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# High-Throughput, High-Sensitivity Genetic Mutation Detection by Tandem Single-Strand Conformation Polymorphism/Heteroduplex Analysis Capillary Array Electrophoresis

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**We present the first optimization of linear polyacrylamide (LPA)-based DNA separation matrixes for an automated *tandem* microchannel single-strand conformation polymorphism (SSCP)/heteroduplex analysis (HA) method, implemented in capillary arrays dynamically coated with poly(*N*-hydroxyethylacrylamide) (polyDuramide). An optimized protocol for sample preparation allowed both SSCP and HA species to be produced in one step in a single tube and distinguished in a single electrophoretic analysis. A simple, two-color fluorescent sample labeling and detection strategy enabled unambiguous identification of all DNA species in the electropherogram, both single- and double-stranded. Using these protocols and a panel of 11 p53 mutant DNA samples in comparison with wild-type, we employed high-throughput capillary array electrophoresis (CAE) to carry out a systematic and simultaneous optimization of LPA weight-average molar mass ($M_w$) and concentration for SSCP/HA peak separation. The combination of the optimized LPA matrix (6% LPA, $M_w$ 600 kDa) and a hydrophilic, adsorbed polyDuramide wall coating was found to be essential for resolution of CAE-SSCP/HA peaks and yielded sensitive mutation detection in all 11 p53 samples initially studied. A larger set of 32 mutant DNA specimens was then analyzed using these optimized tandem CAE-SSCP/HA protocols and materials and yielded 100% sensitivity of mutation detection, whereas each individual method yielded lower sensitivity on its own (93% for SSCP and 75% for HA). This simple, highly sensitive tandem SSCP/HA mutation detection method should be easily translatable to electrophoretic analyses on microfluidic devices, due to the ease of the capillary coating protocol and the low viscosity of the matrix.**

Single-strand conformation polymorphism (SSCP) is based on the principal that, even in DNA fragments of equal length, an altered DNA nucleotide sequence caused by a mutation can affect single-strand DNA (ssDNA) folding and, hence, can be detected as a difference in electrophoretic mobility.[1,2] Similarly, heteroduplex analysis (HA) is based on the premise that dsDNA fragments of a given length consisting of perfectly complementary strands (homoduplexes) often exhibit electrophoretic mobilities different from those of dsDNA that contain sequence mismatches (heteroduplexes).[3] Generally, both SSCP and HA are relatively difficult DNA separations to achieve. Traditionally, the two methods are performed separately and involve PCR amplification of the DNA region of interest with $^{32}$P-labeled (radioactive) primers, thermal denaturation, and cooling of the resulting dsDNA to form either single-strand conformers or mixed homo- and heteroduplexes, electrophoresis in highly resolving cross-linked polyacrylamide slab gels, and finally autoradiographic detection. The major practical weakness of SSCP is the perceived need to conduct the analysis under more than one set of experimental conditions to obtain a high sensitivity of mutation detection, while the major disadvantage of HA is its generally low sensitivity for the detection of single-base substitutions.[1] Another weakness of SSCP as a diagnostic tool is its empirical nature, which results from the inability to predict ssDNA folding and to correlate it with the electrophoretic mobility of the resulting single-strand conformers.[4]

More recently, there has been interest in performing SSCP and HA in tandem. If a mixture of the wild-type and mutant dsDNA species is thermally denatured and rapidly cooled to form ssDNA conformers under the correct buffer conditions, a significant portion of the mixture also reanneals to form homo- and heteroduplexes, allowing one to conduct both SSCP and HA *simultaneously* using a single sample. The sensitivity of mutation detection with this combined methodology exceeds that of SSCP or HA alone (60−90% typically) and can approach 100%, as evidenced by several slab gel electrophoresis studies.[5−8] These studies

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also document the high specificity of combined SSCP/HA and alleviate some earlier concerns that fast hybridization kinetics might lead to partial reannealing of the dsDNA, which could result in spurious peaks and false positives.[9]

