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Analysis of Nucleic Acids by Capillary Electrophoresis

CHROMATOGRAPHIA CE Series

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4 Influence of Polymer Concentration and Electric Field

Experimental Study and Comparison with Theory

Annelise E. Barron and Christoph Heller

4.1 Introduction

4.1.1 Slab Gel vs. Capillary Electrophoresis of DNA

DNA molecules cannot be separated by free-solution electrophoresis [1], because in free solution the ratio of net molecular charge to friction coefficient (the electrophoretic mobility) is nearly equal for all DNA molecules regardless of their chain length [2, 3]. However, it was discovered in 1967 [4] that if electrophoresis is performed within a properly-formulated slab gel matrix (*e.g.*, agarose or crosslinked polyacrylamide), it is possible to separate differently-sized DNA molecules into distinct zones, where electrophoretic mobility is a decreasing function of DNA chain length. In addition to providing size-based separation of DNA, the crosslinked polyacrylamide or agarose network of a slab gel serves as a physical support during electrophoresis, substantially reducing diffusion and convection of migrating DNA

molecules. This allows the separated zones of differently-sized DNA molecules to remain relatively sharp, if gel formulation and electrophoresis conditions are chosen properly.

Although slab gel electrophoresis is a powerful technique with many important applications in biochemistry and molecular biology, it is also time-consuming to set up and run, difficult to fully automate or quantitate, and potentially hazardous to the scientist, due to the common use of neurotoxic acrylamide monomers to formulate gels, radioactive DNA labeling, and/or large amounts of buffer containing mutagenic DNA-intercalating dyes.

All of these drawbacks can be significantly reduced or eliminated when electrophoresis is performed within a microbore capillary (inner diameter 20-100 μm). Capillary electrophoresis (CE) is at least an order of magnitude faster than slab gel electrophoresis, may be completely automated, allows easier and more precise quantitation of results, and due to the small scale and self-contained nature of the apparatus, reduces health risks from toxic chemicals. Of course, along with the many advantages of miniaturization have arisen new challenges as well. The initial inconveniences of CE included the high-field instability of the crosslinked polyacrylamide capillary gels which first were used for DNA separations; the inability to run more than one "lane" in parallel using a capillary format; the inability to load large sample volumes (> 10 μL) onto a capillary, such as can be applied to a slab gel; and the difficulty of post-electrophoretic sample collection. We are happy to report, however, that these disadvantages of CE are rapidly being overcome (for technical details, please see the recent review by Barron and Blanch [5]). Although CE will most likely never completely replace preparative slab gel electrophoresis, it is an important complementary technique which will serve molecular biologists well in the coming years.

4.1.2 Capillary Electrophoresis of DNA: The Transition from Crosslinked Gels to Uncrosslinked Polymer Solutions

Soon after the advantages of performing biomolecule electrophoresis within a capillary rather than on a slab gel had been demonstrated [6], *gel-filled* capillaries were applied to the separation of nucleic acids. Many groups have investigated the use of capillaries filled with crosslinked polyacrylamide gels for DNA separations (*e.g.*, [7-21]. Excellent capillary electrophoretic separations of small DNA fragments may be obtained in crosslinked polyacrylamide. Single-base resolution is achieved for single-stranded (ss) DNA ranging from 15 to more than 500 bases (for DNA sequencing) [21], often with astoundingly high plate counts (*e.g.*, 15-30 million theoretical plates/meter [7, 22, 23]). However, the investigation of DNA separations in crosslinked gel-filled capillaries has been hindered by the difficulty of polymerizing gels of adequate quality, as well as by the persistent occurrence of gel breakdown and bubble formation during prolonged runs at high voltage [9-11, 24-28].

At some cost to DNA resolution, the difficulties associated with crosslinked intracapillary separation media can be avoided simply by omitting the crosslinking step. This is possible because in a fused-silica capillary, the anti-convective and DNA-separating roles of the electrophoresis matrix can be effectively decoupled. Even in the absence of a dense gel matrix, only very minimal thermal convection and diffusion of analyte molecules occurs during capillary electrophoresis. This is not only because of the capillary's minute dimensions, but also because the high electrical resistance of fused silica results in very low current

generation in typical electrophoresis buffers (even under high electric fields); hence only minimal Joule heat is generated. Due to the capillary's large surface area-to-volume ratio, the small amount of Joule heat which *is* generated can be rapidly and evenly dissipated with convected air [29], liquid cooling [30], or a Peltier device [31].

The anti-convective properties of a fused silica capillary allow greater freedom to the researcher in the choice of DNA separation matrix. A rigid anti-convective gel structure is not neccessary; one must simply find a matrix which imparts a size-dependence to the frictional forces which act on the DNA molecules. Contrary to original assumptions, crosslinking of the polymer matrix is in fact *not necessary* for DNA separation to occur when electrophoresis is performed within a capillary. Rapid, high-resolution DNA separations are routinely achieved in capillaries filled with uncrosslinked polymeric media, under potential gradients which would be unthinkable in a typical slab format (*e.g.*, 300 V/cm).

One of the primary advantages of using uncrosslinked matrices for DNA separation in capillaries is the relative ease of their preparation and use, compared to crosslinked matrices. Often, no gelation or chemical reactions are required for the preparation of uncrosslinked matrices; polymer solutions with moderate viscosities are simply pumped into the capillary, and can be rapidly and automatically replaced for each new separation. DNA molecules migrate much more rapidly through polymer solutions than they do through crosslinked polyacrylamide gels, dramatically shortening analysis times [32]. Perhaps most importantly, even high-concentration uncrosslinked matrices generally remain stable under the high potential gradients which are routinely employed for CE.

4.1.3 Polymers Used for CE Separations of DNA

In 1989, Zhu *et al.* [33] were the first to demonstrate the feasibility of using uncrosslinked polymer solutions for CE, employing both methyl cellulose and hydroxypropylmethyl cellulose for the separation of double-stranded (ds) DNA up to 4.2 kilobase pairs (kbp) in size. A year later, Heiger *et al.* [34] published a study demonstrating the use of sparsely-crosslinked and uncrosslinked linear polyacrylamide for high-resolution CE separations of both dsDNA and single-stranded (ss) DNA fragments. Strege and Lagu [35] later showed that low-viscosity methyl cellulose solutions could be used to separate dsDNA as large as 23 kbp in size. Since 1991, other researchers have investigated DNA separations using solutions of these and several other types of hydrophilic polymers. A brief but representative list includes hydroxyethyl cellulose [36-39], hydroxypropyl cellulose [40-42], hydroxypropylmethyl cellulose [40, 43-46], and polyvinyl alcohol [44, 47, 48], as well as liquefied agarose [49], linear polyacrylamide [28, 34, 46, 48, 50-52], and linear polyacryloylaminoethoxyethanol [53] (for more details, see Chapters 6 and 11).

One reason that researchers have attempted DNA separations using so many different types of polymer matrices for CE is that the relative importances of polymer properties as hydrophilicity, persistence length (chain stiffness), polydispersity, and chain length are only beginning to be understood; hence it has been necessary to mainly take an empirical approach to formulating a high-performance DNA separation matrix.

At this point we should mention that another, unexpected advantage of uncrosslinked matrices for CE has emerged along with the aforementioned ease-of-use issues: Experimen-

tal findings using uncrosslinked media have revealed new information about the mechanisms which will provide electrophoretic DNA separation. In particular, researchers have gained a new understanding of the matrix characteristics which are *necessary* for DNA separation to occur in CE, and in a related but separate question, those matrix characteristics which favor high-resolution separations of ssDNA and dsDNA in various size ranges of interest. As will be discussed below, this recent work should help to speed the process of matrix selection for the various applications of DNA electrophoresis.

4.1.4 A Brief Overview of DNA Electrophoresis Theories

As discussed in Chapter 2, experimental and theoretical investigations into the mechanism of low-field electrophoretic DNA separations in agarose and crosslinked polyacrylamide slab gels have led to the formulation of two well-known models for DNA electrophoresis: the Ogston model [54-56], and the reptation model [57-67]. In the Ogston model, electrophoresing molecules are treated as spherical coils moving through the voids in a random fiber matrix. According to the model, a molecule may only move forward if it encounters a void (or "pore") which is large enough in volume to accommodate its passage. The Ogston model is applicable to the case in which DNA molecules have radii of gyration less than or equal to the average pore radius of the gel. On the other hand, the reptation model is applied when the radius of gyration of the undeformed DNA coil would exceed the average pore radius of the gel. In this case, DNA must deform from its random-coil conformation to enter and migrate through the gel matrix. The reptation model describes the conformation of electromigrating DNA as extended and snake-like, having head-and-tail character, with its forward motion confined to "reptation tubes" which are formed by the network of gel pores. During the last years, the original reptation concept has been refined to take into account the influence of the electric field (Biased Reptation Model, BRM) and also the influence of tube length fluctuation (Biased Reptation with Fluctuations, BRF, see Chapter 2).

In their application to DNA electrophoresis, both the Ogston and reptation models were originally formulated to capture the essential physics of DNA migration in true gels (such as agarose or crosslinked polyacrylamide), and hence rest upon the assumption that DNA migrates through an undeformable network of topologically static "pores" having a certain average pore radius.

To our knowledge, the earliest theoretical paper dealing with electrophoresis in deformable, non-rigid media is due to Bode [68]. Based on his finding that linear polymer-supplemented agarose slab gels provide improved separations [69, 70], Bode suggested an alternate approach to understanding gel electrophoresis of poly-anions such as DNA and SDS-complexed proteins [68]. Using a simple mathematical model, he was able to demonstrate that experimental results from polyacrylamide gel electrophoresis of macro-ions, which had been interpreted earlier as substantiation of the "rigid-pore" concept of a gel, could be equally well-explained by a microscopic viscosity model based on the assumption that solvated polymer molecules incorporated into the gel represent obstacles which must either be deformed or cleared aside by the electrokinetic pressure of the migrating macro-ions [68]. In Bode's model, a polyacrylamide gel is represented as a "viscosity emulsion" consisting of two types of interpenetrating regions, one of which allows DNA to migrate at

its free-solution mobility, and the other of which presents more resistance to straight-on DNA motion; this additional resistance is modeled as an increased viscosity in the latter region. Bode's "viscosity model" did not gain wide acceptance, primarily because it was quickly eclipsed by the successes of the reptation model of DNA electrophoresis, the concepts of which were first introduced in 1982 [57]. However, the basic idea behind the Bode model, that constrictive gel pores are not necessary for DNA separation, was to become important again in the 1990s with the advent of CE in uncrosslinked polymer solutions.

