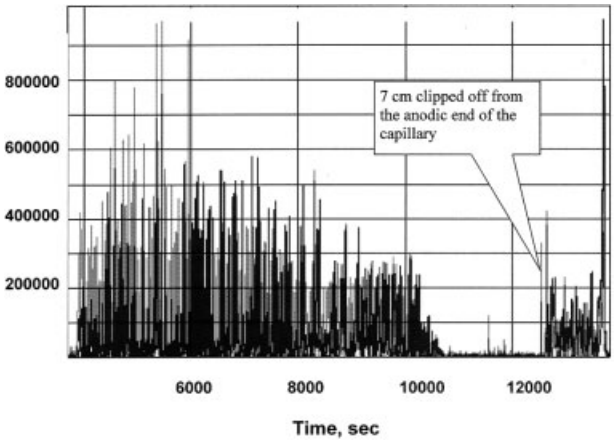

Figure 5. Electropherogram of the two-stage separation process. In the first stage, the resistive region ~6 cm long was formed at the anode. The motion of the peaks was completely extinguished. Before the second stage, the anodic end of the capillary (7 cm long) was clipped off, and the separation voltage applied again. The peaks resumed their motion.

rescence through the second window. After removal of the affected region the peaks started moving again and the run was completed (electropherogram in Fig. 5).

3.5 Behavior of the DNA peaks in high electric fields

This experiment was designed to observe the behavior of separated peaks under extremely high electric fields similar to those observed in the resistive region (the maximum field in the region is on the order of 1.7 kV/cm). The built-in power supply in our sequencer can reach only to 15 kV. With an external power supply (Spellman CZE1000R) we can produce 25 kV. Still, to achieve the desired fields we had to limit the length of the capillary to 15 cm. This length is not sufficient to have both ends of the capillary in their respective vials (the minimum capillary length of our instrument is 33 cm). To overcome this obstacle, we first carried out our standard separation run (60 cm, 12 kV), with the 75% POP solution in the anode vial. When we observed regular, well-shaped large peaks (150–160 bp) appearing at the detection window we turned off the voltage and very carefully nicked and broke the capillary to the desired length of 15 cm. This length of the capillary contained already separated zones. Prior to the run, the capillary was threaded through a tight hole in a small plastic cup, and after the capillary was trimmed and stood upright, the cup was filled with the cathode buffer (the anode end was still in its vial with the POP-5/buffer mix). A Pt wire was immersed in the cup and its other end was connected to the output of the 25 kV power supply. We observed the peaks that were contained in the 15 cm of the capillary as they passed with a very high speed by the detection window. The peaks looked scrambled, washed out and distorted but nevertheless much alive, distinguishable and even readable.

4 Discussion

Below we shall summarize the results of the experiments described in the previous section and suggest a model that agrees with our observations. We observe that when the anodic vial is filled with buffer, the current slowly drops to as low as 1/3 of its original value. This is consistent with the continuous increase in the total resistance of the capillary due to the formation of the resistive zone at the anode (Sections 3.1 and 3.2). At these conditions we also observe a deceleration of the separation process (compare the time of peak 400 bp in Figs. 1a and b). This is expected because at a constant voltage the electric field in the expanding resistive region increases at the expense of the field in the rest of the capillary.

Later in the separation process we observe an abrupt degradation of the peak amplitudes, followed by the complete disappearance of the peaks. The drop in the field in the "healthy" remainder of the capillary from 200 to 80 V/cm cannot explain the complete cessation of the peak motion. We notice that the moment of the abrupt diminishing of the peaks coincides with the time necessary for the resistive region to migrate from the anode to the observation window (6 cm, about 2.5 h from high-voltage application, including 10 min cleaning and 40 min to the first peak appearance). Moreover, when the resistive region moves above the window, the peaks disappear. This suggests that the peaks either disappear in or never enter the resistive region.

It is known that high electric fields can detrimentally affect the resolution of the separation. Electric field can affect the peak separation *via* several mechanisms. The band broadening due to local Joule heating and strong zone diffusion causes a considerable loss of separation, especially for longer nucleotides, even at relatively moderate fields of 300–400 V/cm [13–15]. Another band broadening mechanism is the radial temperature nonuniformity. In the capillary the heat is dissipated radially through the glass walls. The resulting temperature gradient across the capillary cross-section nonuniformly alters the viscosity of the polymer such that the electrophoretic mobility of the bands is higher along the capillary axes [16–18]. This effect is insignificant for separation fields of 200–250 V/cm [14], but at higher fields may become important [13]. In addition, turbulent motion in high fields may also cause intermixing of the zones.