Genetic analyses designed for slab gels can often be accomplished more efficiently using microchannel electrophoresis formats such as capillary electrophoresis (CE), capillary array electrophoresis (CAE), and microchip electrophoresis (ME).[10−12] In C(A)E, electrophoretic separation is carried out within microbore fused-silica capillaries, while in ME, electrophoretic separation is conducted in tiny grooves manufactured on the surface of glass or plastic chips. Both C(A)E and ME formats offer access to process streamlining, including automated loading of separation matrix between runs and automated injection of DNA samples, and provide much faster separations than slab gels due to the higher electric fields applied.[10] C(A)E and ME can be equipped with laser-induced fluorescence (LIF) detectors that eliminate the inconvenience of radioactive labeling while maintaining high sensitivity of analyte detection. Finally, ME offers the potential of so-called "lab-on-a-chip" devices that are capable of integrating all of the steps required for genetic screening on a single microfluidic chip, including DNA extraction, purification, PCR amplification, and electrophoretic separation.[13]

In order for C(A)E and ME technologies to work well for genetic screening by tandem SSCP/HA, it is critical that optimal DNA separation matrixes for these types of samples be developed for microchannel formats. In slab gel electrophoresis SSCP, such optimization studies have focused on the ratio of *N*,*N*′-methylene bisacrylamide (cross-linker) to acrylamide monomer and have yielded matrixes with a low ratio of the former to the latter, which are more flexible and therefore more efficient for mutation detection.[14] In CE-SSCP and CE-HA, which employ non-cross-linked (linear) entangled polymer matrixes, such optimization must focus on the chemical (i.e., composition and hydrophobicity) and physical properties (i.e., molecular weight, persistence length, and polydispersity) of the polymers that form DNA separation matrixes. These chemical and physical polymer characteristics are important since they determine the properties of the entangled polymer network that is formed and, hence, the matrix "mesh size" and ability to provide high-resolution DNA separations.[15] Previously, several polymers such as hydroxyethylcellulose (HEC), poly(vinylpyrrolidone) (PVP), methylcellulose (MC), ABI POP, and linear polyacrylamide (LPA) were applied to CE-SSCP or CE-HA with varying degrees of success, and these studies have shed some light on the impact of polymer chemical composition on the performance of these analyses.[16]

Only one study touched upon the impact of polymer physical properties on the sensitivity of mutation detection by CE-SSCP and CE-HA. Notably, Ren et al. demonstrated that DNA separation matrixes based upon short-chain LPA of unspecified average molar mass can be used for CE-SSCP mutation detection and are easier to load into capillaries than those composed of long-chain LPA because of the lower polymer solution viscosity of the former.[17,18] But in order to achieve a systematic study of the influence of polymer physical characteristics on CE-SSCP and CE-HA performance, it is necessary to both synthesize and characterize polymers suitable for CE. The majority of the polymers studied for this purpose to date are commercially available only in certain chain lengths, which for naturally occurring polymers (i.e., HEC and MC) also tend to vary from batch to batch, precluding precise and reliable evaluation of the impact of their physical nature on CE separations. In the absence of a large choice of polymers, some efforts have been directed at optimizing the concentrations of commercially available polymers for CE-SSCP, primarily POP, a DNA sieving polymer supplied by Applied Biosystems. Not surprisingly, more concentrated POP matrixes have yielded better results, but also complicated capillary loading due to their high viscosities.[19−21]

Research projects in our group place emphasis on the controlled synthesis of polymers suitable for CE applications and on their complete and precise physical characterization by gel permeation chromatography (GPC) in tandem with refractive index and multiangle laser light scattering (MALLS) detection, resulting in the determination of both polymer molar mass distribution and the average radius of gyration in the solvent of interest.[22] With this knowledge in hand, we recently carried out detailed studies of the impact of polymer properties on DNA sequencing separations by capillary electrophoresis.[23] Herein, we extend our correlations of matrix properties with CE performance of genetic analysis and describe the first thorough investigation of the combined impact of the weight-average molar mass ($M_w$) of LPA and its concentration in the DNA separation matrix on the resolution of tandem CAE-SSCP/HA, resulting in the formulation of an optimized DNA separation matrix for this important application. The dynamic coating we used for passivation of bare fused-silica capillary arrays is based upon polyDuramide (poly-*N*-hydroxyethyl acrylamide), a novel polymer developed in our laboratory in collaboration with BioWhittaker Molecular Applications (Walkersville, MD).[24] Precoating the capillaries with a dilute solution of polyDuramide provides a low-cost solution for the lack of self-coating ability of LPA and allows for highly efficient and reproducible electrophoretic peak patterns of SSCP and HA