4.1.5 Dilute and Semi-Dilute Polymer Solutions: The Entanglement Concept and its Relevance to the Mechanism of DNA Separation in Polymer Solutions

It is helpful to preface further discussion with a clarification of what is meant when we refer to dilute, semi-dilute, and entangled polymer solutions (for more details, see also Chapter 1). In a dilute solution, polymer concentration is low enough and there is essentially no interaction between solvated polymer molecules. In this regime, viscosity increases in direct proportion to polymer concentration, and the slope of a log-log plot of viscosity *vs.* concentration is constant at about 1.0. The *semi-dilute* concentration regime is entered when the concentration is raised sufficiently that polymer coils begin to interact and overlap in solution. If the polymers are of sufficient length, they will begin to entangle with each other as their coils overlap. This transition from a dilute to a semi-dilute polymer solution occurs at "the overlap threshold concentration", or c^*, defined as the concentration at which the polymer coils just begin to touch in solution. Using this definition, c^* can be calculated using the geometrical formula [71] (same abbreviations as in Chapters 1 and 2 are being used here):

$$c^* \cong 3\, M_W / 4\pi N_A\, R_{gp}^3 \cong 1.5\, [\eta]^{-1} \qquad (1)$$

The overlap threshold concentration can also be measured directly from viscosity *vs.* concentration data. The formation of an incipient entangled polymer network in solution [72] is evidenced by a large increase in viscosity, with a corresponding increase in the slope of the log viscosity *vs.* log concentration curve (for example, Figure 4.1 shows this type of plot for a sample of hydroxypropyl cellulose dissolved in Tris-Borate-EDTA, a common DNA electrophoresis buffer).

In early publications demonstrating DNA separations in polymer solutions, researchers often discussed their results with the implicit assumption that the mechanism of high-field CE separation of DNA in uncrosslinked polymer solutions is very similar to that observed under low electric fields in slab gels made of agarose or crosslinked polyacrylamide (*e.g.*, [34, 40, 47, 48, 74, 75]). That is, it was often presumed that polymer solutions must be concentrated enough to be "gel-like" in nature if they were to provide DNA separation.

It was pointed out by Grossman and Soane in 1991 [74] that an uncrosslinked polymer solution could be qualitatively compared to a crosslinked polyacrylamide gel if, and only if, the polymer concentration was sufficiently high that the polymer chains formed an entangled network, *i.e.*, a sort of "dynamic mesh" with "fluctuating pores." They proposed that in a

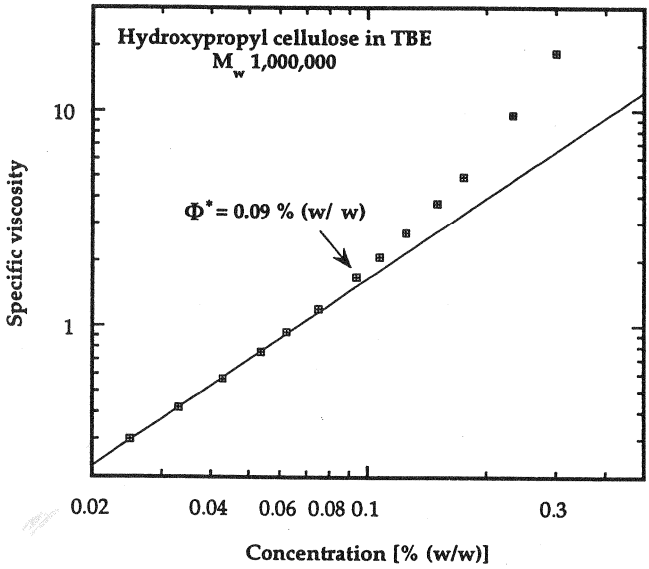

Figure 4.1 A log-log plot of the specific viscosity of an aqueous polymer solution as a function of polymer concentration. The point of departure of the data from linearity is the approximate concentration at which an entangled polymer network is formed within the solution. In this case, the polymer is hydroxypropyl cellulose, with a weight-average molecular mass of about 1,000,000 g/mol, dissolved in an 89 mM Tris-89 mM borate-5 mM EDTA buffer, pH 8.15. The entanglement threshold concentration, c^*, is approximately 0.09% (w/w) HPC. Viscosity measurements were taken at 25°C with an automated Ubbelohde viscometer.

highly entangled polymer solution, DNA could be separated by the Ogston and/or reptation mechanisms which were postulated for rigid, porous slab gels. The systematic studies of the dependence of DNA electrophoretic mobility on polymer concentration and electric field address these questions directly.

4.2 DNA Mobility as a Function of Polymer Concentration, Molecular Weight and Applied Electric Field: Systematic Experimental Studies and Comparisons with Theory

As we have discussed, high-resolution CE separations of DNA in uncrosslinked polymer solutions were first demonstrated in 1989 [33]. However, to understand and verify the underlying DNA separation mechanisms which prevail in different types of matrices, systematic studies are necessary. Such studies were undertaken by Barron *et al.* [37, 38, 42], who worked with dilute and semi-dilute polymer solutions, and by Mitnik *et al.* [41], who mainly focused on the use of semi-dilute (i.e., entangled) polymer solutions.

4.2.1 DNA Separation in Ultra-Dilute and Dilute Polymer Solutions

Influence of Polymer Concentration and Size

In the dilute to semi-dilute concentration regime, a variety of polymer types, molecular weights, and concentrations have been tested systematically for their ability to separate dsDNA fragments. In several related studies by Barron et al. [37, 38, 42], hydroxyethyl

cellulose, hydroxypropyl cellulose, and linear polyacrylamide, each of at least two different molecular weights, were each employed for CE separation of dsDNA, at concentrations which ranged from extremely dilute to semi-dilute (entangled).

It was first demonstrated in 1993 that DNA separation is possible in uncrosslinked polymer solutions having concentrations *well below* the polymer overlap threshold [37], experimentally confirming the theoretical prediction of Viovy and Duke [71]. In a 1994 study, Barron *et al.* [38] systematically measured the mobility of double-stranded DNA restriction fragments ranging in size from 72 bp to 23 kbp (using a mixture of the ΦX174-*Hae*III and the λ-*Hind*III restriction digests) as a function of hydroxyethyl cellulose (HEC) concentration for HEC of two different average chain lengths. The weight-average molecular weights of these samples were measured by light scattering [76] to be M_W 139,000 for the short-chain HEC sample, and M_W 1,315,000 for the long-chain sample. Viscosity measurements were performed to determine the overlap threshold concentrations of these HEC samples [37]: for HEC (M_W 139,000), $c^* \approx 1.80\%$, while for HEC (M_W 1,315,000), $c^* \approx 0.37\%$. To measure DNA electrophoretic mobilities, the technique of counter-migration CE (CMCE) in uncoated fused silica capillaries was used, in which electroosmotic flow is exploited to drive electromigrating DNA fragments past the UV absorbance detector. Hence, DNA fragments pass the detector in the order of largest (lowest electrophoretic mobility) to smallest (highest electrophoretic mobility). DNA electrophoretic mobility can be reliably determined using either polyacrylamide-coated capillaries (which exhibit no electroosmotic flow) or uncoated capillaries. For further explanation of CMCE, and a comparison of separation in polyacrylamide-coated capillaries to that in uncoated capillaries, see reference [77].

Experimental results show that molecular weight is a very important factor in the efficiency of DNA separations in dilute polymer solutions. Low-molecular weight HEC (M_W 139,000) will provide reasonable separation of small DNA fragments at dilute concentrations (*i.e.*, at concentrations well below the measured c^* of 1.80%), but does not provide good separation of larger fragments in the ΦX174-*Hae*III restriction digest (872, 1078, and 1353 bp) at any concentration. Figure 4.2a depicts the CE separation of restriction fragments up to 23.1 kbp in length, using a 0.30% solution of low-molecular weight HEC polymers (M_W 139,000). 0.30% is roughly the optimum concentration, for this particular HEC sample, to separate DNA larger than 1 kbp. Yet still the DNA fragments larger than 872 bp are not separated with good baseline resolution. This illustrates the relative uselessness of low-molecular weight cellulosic polymers for the separation of DNA larger than 1 kbp.

Increasing the concentration of short-chain HEC results in progressively poorer resolution of larger DNA fragments, compared to that achieved at 0.30% HEC. Figure 4.2b shows the separation of ΦX174-*Hae*III restriction fragments in 0.90% HEC (M_W 139,000). While resolution of the smaller DNA fragments does improve markedly as HEC concentration is increased, 10-base pair resolution of the 271 bp/281 bp fragments was not achieved using this low molecular weight-HEC. At a higher concentration of 3.0% HEC (M_W 139,000) ($c > c^*$), the three largest DNA fragments eluted as one poorly-shaped peak, and appeared to have anomalously low mobilities, as though they were becoming trapped in the HEC matrix while the smaller fragments were not (electropherogram not shown). This can be seen clearly in Figure 4.3, which is a plot of DNA electrophoretic mobility *vs.* low-molecular weight HEC concentration. The mobilities of the larger restriction fragments (> 603 bp) are so close on this plot as to be indistinguishable, although the peaks can be clearly discerned in Figures

