Manipulation of Polymer Chain Entanglement and Self-Assembly for DNA Capillary Electrophoresis

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Summary: A conceptual model has been developed and tested experimentally on five different aspects, relating to the architecture and topology of the polymers making up the transient network that constitutes the separation matrix. The concept can be applied for DNA sequencing analysis and separations of short fragments double-stranded DNA as well as oligonucleotides. Examples are provided to illustrate the success of the concept.

Keywords: copolymers; DNA; efficient separation matrix; electrophoresis

Introduction

Capillary electrophoresis (CE), including capillary array electrophoresis (CAE) and microfabricated chips, is a proven method for high-throughput DNA sequencing and separation analysis. Separation media, where replaceable polymer solutions, acting as a dynamic quasi-network for sieving the charged macromolecular fragments, are widely used and can dramatically influence the CE performance, in terms of resolution, efficiency, speed and read-length. A range of hydrophilic polymers has been developed and tested as DNA separation media with varying degrees of success. These could include linear polyacrylamide (LPA),[1-7] poly(N,N'-dimethylacrylamide) (PDMA),[8, 9] polyethylene oxide (PEO),[10, 11] poly(vinyl-pyrrolidone),[12, 13] and poly(N-hydroxyethylacrylamide) (polyDuramide).[14]

To date, LPA shows the best performance. The LPA-type separation media have been commercialized as *CEQ separation gel I*™ by Beckman Coulter, and as *LongRead*® *Matrix* by Molecular Dynamics. Recently, Applied Biosystems has introduced POP7, in addition to the popular POP6 separation matrix, with substantial improvements in DNA sequencing performance.

It has been recognized that the best-known DNA sequencing medium, LPA, has two major drawbacks, i.e., (1) high molecular weight LPA, which is used to increase the read length, has a very high viscosity and (2) its dynamic coating ability is poor. Therefore, a coated capillary is required if only LPA homo-polymers are used. The first drawback is a

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general property that all polymer solutions have to obey, i.e., the viscosity of a polymer solution increases with increasing polymer molecular weight at fixed polymer concentrations, while the second drawback is a specific deficiency for LPA.

One should recognize that due to the difference in the persistence length between double-stranded (ds) and single-stranded (ss) DNA as well as the needs for ssDNA sequencing analysis and dsDNA fragment separations/identifications, e.g., in forensic science, the *optimizations on separation media should be tailored to specific needs.* Thus, it is difficult for homopolymers to possess all the properties that can meet the different challenges. However, *copolymers,* including miscible polymer mixtures, are able to combine the desirable properties of different monomers or segments, yielding useful and essential features for a successful separation medium not accessible to any known homopolymers. The copolymer topology and architecture now plays a crucial role in determining the CE performance. Several categories according to the molecular architecture of the polymer separation matrix have been tested: (1) random copolymers of PDMA/N,N'-diethylacrylamide,[15,16] LPA/PDMA,[17] LPA/sugar,[18,19] hydroxylated PDMA;[20] (2) graft copolymers of LPA-g-poly(N-isopropylacrylamide) with PNIPAM = poly(N-isopropylacrylamide),[21] LPA-g-PDMA;[22] (3) block copolymers (or polymer surfactants) of $PEO_{106}PPO_{70}PEO_{106}$[23-26] (with PEO and PPO denoting, respectively, polyethylene oxide and polypropylene oxide), polyethylene glycol end capped with fluorocarbons,[27] *n*-dodecane-PEO-*n*-dodecane,[28] *n*-alkyl polyoxyethylene ethers (e.g., $C_{16}PEO_8$);[29] (4) mixtures of hydroxy-propylcellulose(HPC) and hydroxyethylcellulose (HEC).[30,31] To improve the desired properties of *dynamic coating ability, low viscosity, and high sieving ability*, the use of copolymers and polymer mixtures is clearly a logical alternative. The above references have not included the recent contributions from Stony Brook.[32-53]

A quasi-interpenetrating network (quasi-IPN) of two weakly incompatible polymers of LPA and PDMA that are miscible at low polymer concentrations (~2-3 wt% total polymer concentration) and random copolymers of LPA and PDMA have been developed, synthesized, characterized, and tested, respectively, for ssDNA sequencing and dsDNA fragments analysis.