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Table 1. Data Detailing Preparation and Characterization of a Set of LPAs

| | monomer, g | $H_2O$, mL | 2-propanol, mL | APS, 10% (w/v) aq, mL | TEMED, 10% (v/v) aq, mL | $M_w$, kDa | $R_g$, nm | PDI |
|---|---|---|---|---|---|---|---|---|
| LPA200K | 10 | 110 | 5.8 | 0.625 | 0.625 | 200 | 63 | 1.51 |
| LPA600K | 10 | 110 | 0.7 | 0.5 | 0.5 | 600 | 64 | 1.8 |
| LPA1M | 10 | 110 | none | 0.4 | 0.4 | 1000 | 73 | 1.94 |
| LPA2.4M | 10 | 110 | none | 0.625 | 0.625 | 2400 | 116 | 2.91 |

samples. The optimized LPA matrix in combination with polyDuramide dynamic coating was utilized for the screening of 32 mutant DNA specimens of the p53 gene, which is the most frequently mutated gene in human cancer.[25,26] When used in concert with two-color LIF detection at 27 °C and optimized sample preparation protocols, tandem CE-SSCP/HA afforded 100% sensitivity of mutation detection coupled with complete and unambiguous peak assignments in the resulting electropherograms. On the other hand, CE-SSCP and CE-HA applied individually to the same set of p53 gene samples revealed only 93 and 75% of existing mutations, respectively, making these techniques unsuitable for implementation in a clinical setting, for which the NIH presently recommends a sensitivity of at least 97%.

## EXPERIMENTAL SECTION

**Reagents and Equipment.** The following materials and reagents were purchased from commercial suppliers and were used as received: boric acid; ethylenediaminetetraacetic acid tetrasodium salt (EDTA), tris(hydroxymethyl)aminomethane (Tris), ethidium bromide, ammonium persulfate (APS), TEMED, acrylamide (all from Amresco, Solon, OH); 2-propanol, HCl, NaOH (Fisher, Brightwaters, NY), dialysis cellulose ester membranes (MWCO 100K, Spectrum Laboratories, Rancho Domingues, CA); compressed $N_2$ (Air Products and Chemicals, Allentown, PA), and glycerol (Aldrich, Milwaukee, WI). Mutant DNA specimens of the p53 gene (exon 7, size 139 bp; exon 8, size 200 bp) were supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, MD) in the form of PCR amplicons and used as stock solutions of SSCP and HA samples without further purification. P53 cell lines (genomic DNA) that contain mutations in the p53 exon 7 were purchased from American Type Culture Collection (ATCC, Manassas, VA). These were PCR-amplified (amplicon size 300 bp) with forward (FAM-CCT GCT TGC CAC AGG TCT) and reverse (JOE-GTG ATG AGA GGT GGA TGG GT) primers, purified as described elsewhere[27] and then used as stock solutions in this study. PolyDuramide polymer ($M_w$ 4 MDa), which was used for dynamic capillary coating, was prepared, purified, and characterized in our laboratory as described by Albarghouthi et al.[24]

The following equipment was used in this study: a GPC 2690 Separations Module (Waters, Milford, MA) with Shodex (New York, NY) OHpak columns SB-806 HQ, SB-804 HQ, and SB-802.5 HQ connected in series, a MALLS DAWN-Optilab system (Wyatt Technology, Santa Barbara, CA), a PTC200 DNA Engine thermocycler (MJ Research, Waltham, MA), and a MegaBACE DNA sequencing system with uncoated fused-silica capillary arrays (Molecular Dynamics, Sunnyvale, CA).