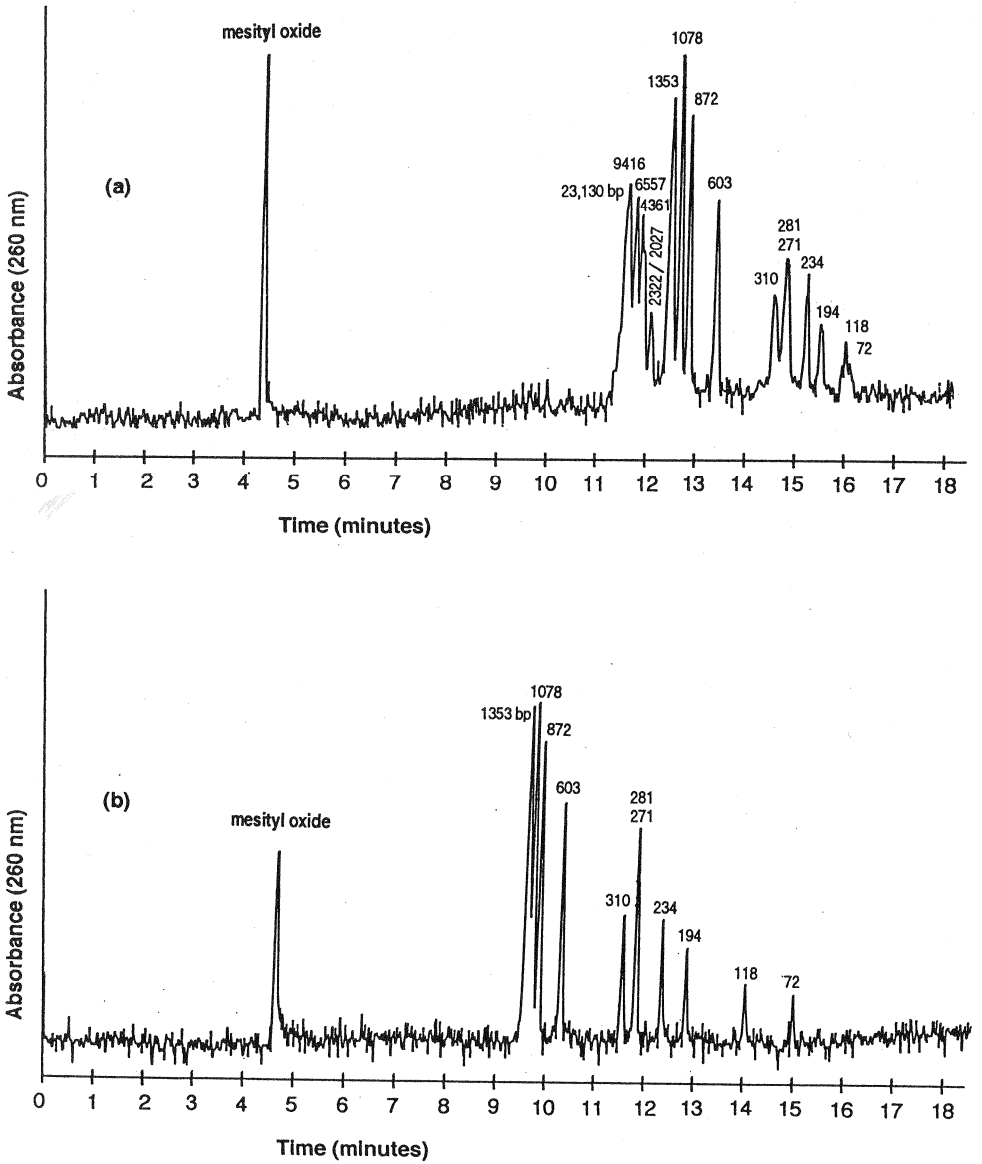

Figure 4.2 Separation by counter-migration capillary electrophoresis of λ–*HindIII* and ΦX174-*HaeIII* restriction fragments (in non-stoichiometric mixture), in (a) 0.30% HEC (M_w 139,000), and (b) 0.90% HEC (M_w 139,000). Buffer: 89 mM Tris(hydroxyethylaminomethane), 89 mM boric acid, 5 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.15. Capillary: 51 μm i.d., 50 cm total length (35 cm to detector); temperature, 30 ± 0.1°C. Detection was by UV absorbance at 260 nm. Injection was hydrodynamic. Electrophoresis conditions: field strength 265 V/cm, current was (a) 8.0 μA, (b) 9.6 μA.

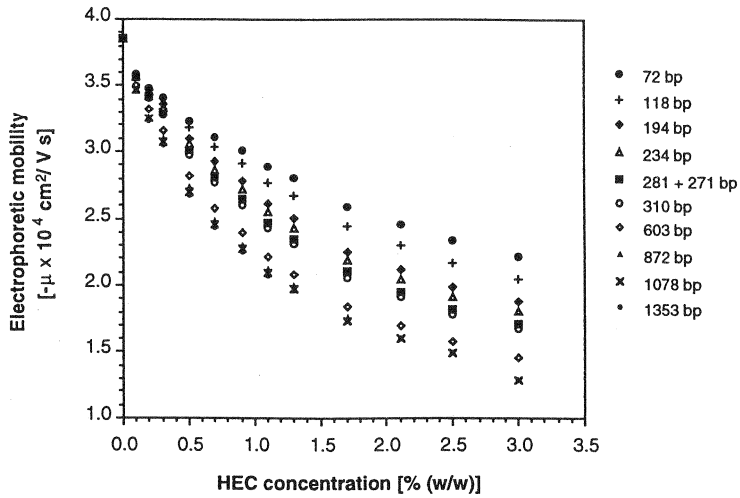

Figure 4.3 A plot of DNA electrophoretic mobility *vs.* HEC concentration (M_W 139,000) for DNA restriction fragments ranging from 72 bp to 23130 bp in length. Data points at each HEC concentration are the average of 3 – 5 individual determinations. Average run-to-run variation in calculated electrophoretic mobilities: ± 0.33 %. DNA electrophoretic mobility was calculated by subtracting the electroosmotic mobility, calculated from the elution time of a neutral marker, from the *apparent* electrophoretic mobility of the DNA fragments, as DNA electrophoretic motion was opposite in direction to the electroosmotic flow which was used to drive it past the UV absorbance detector. The electrophoresis buffer, all conditions, and DNA sample, same as in Figure 4.2.

4.2a and 4.2b. However, if one follows the smooth curve of the data in Figure 4.3 for the 1353/1078/872 bp fragments, a sudden drop in mobility can be seen at 3.0% HEC. Although the measured overlap threshold concentration for this HEC sample is ≈ 1.80% [37], the only discernible change in the mobility data observed at or near this concentration is the complete loss of resolution for the three largest DNA fragments. It is clear, therefore, that entanglement of the HEC chains with each other is not an important factor in achieving DNA separations, and may in fact even be detrimental to the resolution of larger fragments. Furthermore, low-molecular weight HEC polymers are not effective for separations of DNA larger than 1 kbp, although relatively poor resolution may be achieved at low concentrations.

Much better separation of large DNA fragments is achieved in solutions of high-molecular-weight HEC polymers. An HEC sample with a weight-average molecular weight of 1,315,000 g/mol, with chain lengths on average ten times greater than the previously discussed sample, was determined to have a c^* of approximately 0.37%. A systematic study was undertaken to determine the minimum long-chain HEC concentration required for DNA separation. Figure 4.4a depicts the separation by CE of the mixture of ΦX174-*Hae*III and λ-*Hind*III DNA restriction fragments, in a 0.15% HEC (M_W 1,315,000) solution. Clearly, this longer HEC provides better resolution of large DNA restriction fragments than the short-chain HEC, as well as partial resolution of the 271 and 281 bp fragments. With a more sensitive detection system (such as laser-induced fluorescence) smaller sample volumes could be used and the 271 and 281 bp fragments likely would be resolved to baseline. Figure 4b

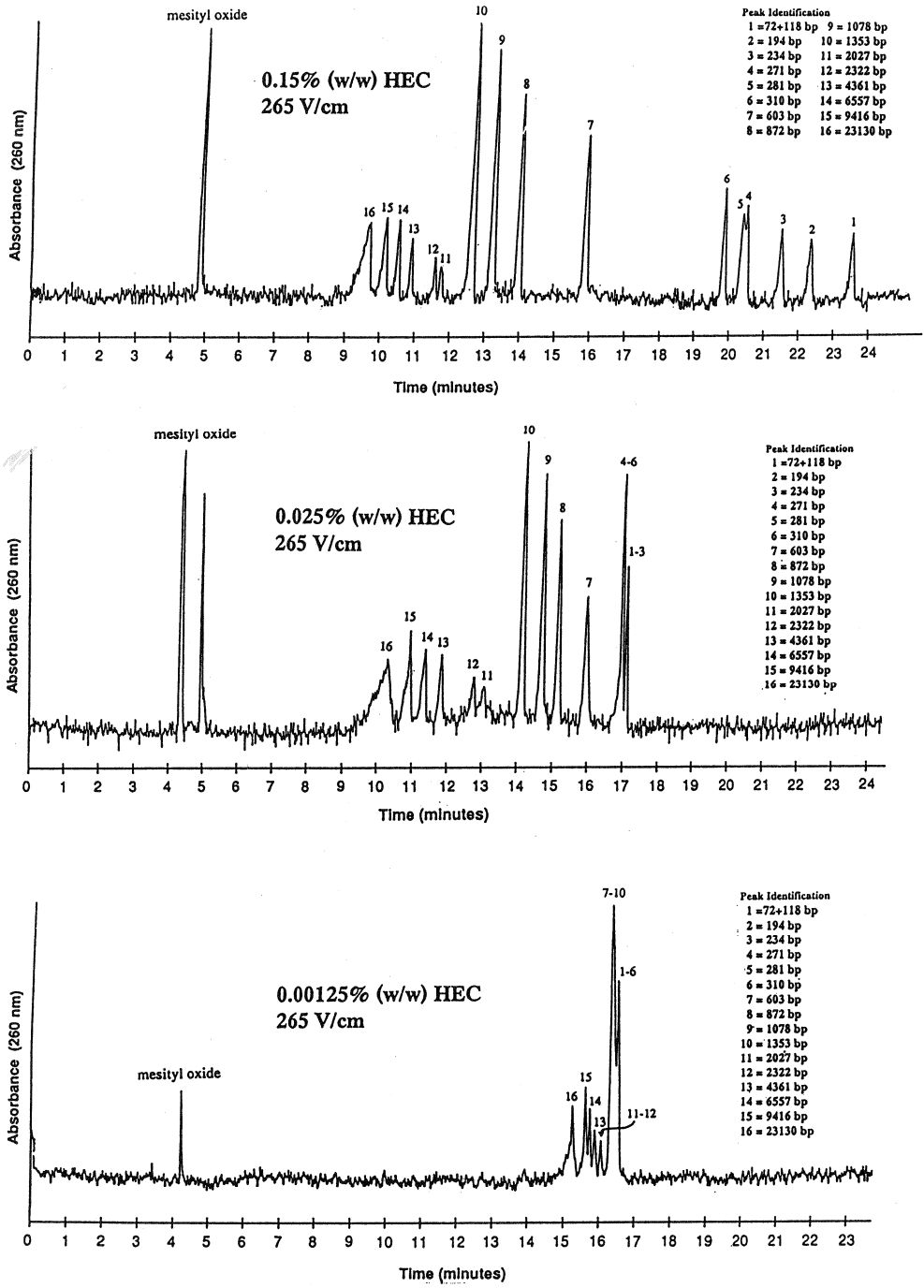
mesityl oxide
0.15% (w/w) HEC
265 V/cm
Peak Identification
1 =72+118 bp
2 = 194 bp
3 = 234 bp
4 = 271 bp
5 = 281 bp
6 = 310 bp
7 = 603 bp
8 = 872 bp
9 = 1078 bp
10 = 1353 bp
11 = 2027 bp
12 = 2322 bp
13 = 4361 bp
14 = 6557 bp
15 = 9416 bp
16 = 23130 bp
Absorbance (260 nm)
Time (minutes)
mesityl oxide
0.025% (w/w) HEC
265 V/cm
4-6
1-3
Peak Identification
1 =72+118 bp
2 = 194 bp
3 = 234 bp
4 = 271 bp
5 = 281 bp
6 = 310 bp
7 = 603 bp
8 = 872 bp
9 = 1078 bp
10 = 1353 bp
11 = 2027 bp
12 = 2322 bp
13 = 4361 bp
14 = 6557 bp
15 = 9416 bp
16 = 23130 bp
Absorbance (260 nm)
Time (minutes)
7-10
1-6
11-12
0.00125% (w/w) HEC
265 V/cm
mesityl oxide
Peak Identification
1 =72+118 bp
2 = 194 bp
3 = 234 bp
4 = 271 bp
5 = 281 bp
6 = 310 bp
7 = 603 bp
8 = 872 bp
9 = 1078 bp
10 = 1353 bp
11 = 2027 bp
12 = 2322 bp
13 = 4361 bp
14 = 6557 bp
15 = 9416 bp
16 = 23130 bp
Absorbance (260 nm)
Time (minutes)