Conceptual Model

Figure 1 shows the schematics of a conceptual model for separation media based on molecular architecture and topology of copolymers.[40] It can be divided into five categories. We have experimentally tested each feature presented in the model. The references from our group as well as those reported in the literature have been noted. In brief, our findings can be summarized as follows.

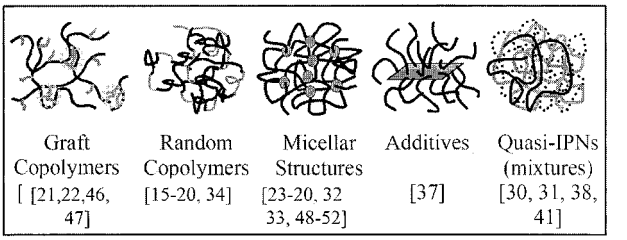

Fig. 1. Schematic model for separation media based on molecular architecture and topology.

- In order to slow down the chain dynamics, we introduced graft copolymers with short grafted chains and graft densities appropriate for the mesh size of the polymer network. The graft copolymers showed better separation properties than those of homopolymers of comparable size range.[46, 47] Later studies by Viovy et al[21, 22] confirmed this finding.
- Random copolymers of LPA and PDMA were synthesized to test the combined advantages of the high sieving ability of LPA and the dynamic coating ability of PDMA.[34] This combination was successful but also pointed to the direction that could alleviate the phase separation problem for less compatible polymers or the mixing of two polymers at higher polymer concentrations. The performance of random copolymers of LPA and PDMA was comparable to that of LPA of comparable molecular weight, even without substantial tuning of the chemical composition.
- Micellar structures of tri-block[32,33] suggested that the efficiency of the separation matrix depended on the polymer network structure. For example, core-shell micelles were less effective than micellar structures exhibiting a more open network structure. Two key points are noteworthy, i.e., the mesh size formed by the least amount of the polymer chains in the network is most efficient and the use of PPO block has unique advantages. In aqueous solution, PPO is hydrophilic at low temperatures but becomes hydrophobic at temperatures higher than about 15 °C in aqueous solution without urea. The presence of urea could increase this transition temperature. The first point

suggested the advantages of interpenetrating networks, while the second point revealed the practical aspect of using the self-assembly behavior to change the viscosity of the separation matrix and to increase the effective polymer molecular weight.

- An effective separation matrix requires high molecular weight polymers (or long chains) to produce entanglements that are responsible for large-fragment DNA separations. Thus, we intentionally used a low molecular weight (470k) PDMA that is not suitable as a good separation matrix for DNA analysis. However, by adding only about 10^{-5} g/ml of exfoliated clay sheets, an effective separation matrix was created[37]
- The central idea in creating an interpenetrating network (IPN) is the ability to use two different types of polymers that are miscible but do not like each other so much that the polymer chains tend to avoid each other. Thus, we can take advantage of using different polymers for their intrinsic properties and at the same time to have the chains becoming more expanded because they try to avoid each other. Such a polymer network, known as quasi-IPN,[38, 41] has no permanent chemical cross-linking points but can be very effective, as we shall present in some of the experimental findings below.
- We have also compared the difference between polymer mixtures of LPA and PDMA[35] with the corresponding quasi-IPN. In a polymer mixture in which the low molecular weight PDMA component played only the role for dynamic coating, the short PDMA chains were dispersed in the LPA matrix. Thus, it was not as effective as the quasi-IPN in which each polymer (LPA and PDMA) had concentrations above its own overlap concentration.
- We have examined the effects of solvent (glycerol)[39] and of dye (ethidium bromide)[45] on the DNA separations. Both glycerol and dye changed the DNA conformation and effective charge density. For large DNA fragments, the resolution could be increased at the expense of longer analysis time.
- In trying to increase the resolution of existing separation matrix, we introduced a concentration gradient by taking advantage of techniques in micro-fluidics. The results[42] suggested that the concentration gradient could indeed improve the resolution, not available by any other means. However, the specialized technical

knowledge required was likely to make such an approach difficult to implement in a biology laboratory.