**Polymer Synthesis and Characterization.** Two samples of short-chained ($M_w$ 200 and 600 kDa) and two samples of long-chained linear polyacrylamide ($M_w$ 1 and 2.4 MDa) were synthesized by radically induced aqueous polymerization. In a typical procedure, a solution of acrylamide (10 g) in deionized water (110 mL) was placed into a 300-mL airtight flask, immersed in a water bath kept at 50 °C, and degassed by bubbling $N_2$ for 30 min (for more details see Table 1). Next, 2-propanol (2.5−5 mL), APS (0.625 mL, 10% w/v aqueous), and TEMED (0.625 mL, 10% v/v aqueous) were added in a consecutive fashion, and the resulting mixture was kept at 50 °C under $N_2$ flow for 1.5 h to ensure anaerobic conditions. During this time, the acrylamide monomer underwent radically induced polymerization, as evidenced by the increased viscosity of the resulting LPA solution. LPA was then loaded into a tubular cellulose ester membrane and dialyzed against distilled, deionized water for 10 days with daily water changes. Purified LPA was lyophilized and characterized by tandem GPC-MALLS as described elsewhere (see Table 1 for polymer data).[28] Dry polymers were dissolved at 2, 4, and 6% (w/v) in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 10% glycerol to afford a set of 12 different DNA separation matrixes (stored at 4 °C throughout the present study).

**SSCP/HA Sample Preparation and CE.** Fluorescently labeled DNA amplicons (forward strand, FAM; reverse strand, JOE) of exons 7 and 8 of the p53 gene were diluted 10×−40× with Tris-HCl (10 mM, pH 8.5). The diluted PCR products or their mixtures were loaded onto a 96-well plate (5 μL into each well), denatured at 95 °C for 3 min using a PTC200 thermocycler, and snap-cooled on ice. Prior to CE-SSCP/HA runs, uncoated MegaBACE capillary arrays (total length 64 cm, effective length 40 cm, inner diameter 75 μm) were conditioned and dynamically coated by rinsing them with the following: NaOH (aqueous, 0.1 M, 4 mL) for 30 min at low pressure (100 psi), deionized water (2 mL) for 30 s at high pressure (1000 psi), HCl (aqueous, 0.1 M, 4 mL) for 30 min at low pressure (100 psi), water (2 mL) for 30 s at high pressure (1000 psi), polyDuramide (coating reagent, aqueous, 0.5%, 0.3 mL) for 30 min at low pressure (100 psi), and water (2 mL) for 30 s at high pressure (1000 psi). Throughout this study, the arrays were reconditioned by this procedure every 10−14 runs to ensure high-performance separations. The capillary arrays were loaded with the LPA-based DNA separation matrixes for 0.5−1.5 min and temperature-equilibrated for 20 min at 27 °C. Pre-electrophoresis was performed for 5 min at 140 V/cm. The DNA amplicons were injected at 230 V/cm for 25 s and

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Table 2. Results of Genetic Screening of the P53 Gene by Tandem CAE-SSCP/HA, Utilizing an Optimized DNA Separation Matrix[a,b]

| | p53 exon 7 (size 139 bp) | | | | p53 exon 8 (size 200 bp) | |
|---|---|---|---|---|---|---|
| codon no. | 242 | 245 | 248 | 249 | 273 | 282 |
| wild-type sequence | TGC | GGC | CGG | AGG | CGT | CGG |
| mutant DNA specimens | AGC (+/+) | AGC (+/+) | AGG (++/++) | AGC (++/++)[c] | AAT (+/++) | *GGG (−/+)* |
| | AAC (+/+) | CGC (++/++)[c] | CAG (++/++) | *AGT (−/++)* | ACT (+/+)[c] | *AGG (−/+)*[c] |
| | ACC (++/+)[c] | GCC (+/++) | GGG (++/++)[c] | | *AGT (−/+)* | **TGG (+/−)** |
| | ATC (++/+)[c] | *GTC (-/++)* | TGG (+/++) | | ATT (+/++) | |
| | CAC (++/+) | *TGC (-/++)* | | | *CAT (−/+)*[c] | |
| | CTC (++/+) | | | | CTT (+/+) | |
| | GGC (+/+) | | | | TAT (+/++) | |
| | TCC (+/+) | | | | **TCT (+/−)[c]** | |
| | TTC (+/+) | | | | *TGT (−/+)* | |
| no. of samples | 9 | 5 | 4 | 2 | 9 | 3 |
| | | | | | total no. of samples: 32 | |