At extreme fields of our experiment the loss of separation may also occur due to the transition from the regime of reptation without orientation, where the fragments mobility is size-dependent, to the regime of reptation with orientation where the fragments mobility is size-independent [19]. Generally, sequencing experiments are usually carried out at electric fields below 300 V/cm. It is known that the best resolution is achieved at about 200 V/cm, and deteriorates at higher fields [13]. Available data in the literature usually deal with fields below 500 V/cm. In one work, the authors report results obtained at 1200 V/cm [20], however, their experiments were carried out in cross-linked polymer. No information is available on the zone behavior in the noncross-linked polymer at fields of the order that occur in the resistive region. One of our experiments (Section 3.5) was carried out to observe the behavior of the separated peaks under the field of 1.7 MV/cm. The results indicate that such field indeed distorts, smears, and misshapes the peaks. But it does not make them disappear. Therefore, the extreme electric field in the resistive region is not responsible for the cessation of the peaks.

The other possibility is that the peaks never enter the resistive region. We speculate that there is an electrostatic barrier for the motion of DNA near the boundary between the resistive region and the rest of the capillary. Such a barrier may be formed by a charged layer at the boundary region. Spencer's model [3,4], which neglects diffusion, assumes that this layer does not exist. However, this assumption breaks down when diffusion is included. At the boundary between a low-field and a high-field region the ion distribution is highly nonuniform and is accompanied by a net charge buildup and a local potential maximum. This situation is familiar from transport properties of semiconductor devices with highly nonuniform carrier concentration. The existence of a potential maximum does not affect the continuity of the current due to majority carriers (ions of the electrolyte), which is maintained by diffusion. On the other hand, the "test" charges (DNA) get trapped by the potential barrier. This effect naturally explains the complete disappearance of the fluorescent peaks once the resistive portion of the capillary extends beyond the detection window; this clearly observed fact cannot be explained in the original Spencer model. Clipping off the resistive region at the anodic end of the capillary resumes the motion of the peaks towards the anode (see Sections 3.1 and 3.4).

In our experiments, the injected DNA fragments and the boundary of the resistive region are moving toward each other and eventually collide. In contrast, when the resistive region forms at the cathode, the DNA peaks and the boundary of the resistive region are moving in the same direction. However, the injected plug moves out of the budding resistive region faster than the region is formed (even the longest fragments move with a speed of several mm/min, while the resistive region expands at the rate of 2 cm/h). When the peaks leave the boundary behind, they can still be affected by the existence of the resistive region through the electric field redistribution in the capillary, but the main effect is the increase in the total resistance of the capillary. In some reported cases when the resistive region at the cathode was formed prior to the injection (for example, during the preseparation cleaning [5,8]), no injection occurred, because during the cleaning a short resistive region was formed and the injected DNA was held at its boundary. When the offending end was clipped, the DNA injection and separation proceeded normally. Note that the presence of just a high field at the capillary entrance by no means would stop the injection. Quite the contrary, the injection would be enhanced by the high field at the capillary entrance.

In conclusion, we have studied properties of the resistive region formed during the electrophoretic DNA separation. The main distinctive feature of our experiments is that the anomalous region with a high resistivity is formed at the anode, at the detection site. As the depleted region extends beyond the detection window, it becomes accessible to direct observation by fluorescent detection. We have found that DNA fragments do not enter the resistive region, but are detained at the boundary. When the resistive region is clipped off the peak motion resumes. These results suggest the existence of a potential barrier at the resistive layer boundary that prevents the drift of fragments towards the anode. Quantitative description of this barrier requires augmenting Spencer's model to include diffusion and the local deviation from neutrality of the electrolyte. This can be done by a numerical simulation, combined with further experimentation, and we are continuing our research in this direction. We encountered the effect of anomalous conductivity while testing a new DNA sequencing instrument built in our laboratory. For the ABI chemistry we found conditions at which this effect is eliminated, that is, the transference numbers for cations and anions are equated. It corresponds to the polymer/buffer mix of 75% and 50% for POP-5 and POP-4 ABI polymers, respectively.

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