- Finally, we tried an alternative separation matrix that could remain stable in alkaline conditions. At high pH values, the hydrogen bonds in dsDNA should break down and the use of urea could possibly be minimized. Furthermore, in looking ahead on separation media for proteomics, the pH change could play an important role. Two papers[43, 44] were published on modified polyvinyl alcohol.

Based on our schematic model, as shown in Figure 1, the formation of quasi-IPN provides a practical approach to create new novel DNA separation media consisting of two or more essentially miscible polymers that can tolerate a small amount of incompatibility. In the following sections, we present the improved performance of the first-generation LPA/PDMA quasi-IPN for DNA sequencing analysis. It is noted that, in making comparisons with commercial separation matrices, we used electrophoresis conditions recommended by each manufacturer for the specific product, not the running conditions optimized for our own quasi-IPN, because we want to take each separation matrix, including a commercial product of unknown chemical composition and molecular weight under its own best recommended operating conditions.

Comparison of Electrophoresis Results

1. DNA Sequencing Analysis

To verify the idea that quasi IPN could be used for DNA sequencing analysis, a quasi-IPN of LPA and PDMA was synthesized by polymerization of DMA monomers in a LPA solution matrix at concentrations above the LPA overlap concentration. Inverse micro-emulsion polymerization was used in the synthesis for LPA to ensure a high LPA molecular weight.

The choice of LPA and PDMA to form the quasi-IPN could combine the advantages of both polymers, i.e., the high sieving ability of LPA and the dynamic coating ability of PDMA. To examine the ssDNA separation ability of LPA/PDMA quasi-IPN, two different instruments: a lab-built single-color sequencing analyzer (**A**) and an ABI 310 sequencing analyzer (**B**), were used for ssDNA capillary electrophoresis. In figures below, *symbol c denotes DNA sequencing.*

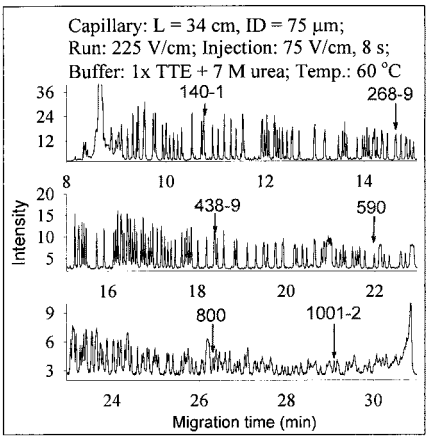

Fig. c-2. Separation of DNA [pGEM-3Zf(+) from the –21M13 forward Primer] up to 1000 bases by using 2% quasi-IPN. Instrument **A**.

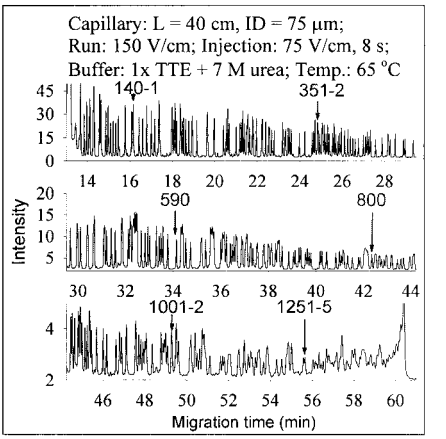

Fig. c-3. Separation of DNA [pGEM-3Zf(+) from the –21M13 forward Primer] up to 1200 bases by using 2% quasi-IPN. Instrument **A**.

In Figure c-2, the sequencing analysis data shows that the separation of one thousand bases with one-base resolution (by eye only) could be completed *within 30 min*. To increase the throughput of DNA separation, fast separation with a longer read length is one of the ultimate aims. In Figure c-3 [from Figure 5 of reference 53, with permission], the sequencing analysis data shows that DNA sequencing up to 1200 bases in 60 min has been achieved. In our illustration, the operating conditions have not been fully optimized. More importantly, we used an IPN with an average molecular weight of about 6 million while the molecular weight in [2] was about 17 million and was in combination with special base-calling software. The performance of our IPN could be further improved if we could increase the average molecular weight of our IPN. Nevertheless, it should be noted that further improvements in performance require the formation of polymer networks with slower chain disentanglement dynamics, i.e., more permanent polymer network formation.