[a] Key: (−/+) homo and heteroduplexes overlap, wild-type (wt) differs from mutant in only one ssDNA strand; (−/++) homo- and heteroduplexes overlap, wt differs from mutant in both ssDNA; (+/+) one separate heteroduplex, wt differs from mutant in only one ssDNA strand; (+/++) one separate heteroduplex, wt differs from mutant in both ssDNA; (++/+) two separate heteroduplexes, wt differs from mutant in only one ssDNA strand; (++/++) two separate heteroduplexes, wt differs from mutant in both ssDNA; (+/−) one separate heteroduplex, wt does not differ from mutant in either ssDNA strand. [b] Shown in italic type are mutations that were not detected by HA; while mutations not detected by SSCP are shown in boldface type. [c] DNA specimens used for LPA optimization.

electrophoresed at 180 V/cm with an associated current of 10−12 $\mu$A at 27 °C.

## RESULTS AND DISCUSSION

**Polymer Synthesis and Characterization.** LPA was selected from among other synthetic polymers suitable for CE for its high hydrophilicity, efficient DNA separation ability, ease of synthesis, and low cost.[15] Several batches of LPA were synthesized by radically induced aqueous polymerization under anaerobic conditions according to a procedure previously established in our laboratory.[23] The amounts of monomer (acrylamide), catalyst (APS), cocatalyst (TEMED), and chain terminator (2-propanol) were systematically adjusted to afford a set of polymers having weight-average molar mass between 200 kDa and 2 MDa. Following the synthesis, the polymers were purified by dialysis, lyophilized, and analyzed by tandem gel permeation chromatography/multiangle laser light scattering (GPC/MALLS), which is a versatile and accurate method of characterization of polymers and copolymers suitable for CE applications.[22] Based on the results of the GPC/MALLS analysis, a set of four polymers, LPA200K, LPA600K, LPA1M, and LPA2.4M, was selected (Table 1). Each of the polymers was dissolved in 1× TBE buffer containing 10% glycerol—the most ubiquitously used CE-SSCP buffer[16]—at 2, 4, and 6% to afford a set of 12 different matrixes (4 $M_w$ × 3 concentrations = 12) that were tested as DNA separation matrixes in CAE-SSCP/HA experiments.

**Fluorescently Labeled p53 DNA Samples.** Seven mutant DNA samples amplified from exon 7 of the p53 gene (size 139 and 300 bp) and four mutant DNA samples from exon 8 of the p53 gene (size 200 bp) were used in this study for LPA matrix optimization. These DNA specimens were selected so that they include three different DNA sizes, both single- and two-base substitutions, and several different mutation positions (Table 2) in order to establish general trends in the impact of polymer physical nature on the resolution of CAE-SSCP/HA samples. The short DNA fragments (size 139 and 200 bp) were used as received from NIST in the form of PCR amplicons that were fluorescently labeled with FAM on the forward strands and with JOE on the reverse strands**.** The longer DNA fragments (300 bp), obtained from ATCC, were PCR-amplified from genomic DNA with FAM- and JOE-labeled fluorescent primers, purified by a preparative slab gel electrophoresis/cellulose membrane method, and partially desalted by filtration through Microcon-PCR filtration device. This labeling scheme allowed for two-color LIF detection and facilitated the unambiguous assignment of DNA peaks in the CAE-SSCP/HA electropherograms. The final concentration of purified DNA mutant specimens was not measured, but was estimated to be 20 pmol/$\mu$L for exon 7 wild-type and 5−20 pmol/$\mu$L for all other amplicons by the brightness of the DNA sample bands during slab gel electrophoresis relative to the brightness of selected DNA samples whose precise concentration had been calculated on the basis of their $OD_{260}$ values.