Figure 4.4 a-c

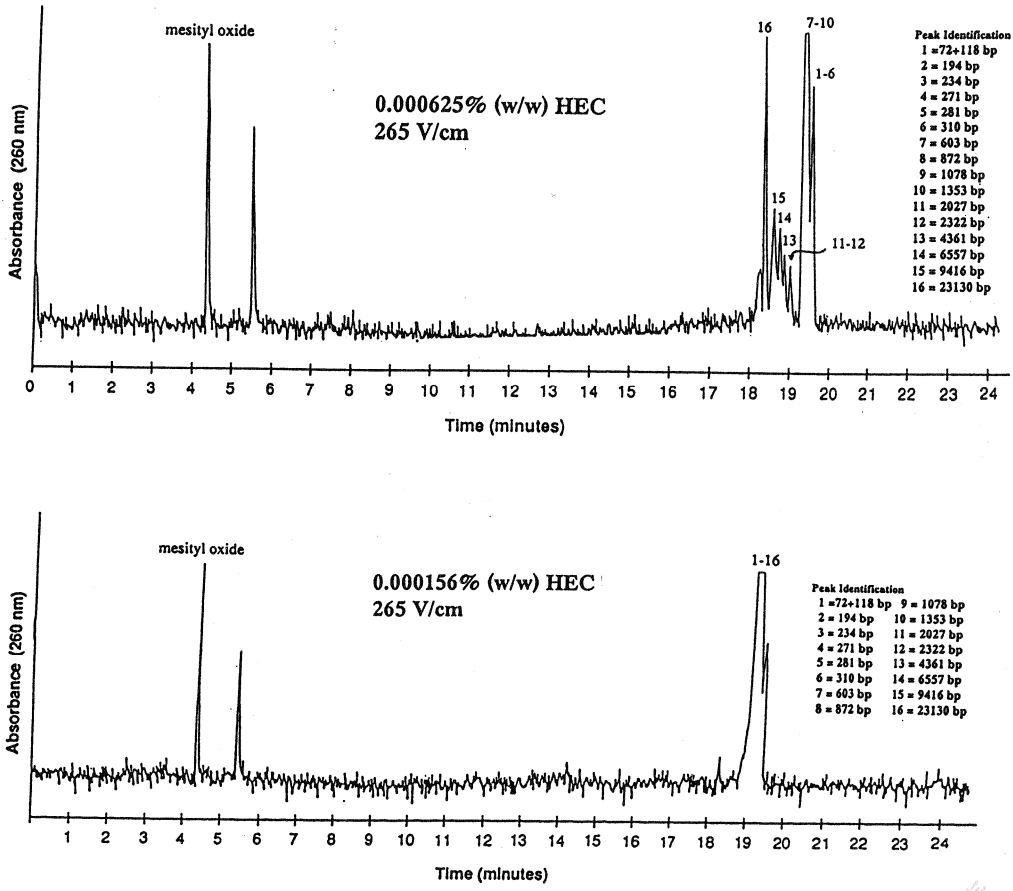

Figure 4.4 Separation by capillary electrophoresis of λ–*Hind*III and ΦX174-*Hae*III restriction fragments (in non-stoichiometric mixture) in (a) 0.15% HEC (M_W 1,315,000), (b) 0.025% HEC (c) 0.00125% HEC (d) 0.000625% HEC and (e) 0.000156% HEC (this extremely low concentration was obtained by successive dilution). Buffer: 89 mM Tris(hydroxyethyl-aminomethane), 89 mM boric acid, 5 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.15. Capillary: 51 μm i.d., 50 cm total length (35 cm to detector); temperature, 30 ± 0.1°C. Detection was by UV absorbance at 260 nm. Injection was hydrodynamic and electric field strength was 265 V/cm,

shows the separation of the same restriction digest in a 0.025% HEC (M_W 1,315,000) solution. At this concentration, resolution is essentially lost for DNA smaller than 603 bp, but retained for the larger restriction fragments. Even at concentrations as low as 0.00125% (12 parts per million) resolution of DNA fragments larger than 2 kbp is achieved (see Figure 4.4c). Resolution is only completely lost when HEC concentration is reduced below 1.56 parts per million, at which concentration the restriction fragments only separate into two large peaks (Figure 4.4d). In free solution, all of the DNA fragments elute as one peak.

Figure 4.5a gives a plot of DNA electrophoretic mobility (μ) as a function of HEC (M_W 1,315,000) concentration for this mixture of ΦX174-*Hae*III and λ-*Hind*III restriction fragments. Although the measured overlap threshold concentration of this HEC (M_W 1,315,000) is ≈ 0.37%, the only distinguishable change in the mobility data at or near this concentration is the rapid loss in resolution of DNA larger than 603 bp. From this plot it is apparent that the larger DNA restriction fragments (603 bp – 23.1 kbp) are best separated at low HEC concentrations, well below the overlap threshold.

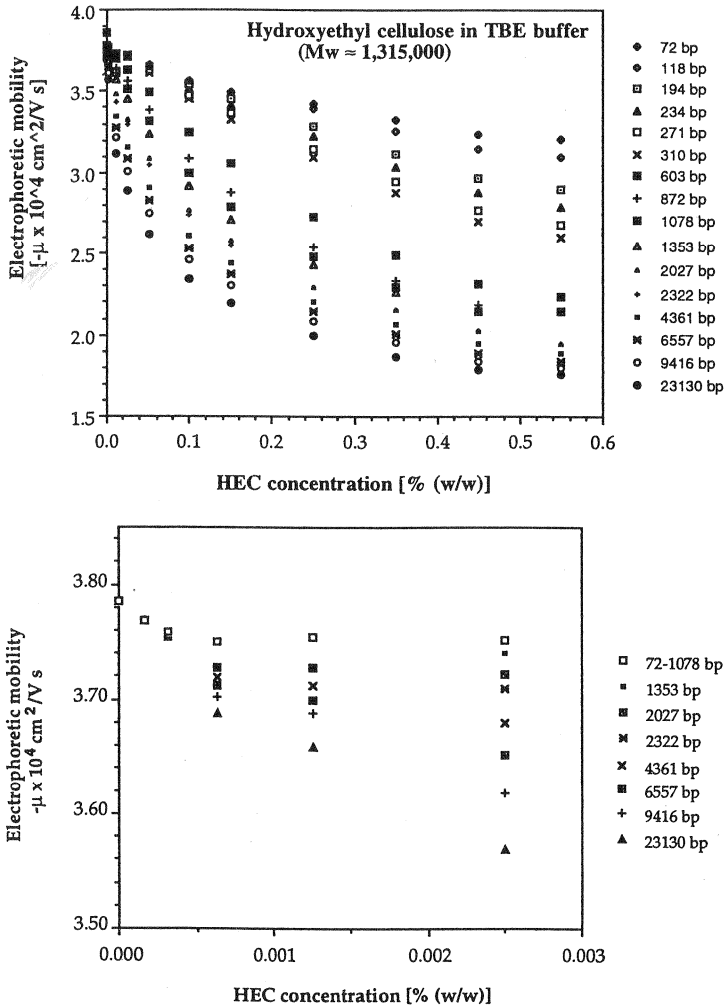

Figure 4.5 (a) A plot of DNA electrophoretic mobility *vs.* HEC concentration (HEC M_W 1,315,000 g/mol) for DNA restriction fragments ranging from 72 bp to 23130 bp in length, normal scale. (b) The same data plotted on an expanded scale to show detail at extremely low HEC concentrations. Data points at each HEC concentration are the average of 3 – 5 individual determinations. Average run-to-run variation in calculated electrophoretic mobilities: ± 0.46 %. DNA electrophoretic mobility was calculated by subtracting the electroosmotic mobility, calculated from the elution time of a neutral marker, from the *apparent* electrophoretic mobility of the DNA fragments. The electrophoresis buffer, all conditions, and DNA sample, same as in Figure 4.2.

Indeed, we observed that at higher concentrations, such as 0.55% HEC, the largest DNA peaks begin to merge and peak shape severely degrades (data not shown). This finding is in direct contradiction to the earlier theoretical predictions [71, 74], which anticipated that it would be necessary to raise the polymer concentration above some minimal value, *significantly higher than the entanglement threshold c**, in order to separate large DNA molecules. Viovy and Duke [71] predicted in 1993 that, for a given high molecular weight polymer, the size of the largest DNA that can be separated should increase roughly linearly with the viscosity of the polymer solution. Clearly, this is not the case, in fact the opposite is true (*i.e.*, the larger DNA is best separated in dilute, low-viscosity solutions). Smaller DNA, on the other hand, *is* best resolved in more concentrated solutions.

Note that for fragments larger than 2 kbp, there is a distinct concave curvature in Figure 4.5a at low HEC concentrations. Figure 4.5b shows the ultra-dilute solution data on an expanded scale; the transition from the equal electrophoretic mobilities of all DNA fragments in free solution, to size-dependent electrophoretic mobilities, is seen to occur at a concentration of about 6 ppm. Note that at the low concentrations depicted on Figure 4.5b, all DNA restriction fragments smaller than 872 bp migrate with essentially the equal electrophoretic mobilities.

Regardless of the polymer type, ultra-dilute polymer solutions – which we want to define here as solutions with concentrations less than two orders of magnitude below c^* – are generally capable of separating large DNA fragments. However, the most effective concentration range for DNA separation does vary somewhat with polymer type. This was demonstrated a 1996 study by Barron *et al.* [42], comparing HEC, HPC, and linear polyacrylamide solutions as DNA separation media for CE. It was found that if the polymers are more flexible (*e.g.*, linear polyacrylamide is about 8 times more flexible than HEC), or form more compact coils due to greater hydrophobicity (HPC is significantly less hydrophilic than HEC), higher polymer concentrations are generally required to achieve a given DNA separation. For ultra-dilute solution separations, the more compact, flexible HPC and polyacrylamide chains must be used at order-of-magnitude higher concentrations to provide the same separation seen in extremely dilute solutions of long-chain HEC. This is illustrated in Figures 4.6a, 4.6b and 4.6c, which show the separation of the ΦX174-*Hae*III/λ*Hind*III fragments in ultra-dilute solutions of high molecular weight HEC, HPC, and linear polyacrylamide, respectively. The difference in the bulk viscosities of these three ultra-dilute solutions is negligible: 0.94 cP, 0.96 cP, and 1.0 cP, respectively, and the electroosmotic flow velocity, as measured by the elution time of a neutral marker, is similar in the three solutions. The salient point which is illustrated by the three electropherograms shown in Figure 4.6 is that more flexible polymers like HPC and PAA must be used at higher concentrations to give the same separation achieved with the more stiff, extended HEC polymers. Figure 4.6 also shows that this separation mechanism generally applies for DNA separation in linear, hydrophilic polymers, and is therefore not likely to be due to specific chemical interactions but to general, physical DNA/polymer interactions.