Comparisons of quasi-IPN with different commercial products used for the separation of DNA demonstrate that the quasi-IPN concept is valid because of the already superior results when compared with any existing commercial separation matrix.

The sequencing analysis data of DNA [pGEM-3Zf(+) from the-21M13 forward Primer] is shown in Figures c-4 and c-5. Figure c-4 shows typical CE results in MegaBACE LPA

gel/1xMegaBACE buffer at 44 °C and 150 V/cm, which were the recommended operating conditions suggested by Amersham Biosciences. Figure c-5 shows the sequencing analysis data of the same DNA, but CE was run in POP6 (Applied Biosystems)/1xABI buffer at 50 °C and 198 V/cm, which were the recommended CE running conditions of POP6.

As a comparison, the migration time of 800 b of DNA sample could be used as a speed criterion of separation. The run was completed within 26.3 min ($t_{800,IPN}$) by using our quasi-IPN as a separation medium (Figure 2), while the same run was completed within 89 min ($t_{800,LPA}$) by using the MegaBACE LPA gel as a separation medium (Figure c-4). The separation speed of DNA in our quasi-IPN was around three times faster than that of DNA in the MegaBACE matrix. The DNA separation in POP6 could not reach 800 b (Figure c-5). If the migration time of 590 b could be taken as a speed criterion of DNA separation, then $t_{590,IPN}$ = ~22 min, $t_{590,LPA}$ = ~70.5 min and $t_{590,POP6}$ = ~89 min by using IPN, MegaBACE and

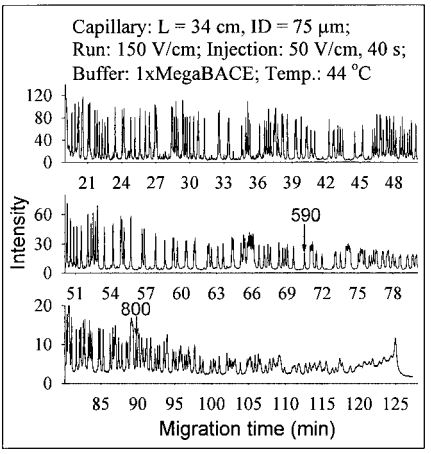

Fig. c-4. Separation of DNA [pGEM-3Zf(+) from the –21M13 forward Primer] by using MegaBACE LPA. Instrument **A**.

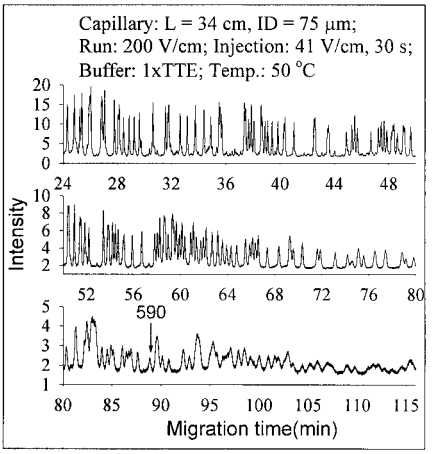

Fig. c-5. Separation of DNA [pGEM-3Zf(+) from the –21M13 forward Primer] by using POP6. Instrument **A**.

the quasi-IPN was about three times faster than that needed in the MegaBACE matrix and about four times faster than that needed in POP6.