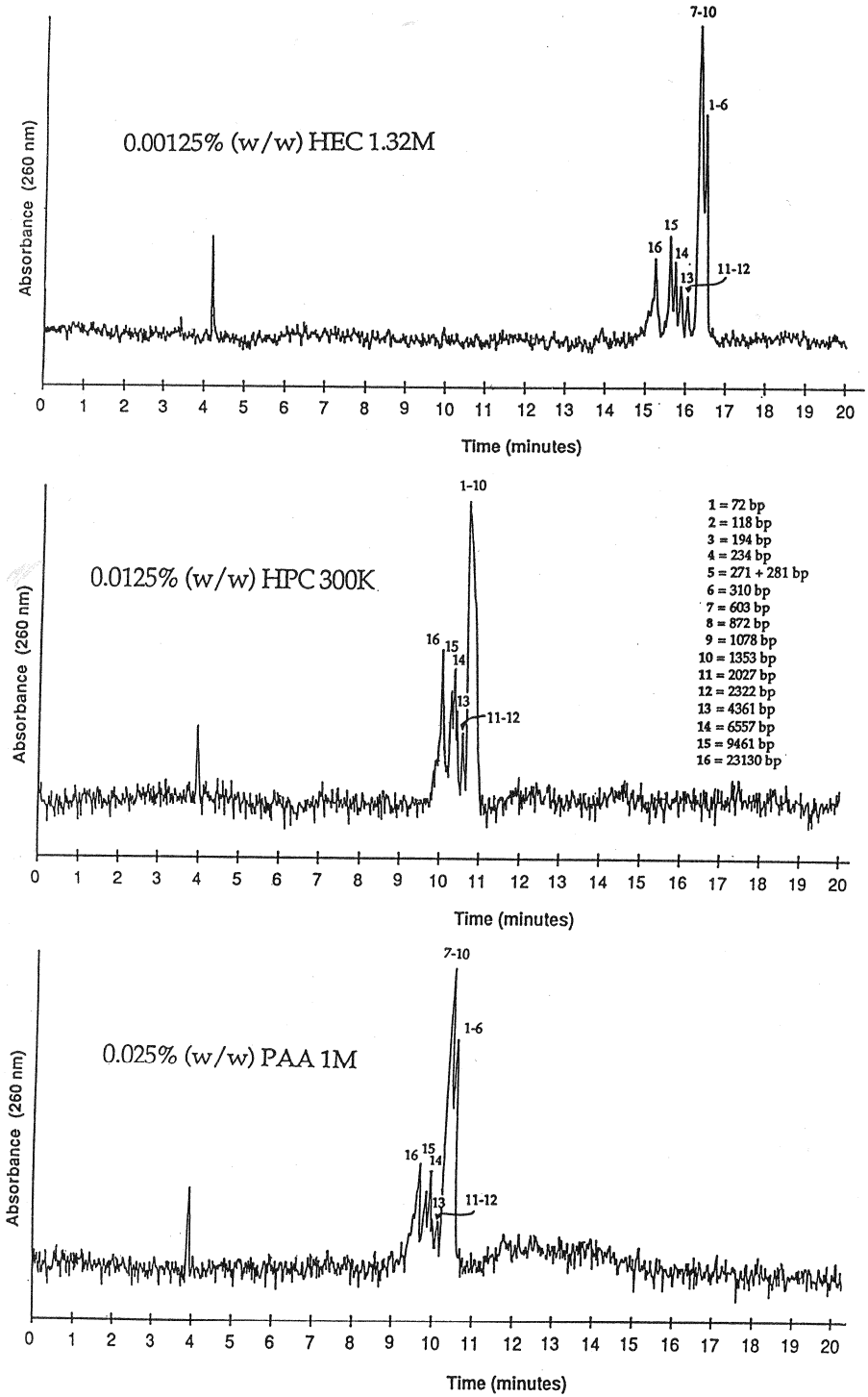

Figure 4.6 Separation by capillary electrophoresis of a mixture of λ–*Hind*III and ΦX174-*Hae*III restriction fragments (in non-stoichiometric mixture) in (a) 0.00125% hydroxyethyl cellulose (M_W 1,315,000), (b) 0.0125% hydroxypropyl cellulose (M_W 300,000), and (c) 0.025% linear polyacrylamide (M_W 1,000,000). Electrophoresis conditions: Field strength 265 V/cm, current was (a) 7.2 μA, (b) 7.2 μA, (c) 7.4 μA. All other conditions and specifications as in Figure 4.2.

The Effects of Polymer Sample Polydispersity

One advantageous property of polyacrylamide and related acrylamide-based polymers like polyacryloylaminoethoxyethanol (p(AAEE)) is that their polymerization can be controlled to give a desired range of molecular mass. Ruiz-Martinez *et al.* [32], Heller and Viovy [85], and Gelfi *et al.* [52] have shown that the molecular mass of linear polyacrylamide may be optimized to provide good resolution at a reasonably low viscosity, so that the solution can be pushed in and out the capillary easily using a syringe. Although cellulose derivatives are also available in a range of molecular masses, they are not synthetically polymerized, but instead are derived from various natural sources. For example, HEC and HPC are made by the high-temperature, high-pressure reaction of alkali cellulose with ethylene oxide and propylene oxide, respectively. The product is purified, to varying degrees, but rarely fractionated with respect to molecular mass. Hence, commercially available derivatized celluloses tend to be quite polydisperse (*i.e.*, $M_W / M_n >> 1$), with the polydispersity varying quite a bit from sample to sample. For a systematic study of the dependence of DNA separating ability on polymer molecular mass, it might be desirable to use a monodisperse polymer sample; but there has been no indication that polydispersity has any deleterious effects on DNA resolution. In fact, in a recent study Chan and Yeung used solutions of mixed-molecular-mass polyethylene oxide (PEO) to show that controlled polydispersity has advantages for the separation of single-stranded DNA sequencing fragments; by mixing long and short PEO chains they were able to strike a balance between two desirable properties, high DNA resolving power and low solution viscosity [86].

To study the effects of polydispersity on DNA separation, Barron *et al.* took advantage of the availability of relatively monodisperse linear PAA samples [42]. A comparison was performed of the DNA separation performance of two PAA samples which both have weight-average molecular masses of about 1 million, but have very different polydispersities. Their results make it very clear that it is the long chains in a polymer sample that are responsible for the separation of large DNA. Further, it shows that small DNA fragments are relatively indifferent to the presence of long polymer chains in the polymer solution. It can therefore be concluded that sample polydispersity has very little effect on the separation of small DNA fragments; instead, overall polymer concentration is the important parameter. However, the separation of large DNA fragments is significantly improved by the presence of a larger fraction of long polymer chains in the solution.

A Transient Entanglement Coupling Mechanism for DNA Separation in Dilute Polymer Solutions

At concentrations two orders of magnitude below the overlap threshold, HEC chains must remain relatively isolated in solution. Although chains collide and interact transiently, the HEC polymers do not form an entangled network. Nothing resembling the "tubes" or "pores" which are assumed to exist in the reptation model would be expected to exist. The Ogston model assumes the existence of an infinite network of crossed linear fibers. Once again, in dilute HEC solutions a polymer network such as this would not be expected to exist. Yet, DNA larger than 2 kbp can be readily separated at these concentrations. This suggests that the mechanism of DNA separation in dilute, uncrosslinked polymer solutions must be quite different from that postulated for agarose and polyacrylamide gels. In an extremely dilute, uncrosslinked polymer solution, no obstacle is permanent on the time scale of DNA motion

[78]. As proposed by Bode in 1979 [68], the controlling factor in DNA electrophoretic mobility would most likely be the local resistance of polymer chains to dislocation and deformation, which would depend on the relative sizes of the DNA and the polymer chains, as well as polymer properties such as stiffness.

Based on the results described above, Barron *et al.* proposed an alternative mechanism of separation, different from those upon which the Ogston and reptation models of DNA electrophoresis are based [38]. This proposed mechanism of separation is based on a consideration of the properties of the separation matrix polymers as well as double-stranded DNA chains. For example, HEC is a linear (*i.e.*, non-branched), uncharged cellulose derivative, having bulky ethylene oxide side chains terminating in hydroxyl groups. In aqueous solution, these hydrophilic side groups force HEC into a stiff, extended conformation. This stiffness is evidenced by a Porod-Kratky persistence length of 8.3 nm, roughly 10 times that of a typical flexible, random-coil polymer [79]. Double-stranded DNA is even more stiff and extended in solution than HEC, with a Porod-Kratky persistence length of 45 nm in 0.2 M buffer [80]. At comparable concentrations, stiff, extended polymers exhibit the effects of entanglement coupling much more strongly than flexible, random-coil polymers [81]. Given this, it is likely that when DNA molecules encounter isolated HEC polymers in a dilute HEC solution, they become transiently entangled with the HEC; the strength of the entanglement interaction must be augmented by the stiffness of the two participants. Hence, DNA molecules will be forced to drag HEC molecules along with them, resulting in a decrease of DNA electrophoretic mobility. In the proper concentration range, larger DNA molecules, with a larger cross-sectional area, will have a higher probability of encountering and entangling with one or more HEC molecules (for illustration of the principle, see Figure 2.9 in Chapter 2)

It has been demonstrated theoretically by Bueche that the molecular friction factor of a polymer in solution is much increased by entanglement coupling with other polymers [82]. Therefore, this type of DNA/HEC entanglement coupling interaction could alter the frictional characteristics of the DNA molecules moving under the influence of the electric field in a size-dependent manner. This physical picture of the separation mechanism was later supported by the videomicroscopy experiments of Shi *et al.* [83] and quantitatively modeled, with some success for short-chain HEC, by Hubert and Slater [84] (see also Chapter 2).

This physical model of DNA/HEC interactions can be used to interpret the results which were obtained with solutions of short-chain HEC, having a weight-average molecular weight of 139,000 g/mol. We found that unlike the long-chain HEC, this smaller HEC does not have the ability to separate DNA larger than 603 bp very well even at low concentrations (Figures 4.2a and 4.2b). This points to the fact that the relative sizes of the HEC and DNA molecules are very important for the size-separation of DNA. When a larger HEC molecule is entangled with a DNA molecule, it causes more frictional drag than a smaller one. It seems likely that if the HEC chains are too small, they are too easily displaced or dragged by the larger DNA restriction fragments, because they form weak points of entanglement and are also too small to significantly hinder DNA electrophoretic motion. Hence, small HEC molecules would be less efficient in introducing size-dependence to the molecular friction factor of larger DNA, as is observed experimentally.

4.2.2 DNA Separation in Dilute and Semi-Dilute Polymer Solutions

Dependence on Electric Field Strength and Qualitative Comparison with Theories

On the other hand, in fully entangled (semi-dilute) polymer solutions, under electric fields which are relatively low for capillary electrophoresis, systematic studies indicate that DNA migration mechanisms are indeed similar to those observed in true gels [41]. However, in comparison to low-field slab gel electrophoresis, the DNA separation mechanism which is observed even in highly entangled, "gel-like" polymer solutions will be complicated by the dynamic nature of DNA/polymer and polymer/polymer interactions [71], as well as by the susceptability of entangled polymer solutions to network rupture under high electric fields.

For experimental testing of the applicability of DNA electrophoresis theories, it is very important to measure the field dependence of DNA electrophoretic mobility. Mitnik *et al.* [41] undertook a systematic study of the effect of electric field as well as polymer concentration (using high molecular-weight HPC), taking a data set which could be extrapolated down to zero field strength, as is rigorously demanded to test both the Ogston and reptation models. Figure 4.7 shows an example for such separations in 0.4% HPC (above $c^* \approx 0.35$ %, $M_w \approx 1$ million) at different electric fields (Please note, that in this case, coated capillaries were used, which suppress the electroosmotic flow and therefore the smaller fragments are eluted first. The type of capillary used has no influence on the true electrophoretic mobility, which can be correctly determined if the EOF is known). Obviously, with increasing electric field, the separation speed is increased considerably. Also, small fragments are well resolved, but not as good as medium sized ones. Large fragments cannot be resolved at all and migrate in a single peak (Figure 4.7a), exactly as observed in agarose gels [87]. However, when increasing the electric field, the separation of large fragments (which gets worse in agarose gels on increasing the field) is recovered! This was an unexpected result and seems to be unique to polymer solutions. Only when going to very high fields (> 500 V/cm), resolution decreases again.

Similar experiments have been performed at different HPC concentrations and the results are shown in Figure 4.8. Such plots of log (mobility) *vs.* log (DNA size) can be used to test adherence to the reptation model; a slope of –1 indicates that DNA is being separated by the reptation mechanism. In the case of a low-concentration, unentangled polymer solution (0.1% HPC solution, below c^*), it is clearly seen that the slope never reaches –1, indicating that in this case, reptation is not the main separation mechanism (Figure 4.8a). Also, at high electric field strengths, the mobility of the larger DNA fragments never totally levels off. This again indicates a mechanism different from pure reptation. Figure 4.8b and c show results obtained using more concentrated, 0.4% and 1% HPC solutions (above c^*), under different electric fields. The double logarithmic mobility *vs.* DNA size plot is very similar to the one obtained when DNA is separated in slab gels, with a well-defined reptation regime (slope = –1). Above a certain DNA size (above 2 kbp), under low electric fields, the curve levels off, reaching a plateau mobility, due to field-induced orientation of the electromigrating DNA molecules. This also explains the observation of Barron *et al.* [38, 42] (and described above) that in general, the resolution of DNA larger than 600 bp is seen to degrade at higher sieving polymer concentrations. The same trend was observed by Mitnik *et al.* [41], and can be interpreted in terms of a shift in the mechanism of DNA separation as polymer concentration is increased and a highly entangled polymer network is formed.

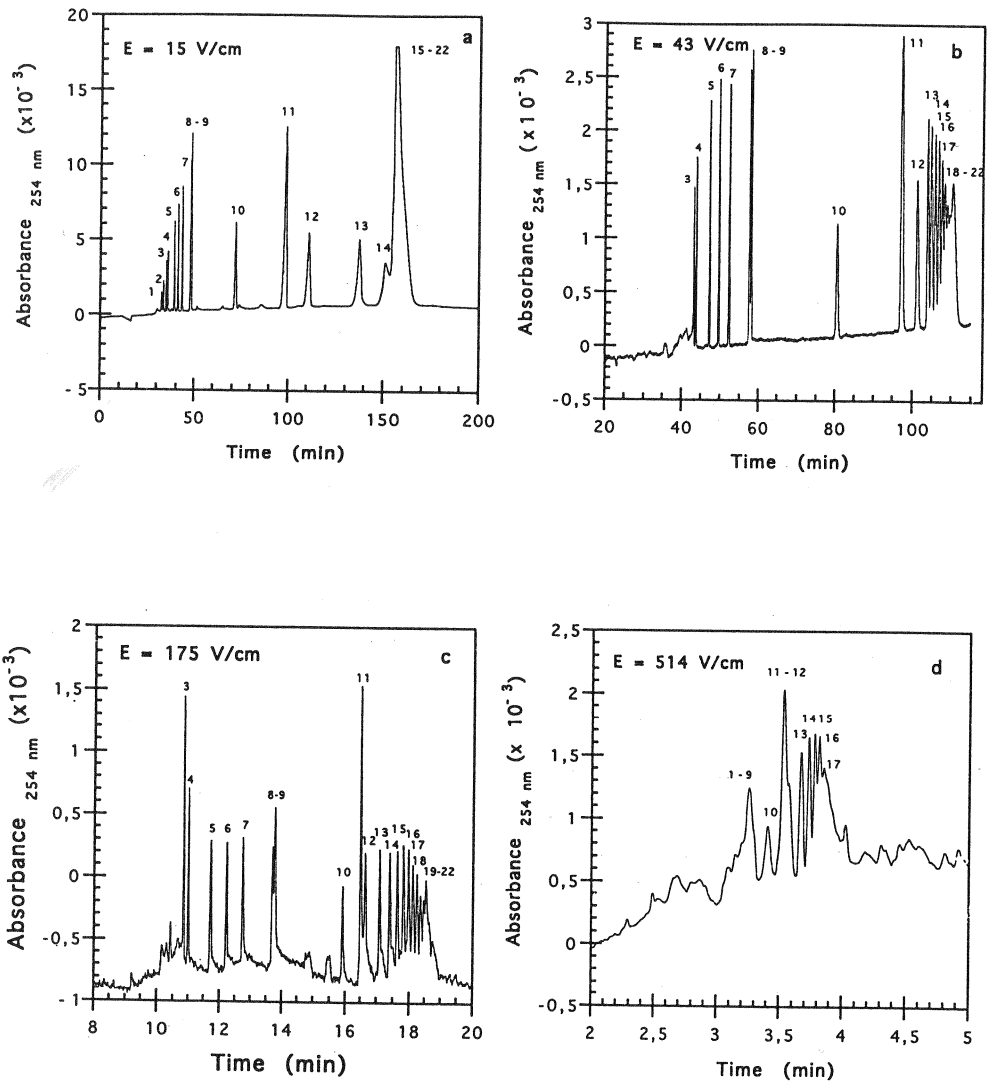

Figure 4.7 Separation by capillary electrophoresis of a 1-kbp ladder in 0.4% hydroxypropyl cellulose (M_W 1,000,000) at (a) 15 V/cm (b) 43 V/cm (c) 175 V/cm and (d) 514 V/cm. Buffer: 89 mM Tris(hydroxyethylaminomethane), 89 mM boric acid, 5 mM Ethylenediaminetetraacetic acid (EDTA), 10 μM ethidium bromide, pH 8.15. Capillary: coated, 100 μm i.d., 37 cm total length (30 cm to detector); temperature 25°C. Detection was by UV absorbance at 260 nm. Injection was electrokinetic. Peaks: 1=134; 2=154; 3=201; 4=220; 5=298; 6=344; 7=394; 8=506 9=517; 10=1018; 11=1635; 12=2036; 13=3054; 14=4072; 15=5090; 16=6108; 17=7126; 18=8144; 19=9162; 20=10 180; 21=11 198; 22=12 216 bp.

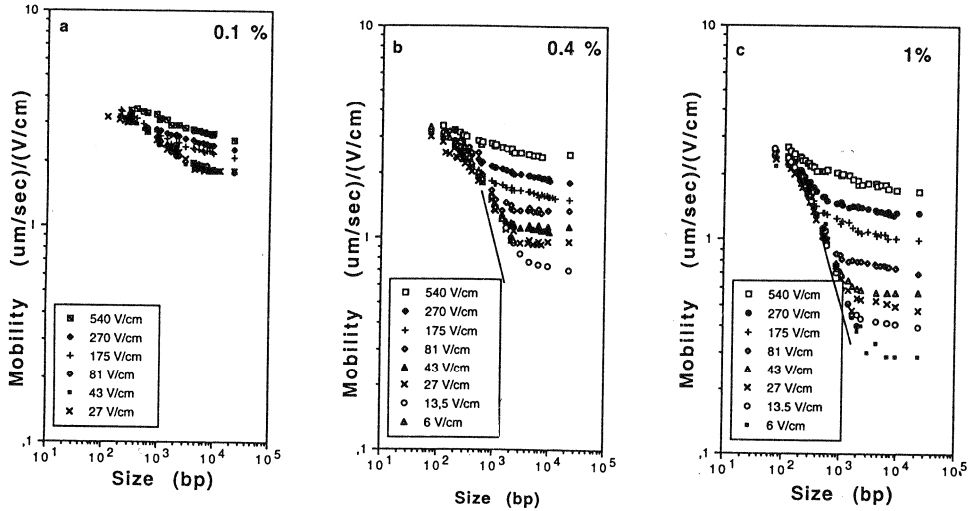

Figure 4.8 Dependence of the electrophoretic mobility of linear double-stranded DNA fragments (ranging from 72 bp to 23130 bp in length) on the molecular weight in (a) 0.1 %, (b) 0.4% and (c) 1.0% HPC (M_W 1,000,000) solutions at different electric field strengths in 1xTBE with 10 µM ethidium bromide at 25°C undergoing CE. Such a plot is useful to test adherence to the reptation model: data falling on a line with a slope of –1 (straight lines) indicate a reptation mechanism.

At higher electric field, however, some separation is recovered for large DNA (a behavior which is opposite to the biased reptation prediction that the limit of separation decreases with increasing electric field strength). This can be explained by considering that electromigrating DNA molecules will be able to rupture the entangled polymer network under high electric field strengths, allowing the transient entanglement coupling mechanism to contribute to DNA separation even in concentrated polymer solutions. This could also explain the rather bad separation at very high fields: The network is strongly disturbed and losing its ability to separate at high resolution.

The same data may be replotted in order to show the dependence of DNA mobility on electric field (Figure 4.9). Again, the separation in 0.4% and 1% HPC is very similar to what is observed in slab gels [87]. Under low electric fields and/or for low-molecular weight DNA fragments, the mobility is essentially constant and independent of electric field. Long DNA chains, however, show a linear dependence of log (mobility) on log (electric field strength) with a slope of 0.4.