The ABI 310 genetic analyzer (**B**) is an automated single-capillary genetic analyzer designed and optimized to support a wide range of sequencing and fragment analysis

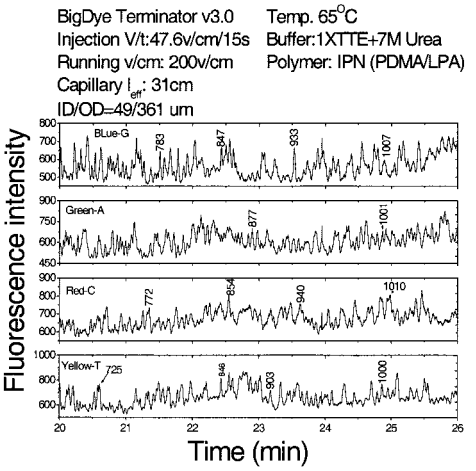

Fig. 6. Last parts (base number from 400 to ~1000) of electrophoretic separation of DNA BigDye Terminator v3.0 by CE (Instrument **B**) in 2% quasi-IPN.

applications. BigDye Terminator v3.0 standard DNA was used to demonstrate the sequencing analysis performance. The resolution of LPA/PDMA quasi-IPN was compared with the resolution of POP6 (Applied Biosystems) and of the MegaBACE matrix from Amersham Biosciences over a range of operating conditions, such as the capillary length, column temperature.

The separation reproducibility of (LPA/PDMA) quasi-IPN was also investigated for the separation of ssDNA. The relative standard deviation of the migration time measured for each DNA fragment was less than 1% in fifteen runs, suggesting that the dynamic coating of the capillary inner wall was quite reproducible.

Figure c-6 shows the base number from (600 to ~1000) of electrophoretic separations of BigDye v3.0. One thousand bases of BigDye v3.0 DNA were separated within about 36 min, using the ABI 310. The injection voltage and time were 27 V/cm and 15 sec., respectively. The running electric field strength was 198 V/cm; running temperature, at 60 °C; capillary effective length, 31 cm and ID/OD = 50/365 (μm/μm). With assistance form Karl Voss of Applied Biosystems, the ABI 310 software for recording the experimental data was modified in order to accommodate the faster run time of our quasi-IPN.

B. Implementation of Separation Media for Micro Fluidic Separation Devices

It has been reported that genotyping analysis on micro-fabricated chips is 10 or 100-fold faster than capillary or slab gel electrophoresis.[54] Recent developments on micro-fabricated technologies showed the potential of increasing the throughput of DNA analysis by orders of magnitude through a combination of massive parallelism and fast assay time.[55] The analytical instruments based on micro-fabricated technologies have been commercialized.

The micro-fabricated chip, used in the 2100 Bioanalyzer and developed by Agilent Technologies represents one of the most successful examples. However, the separation medium used by the Bioanalyzer showed relatively poor separations for small size dsDNA fragments.

B.1 Random Copolymers

The same conceptual model should be applicable to the separation matrix for *small* dsDNA fragments. Here, we have two undesirable effects, i.e., a decrease in mesh size means higher polymer concentration and consequently higher viscosity *and* the mixing of two polymers at high concentrations causes phase separation. The quasi-IPN of LPA and PDMA is no longer practical because phase separation occurs even for LPA and PDMA at total mixture concentrations beyond a few percent. Our experiments could show that the conceptual model remained valid.

A random copolymer of LPA and PDMA (Ran-PAD) should form a homogeneous quasi-network and have dynamic coating behavior. Ran-PAD at different molar ratios of Am/DMA and molecular weight were prepared via the aqueous solution polymerization route using H_2O_2/TMEDA (tetra-methyl ethylene-diamine) as the initiator at 40 °C.

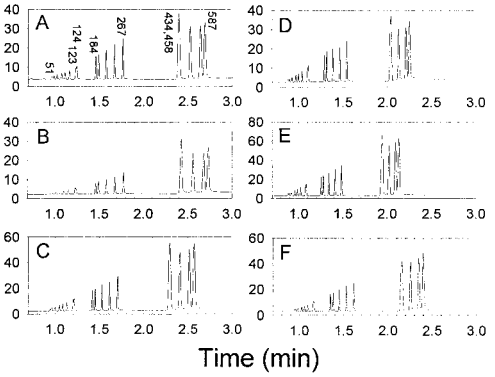

Fig. 7. Electropherogram of pBR322/Hae III digest by using 5 % (w/v) Ran-PAD in 1× TBE buffer solutions at room temperature. The detectable 18 peaks are: 51, 57, 64, 80, 89, 104, 123, 124, 184, 192, 213, 234, 267, 434, 458, 504, 540, 587 bp. Conditions: capillary I.D./O.D. = 25 μm/ 360 μm, effective/total length = 1.2/6 cm, electro-kinetic injection at 50 V/cm for 2 s, running voltage = 125 V/cm. Random copolymers from A to F have different AM to DMA molar ratio and are: 10:1, 6:1, 5:1, 7:3, 4:6, 1:3, respectively. Ethidium bromide was used as the dye.