Semi-logarithmic plots of electrophoretic mobility *vs.* DNA size (Figures 4.10a and 4.10b) allow us to test adherence of the data to the Ogston model, which predicts linearity of this type of plot (called a "Ferguson plot" [88]). For both 0.1% and 1.0% HPC solutions, a considerable curvature is observed above a critical size (smaller than 1 kbp), confirming that the Ogston model does not describe the mobility of DNA in polymer solutions any better than it does in slab gels. Only data for the mobilities of the smallest DNA fragments yield an

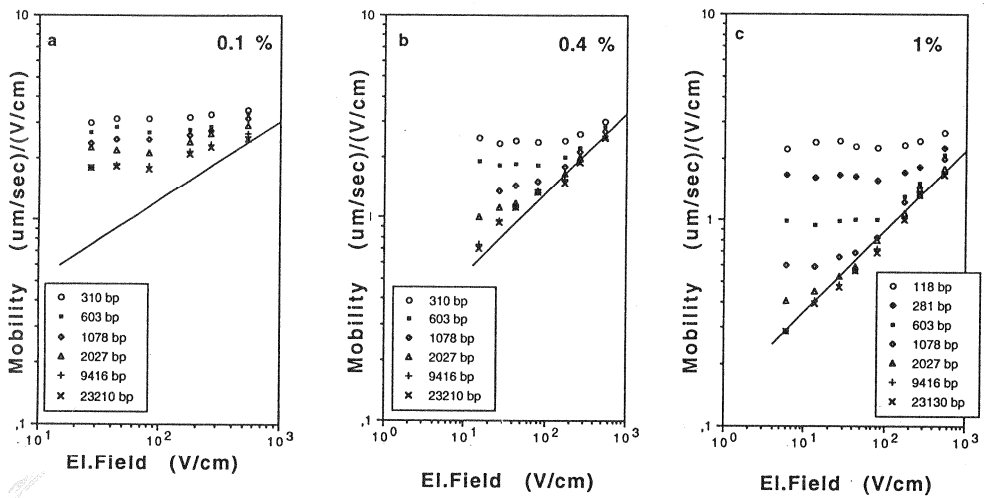

Figure 4.9 Dependence of the electrophoretic mobility of linear double-stranded DNA fragments on the electric field (a) 0.1 %, (b) 0.4% and (c) 1% HPC. The lines have a slope of 0.4. Same conditions as in Figure 4.8.

approximately straight line. The deviation from linearity occurs at around 500 bp in a 1% HPC solution, while for a more dilute, 0.1% HPC solution the cut-off molecular weight is about 1000 bp.

Also, only in 1% HPC, the mobility of small DNA is independent of the electric field (as it should be according to the Ogston model). At low polymer concentrations (0.1%) this is not the case, which indicates that the Ogston model is not valid here.

Effect of Polymer Size

As discussed in Chapter 1 and above, the overlap threshold c^* of a polymer solution depends on both concentration and polymer size. However, once entanglement has occurred, the "pore size" of this mesh is – in principle – only dependent on the polymer concentration and not on the size (for a given type of polymer). Ruiz-Martinez *et al.* [32], Heller and Viovy [85] and Gelfi *et al.* [52] have made use of this effect to separate DNA in entangled polymer solutions of low viscosity.

Figure 4.11 shows a direct comparison of dsDNA separation in solutions of 1% HPC of M_w 100,000 ($c^* \approx 2$ %), M_w 370,000 ($c^* \approx 0.8$%) and M_w 1,000,000 ($c^* \approx 0.35$%). As expected, the separations in HPC 370K and HPC 1 million are very similar (within experimental error) in both, separation speed and performance. In the HPC 100K solution, separation is faster and the sigmoidal shape of the log(mobility) vs log(size) plot is less pronounced. This is not surprising, as the concentration of this solution is below overlap threshold and the transient entanglement mechanism should dominate.

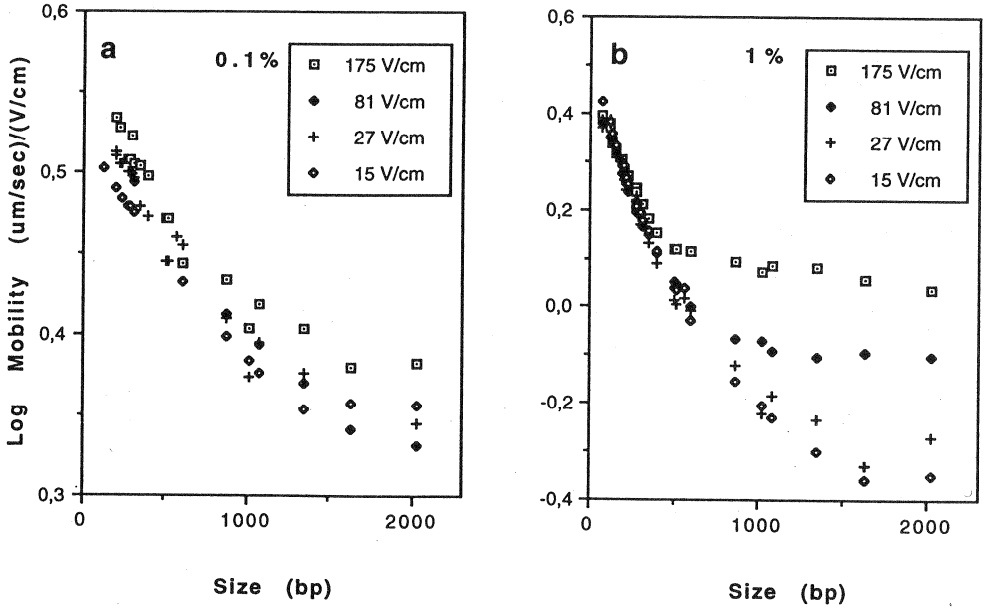

Figure 4.10 Log-linear plot of the mobility of DNA *versus* molecular size, (a) 0.1% HPC, (b) 1% HPC. This kind of plot is used to test for the Ogston model. In this case we can see that the Ogston model can only correctly describe the mobility of small DNA (< 500 bp) in high polymer concentrations ($c > c^*$).

Quantitative Comparison of Experimental Data with Reptation Theory

As explained in Chapter 2, in the BRF model we have to distinguish between the case when the pore size a of the matrix is larger than the Kuhn length b_D of DNA ($\alpha = a/b_D > 1$) and the opposite case, $\alpha < 1$ (so-called "tight gels").

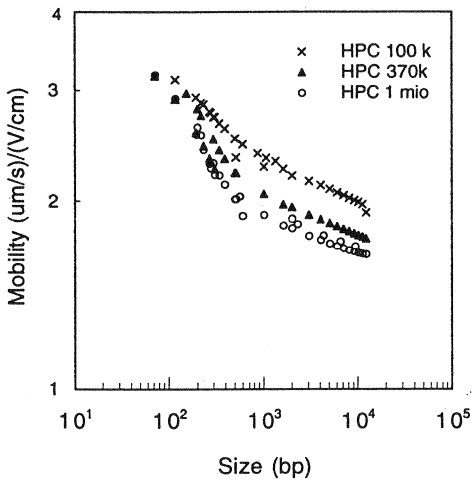

Figure 4.11 Dependence of the electrophoretic mobility of linear double-stranded DNA fragments on the polymer size at a concentration of 1.0% HPC (M_W 100,000; 370,000 and 1,000,000) solutions at 270 V/cm in 1xTBE with 10 µM ethidium bromide at 25°C undergoing CE.

In both cases, we have again to distinguish between two regimes, i.e. small DNA (below a critical size L_D^*) and large DNA (above a critical size).

For $\alpha > 1$, the BRF predicts for the mobility of small DNA:

$$\mu/\mu_0 \approx M_a/3M_D \tag{2}$$

and for large DNA:

$$\mu/\mu_0 \approx \varepsilon\,(1 - M_a/2M_D\,\varepsilon) \tag{3}$$

(see Eqs (28) – (30) of Chapter 2) where μ_0 is the mobility in free solution, M_D is the molecular size of DNA (in bp) and M_a is the portion of a DNA molecule that would fit into one pore and ε the scaled electric field. Obviously, for large DNA and/or high electric fields, Eq. (3) reduces to : $\mu/\mu_0 \approx \varepsilon$.

When $\alpha < 1$, the predictions is:

$$\mu/\mu_0 \approx b_D / M_D\, l_D \tag{4}$$

for small DNA, with b_D being the Kuhn length of DNA and l_D the length of a DNA monomer. In the case of large DNA, three different regimes are predicted (for the limits of the different regimes, see Chapter 2):

$$\mu/\mu_0 \approx \varepsilon/\sqrt{\alpha} \tag{5}$$

$$\mu/\mu_0 \approx (\varepsilon\,\alpha^4)^{2/5} \tag{6}$$

and

$$\mu/\mu_0 \approx \varepsilon^2 \tag{7}$$

One important advantage of reptation models is that only molecular parameters with a clear significance are involved in the calculations. In principle, then, the electrophoretic mobility can be predicted using independently measured quantities, without the use of any adjustable parameters. This is, of course, an ambitious goal, because theoretical modeling always involves some oversimplifications of the true physical picture. For the biased reptation with fluctuations (BRF) model, four parameters are needed: the Kuhn length b_D (which is twice the persistence length); the charge density of the DNA, the pore size of the matrix, a; and the free-solution mobility of the DNA, μ_0 .

The Kuhn length of duplex DNA varies with the ionic strength of the buffer. In 1× TBE, it is equal to 100 ± 20 nm (or about 300 bases). Thanks to several experimental studies, and in particular to Smith and Bendich's videomicroscopy measurements of DNA chain stretching under an applied electric field [89], the effective charge per base pair is now taken to be 0.1 ± 0.05 e^- per base pair.

The pore size of the matrix can be estimated by Eq. (11) of Chapter 1. For 1% HPC, this gives a value of about 32 nm. This means that the experiments shown in Figure 4.8c correspond to the case of tight gels and Eqs. (4) – (7) (see also Eqs. (32) – (36) of Chapter 2) are valid.

The absolute mobility in free solution, μ_0, was evaluated experimentally by Olivera *et al.* [1]. In 1× TBE, it should be around $3 \pm 0.5 \times 10^{-4}$ cm^2/Vs. Substituting these values into Eq. (4) we obtain for the linear regime (small DNA, mobility is size dependent):

$$\mu \approx 3 \times 10^{-4}\ (\mathrm{cm^2/Vs})\ 100\mathrm{nm} / 0.34\mathrm{nm}\ M_D \approx 1/M_D \times 0.1\ \mathrm{cm^2/Vs} \quad (8)$$

with M_D in basepairs.

In the mobility vs. DNA size plot, (Figure 4.8) the linear part of the curve can be associated with the regime in which the mobility is independent of field strength and exhibits an inverse linear dependence on DNA size. In the more concentrated 1% HPC solutions (Figure 4.8c), where gel electrophoresis theories are most likely to apply, we find that

$$\mu \approx 1/M_D \times 0.05\ \mathrm{cm^2/Vs} \quad (9)$$

Considering the fact that the numerical prefactors in the reptation theory are not well known, this is quite good agreement.

For the non-linear regime (large DNA), Eqs. (5) – (7) are valid with the following predictions for the mobility : $\mu \sim E$, $\mu \sim E^{2/5}$ and $\mu \sim E^2$. Therefore our results presented in Figures 4.9b and 4.9c (slope of 0.4) are in excellent agreement with one of the predictions. By using the definitions of Eq. (15) in Chapter 2, we can then calculate the expected mobilities in this regime and the predicted limits of the different regimes (not shown). Mitnik et al. [41] could show that the slope of 0.4 for the field dependence in 1% HPC is observed within the predicted limits.

However, when comparing the absolute experimental values for the mobility in the non-linear regime with the theoretically expected ones (Eq. (6)), Mitnik et al. [41] observed a difference: In the case of 1% HPC, the DNA has a mobility about 3 times higher than expected. Even when taking into account the poor knowledge of prefactors in the theory, we still must assume that this difference is at least partly due to the labile nature of the obstacles, i.e. a rupture of the network.

4.2.3 Summary

To get a more complete picture of what happens in the separation of DNA in polymer solutions, we have tried to summarize the effects described above (see Table 4.1). Basically, we have to know which regime (dilute *vs* entangled polymer solution) we are working in, to be able to predict what will happen upon changing the most important parameters (electric field, polymer concentration). Of course this is a rather simplified picture, as the "crossover" between the two regimes is rather smooth.

From the theoretical discussions and the systematic experimental studies, we can also draw a kind of "phase diagram" (Figure 4.12), showing the different separation regimes in dependence of polymer concentration (probably the most important adjustable parameter for the experimentalist) and DNA size. When working below c^*, (depending on both polymer concentration and polymer molecular weight), DNA separation occurs due to a combination of hydrodynamic and frictional (between DNA and polymer) effects. Theoretical studies to fully understand this regime have only been done recently (see also Chapter 2) and some aspects, like the electric field dependence, have still to be verified. What is very interesting here is that so far, no "plateau" has been observed, neither experimentally nor in the theoretical predictions. In other words, we do not know yet what the largest separable DNA size is. We could imagine, for example, that by using very large polymers, it could be possible to separate very large DNA molecules, however at low resolution (G. Slater, pers. comm.).

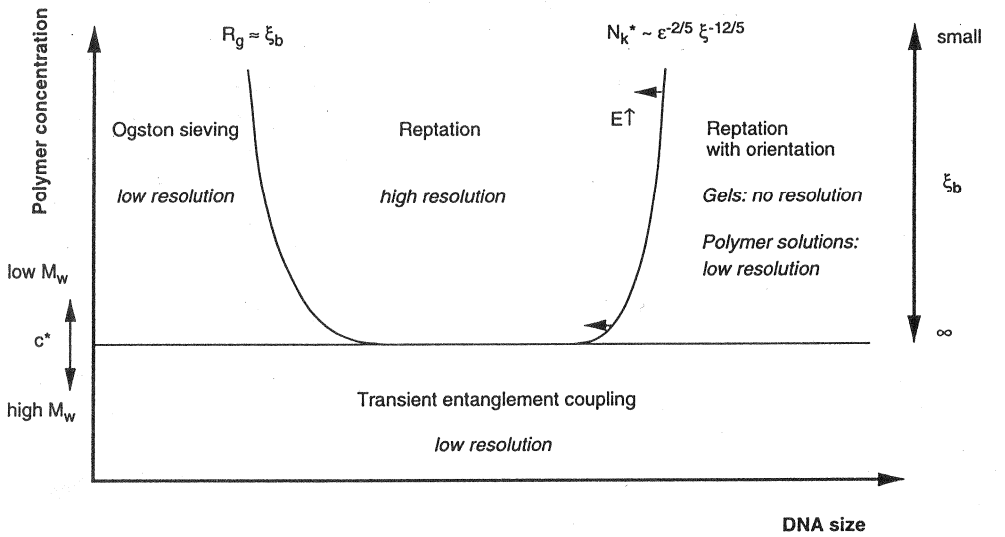

Figure 4.12 "Phase diagram" of the dependence of the separation mechanism (and therefore resolving power) on polymer concentration and DNA size. The transition from transient entanglement to "gel-like" mechanisms occurs when $c = c^*$, the one from Ogston sieving to reptation occurs when the radius of gyration of the DNA is larger than the "mesh size". Orientation occurs above a critical DNA size N_k^* and resolution is lost. In gels (and probably in very highly entangled solutions) this limit is shifted towards smaller sizes with increasing field (arrows). However, in entangled polymer solutions and increasing field, resolution is recovered in this regime, probably due to network rupture.

When working above c^*, we have conditions similar to those in gels, and – not surprisingly – we can observe the same regimes. The main difference here is a different effect of the electric field in two ways: First, the dependence of mobility on the electric field is different, i.e. a slope of 0.4 *vs* a slope of 1 in gels (see Figure 4.9). Second, in gels there is an upper limit of separation (above which all fragments comigrate) and this limit decreases with increasing electric field. In polymer solutions, separation is recovered at high fields (unless very high fields are used) and again, we do not know if there is an upper limit.

Again, this diagram is simplifying the true picture: The lines drawn here must not be regarded as strict "borders", but being thought of as smooth transitions. This can be very well seen, when the results of the studies of Barron et al. [38] and Mitnik et al. [41] are combined (as shown in Figure 4.13 for the case of HPC at 270 V/cm). At the left hand of the concentration scale, we find a separation which is best described by the transient entanglement coupling mechanism. This means that we achieve separation very rapidly over a wide range of DNA sizes, but with rather low resolution. At the other extreme of the concentration scale, we observe separation according to the reptation mechanism, yielding high resolution, but in a rather narrow DNA size range and at the cost of long separation times. These data are also

Table 4.1 Influence of different parameters on the performance of DNA separation in polymer solutions.

Polymer Solution	Separation range	Resolution	Separation speed	Viscosity	Remarks
Dilute	large	low	fast	low	
increasing el. field	n.d.	n.d.	increase	–	
increasing polymer size	increase	increase	decrease	increase	transition to entangled regime
increasing polymer conc.	increase	increase	decrease	increase	transition to entangled regime
Entangled	small	high	slow	high	
increasing el. field	increase*	decrease	increase	–	* due to network rupture
increasing polymer size	–	slight increase	slight decrease	increase	
increasing polymer conc.	decrease	increase**	decrease	increase	** but decrease for larger DNA

in good agreement with videomicroscopic observations of large DNA electromigrating through HEC solutions between two microscope slides [83] (see also Chapter 3), in which a gradual change in migration behavior with changing polymer concentration is observed. It is clear that the "pure" separation mechanisms exist only at the extreme ends of the concentration scale. In between, in the vicinity of c^*, we can assume that a "mixture" of both mechanisms is valid.

This can be described as follows. When an extensive, highly entangled polymer network is formed at concentrations well above c^*, network rupture and polymer dragging involves a high energy barrier for migrating DNA to cross, and there will be a transition to a mechanism of separation which is similar to the one which occurs in true gels (Ogston sieving or reptation). Network rupture will be facilitated by high electric field strengths, causing elements of the transient entanglement coupling mechanism to have a persistent effect even in relatively high-concentration polymer solutions. Network rupture will be less prevalent under low electric fields, allowing a highly entangled polymer network to function similarly to a true gel.

Also, it has to be pointed out here, that most samples of cellulose derivatives are highly polydisperse (as discussed above) which also leads to a smooth transition between the dilute and the entangled regime.

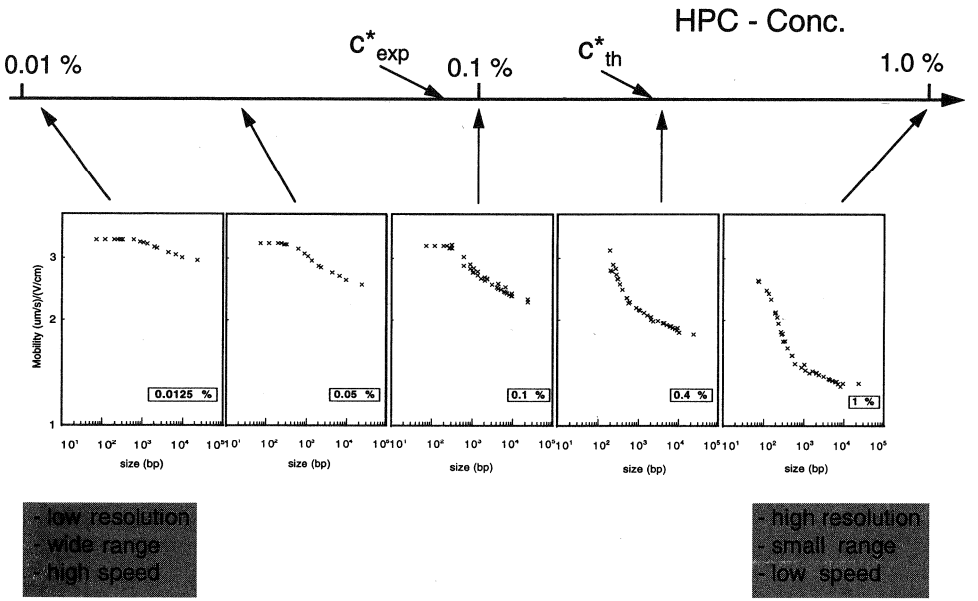

Figure 4.13 Dependence of the mobility of ds DNA on the polymer (HPC) concentration at 270 V/cm. Theoretically estimated (see Eq. (1)) and experimentally observed (see Figure 4.1) entanglement thresholds are indicated. This plot shows the gradual change in separation mechanism, from transient entanglement coupling mechanism at low polymer concentrations to gel-like separation at high concentrations (Ogston sieving and reptation).

4.3 Consequences for the User

These findings have some consequences for the user, depending upon the application and on the DNA sample to be analyzed. If a quick check of the size range of an unknown DNA sample is needed, but a high-resolution separation is not necessary, then we recommend using low polymer concentrations. When working in the non-entangled regime (below c^*), the molecular weight of the polymer can play a major role. For best resolution, a concentration just below c^* should be chosen.

However, if high resolution is necessary, then a rather high polymer concentration should be used. It must be pointed out, however, that not all CE devices can handle such concentrated, viscous solutions. Also, high resolution separations are only possible in a rather narrow DNA size range (about 100 – 2000 bp). For larger DNA, resolution decreases (because of the field-induced orientation of the DNA molecules), and a pulsed-field separation protocol is required (see Chapter 9).

Theory predicts that in fully entangled polymer solutions, the size of the polymer should not be an important factor in determining the "mesh size" of the polymer network, and hence should not strongly affect DNA separation. While the polymer chain length (and hence coil size) determines the concentration at which there is the onset of the overlapping/entangling regime, once the overlap threshold has been passed, the mesh size of the polymer network should depend only on the total polymer concentration [73, 85]. However, as the viscosity of the polymer solution is highly dependent on the average polymer length (and concentration), the following strategy should be adopted: A polymer should be chosen which is large enough to be well entangled at the concentration needed for high resolution, but small enough to yield a manageable viscosity.

The exception to this rule might be that under high electric fields, shorter polymers might form a mesh which is more easily disrupted. Therefore, when working at high electric fields, it should be kept in mind that the use of longer polymer chains might be necessary, which of course has the consequence of higher viscosities.

Therefore, when one is attempting to optimize the separation or when searching for effective separation matrices, the different separation mechanisms (which are only purely valid at the extreme ends of the concentration scale) should be kept in mind. When testing a new polymer, it is of great advantage to know the average molecular weight and the overlap threshold concentration of that particular sample.

DNA sequencing is clearly the most demanding application: single-base resolution of denatured, single-stranded DNA is required. So far, such high resolution could only be achieved in highly entangled polymer solutions, in micellar networks, or in crosslinked gels.

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