Figure 7 shows the performance of a set of random copolymers of ran-PAD in dsDNA separation by using a 1.2-cm long, 25-μm ID capillary whose dimensions are very close to those of the separation channels used in the Agilent microchips in the 2100 Bioanalyzer. As shown in Figure 7, P(AA-co-DMA) with an AA to DMA ratio of 4:6 was able to separate pBR322/Hae III digest with fairly good resolution in about 2 min.

B.2 Self-Assembled Copolymers

For $E_{99}P_{69}E_{99}$ (F127), a (Pluronics) tri-block copolymer of oxyethylene (E) and oxypropylene (P), the viscosity of F127, for example, is about 50 centi-Poise between 10-15 °C at ~21 (w/v) % F127 in 1x TBE buffer, rises to ~5 Poise before reaching 20 °C, and the solution becomes gel-like at temperatures above room temperature. Laser light scattering (LLS) and small angle x-ray scattering (SAXS) show that the gel-like structure has a face-centered cubic unit cell made of core-shell micelles whose size remains constant but whose aggregation number depends on the polymer concentration. It is noted that this type of thermo-reversible gel-like polymer solution at fairly high polymer concentrations (~20 (w/v) %) has a liquid-like viscosity at a conveniently low temperature (10-15 °C) and changes to a 'gel' at room temperatures (≥ 20 °C). The mesh size has a distribution because of the hydrophilic E-chains radiating out from the hydrophobic P-micellar core and is dependent on both the polymer concentration and the molecular architecture of the block copolymer. The distribution in mesh size and the high polymer concentration provides the means to separate DNA from very small sizes to a reasonable size DNA fragment. Thus, such a block copolymer system shows promise for one of the current needs on oligo-nucleotide separations. In an earlier study, by using a 10-cm long capillary, the separations of oligo-nucleotides, together with dsDNA fragments, have been demonstrated. Thus, we can utilize this concept to design separation media for analysis of oligo-nucleotides and very small dsDNA fragments over a fairly broad range of sizes by using EPE-type (or/and EBE-type) block copolymers, as demonstrated in Figure 8.

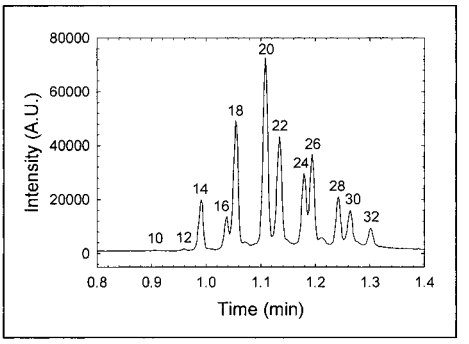

Fig. 8. Fast dsDNA separations using a self-assembled block copolymer (F127), demonstrating that at high polymer concentrations with small mesh sizes, one can take advantage of the thermo-reversible viscosity changes in copolymers to creating new separation media suitable for the new chip format. Operating conditions:
capillary: 50/365 μm, 3/13 cm (effective/total); separation medium: 25 % F127/1xTBE; injection: 300 V/cm, 1 sec; running voltage: 600 V/cm; oligonucleotides: 10-fold dilution by water; OliGreen (Dye): 600-fold dilution by 1 x TBE & mixed with F127 (10 μL: 500 μL).

Conclusion

The use of copolymers offers a wider chemical diversity that can tune the transient polymer network to the desired mesh size with better polymer network stability and controllable effective viscosity. Thus, further improvements on the separation matrix become feasible, not only for DNA sequencing analysis but also for separations of short fragments of single- and double-stranded DNA, including very short oligo-nucleotide fragments with the chip format and fast separation times.

Acknowledgement

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