**Tandem SSCP/HA Sample Preparation.** In the literature, CE-SSCP samples are commonly diluted with 50−95% deionized formamide as well as a small amount of NaOH, without any clear justification for the presence of these additives (that we have found). We recently conducted a systematic study of the effects of formamide on CAE-SSCP and CAE-HA and concluded that it does not provide any benefits for the CAE analysis of the p53 gene, but instead significantly decreases the efficiency of electrokinetic injection and possibly adversely affects the resolution of the homo- and heteroduplexes.[27] In the same study, we also thoroughly evaluated the effects of the salt concentration in the sample on CAE-SSCP and CAE-HA and determined that 1× Tris-HCl (10 mM, pH 8.5) without any other additives is an ideal diluent for the tandem CE-SSCP/HA method.

The diluted DNA samples were denatured at 95 °C for 3 min and snap-cooled on ice. Previously, we noticed that snap-cooling has advantages over allowing the denatured DNA sample to cool naturally, in that it provides faster hybridization kinetics and minimizes the undesired annealing of primers to the SSCP conformers, while at the same time allowing for the formation of homo- and heteroduplexes (vide infra). On the other hand, attempts at slow annealing of the denatured samples over a period

of 10−60 min yields dsDNA exclusively, which can only be used for CAE-HA and not for CAE-SSCP analysis.[27]

Immediately prior to CE, a cold solution of a mutant DNA sample was mixed with a cold solution of the corresponding wild-type DNA species in ∼1:1 ratio, split into three fractions (to allow three repeats of the analysis), and loaded into a 96-well plate. Additionally, chilled solutions of the same mutant and wild-type DNA specimens were each split into three fractions, loaded into the same 96-well plate separately, and used as references for the peak assignments. Successful analyses of mixtures containing only 20% of mutant DNA (one part of exon 7 wild-type at ∼20 pmol/$\mu$L mixed with one part of exon 7 mutant at ∼5 pmol/$\mu$L) confirm the potential of tandem CE-SSCP/HA for sensitive detection of genetic mutations in heterozygous individuals and in DNA samples derived from tumors containing a significant fraction of normal tissue. It is likely that tandem CE-SSCP/HA can detect even lower concentrations of mutant DNA since it is reported elsewhere that CE-HA can detect as little as 1% mutant DNA diluted with 99% of the wild-type DNA,[29] while CE-SSCP can identify mutations in a mixture consisting of 10% mutant and 90% wild-type DNA.[30]

Typically, 2 of the 6 capillary arrays (each consisting of 16 capillaries) available in the MegaBACE instrument were used simultaneously, allowing for screening of 2−3 mutant DNA samples and 1 matrix composition at a time. The high-throughput ability of the MegaBACE instrument proved indispensable for this study, which required only ∼65 separate CAE runs (2000 single CE analyses!) to analyze all of the desired permutations of the polymer $M_w$ and separation matrix concentrations. The already high throughput of the instrument could be further increased for a clinical application of these methods by utilizing all of the 96 capillaries and a 4-color LIF detection mode that would allow for the analysis of two different mutant DNA species labeled with two different sets of dyes in a single capillary (multiplexing).

Tandem CE-SSCP/HA was performed at 27 °C since this temperature falls within the range of those most often recommended for CE-SSCP (20−30 °C)[16] and because it was the lowest temperature possible in the MegaBACE instrument available to us (temperature range 27−44 °C). Although higher temperatures may be beneficial for HA due to larger heteroduplex perturbations at the point of mismatch, leading to larger differences in homo/heteroduplex electrophoretic mobilities and hence higher sensitivity of mutation detection, at least one study reports only a minor improvement in the sensitivity of mutation detection by CE-HA upon temperature increase from 30 to 60 °C.[31] Additionally, the optimum temperature for sensitive mutation detection by both CE-SSCP and CE-HA is believed to be highly sequence-specific and, hence, probably cannot be determined a priori.[4] Because of these considerations, we chose to focus on creating optimized separation matrixes that would allow for high sensitivity of mutation detection without case-by-case fine-tuning of the temperature of analysis, which was fixed at 27 °C throughout our study.

**Dynamic Capillary Coating.** Custom-made bare fused-silica capillary arrays used in this study were coated with adsorbed polyDuramide prior to loading of the LPA separation matrix. Typically, these dynamically coated capillary arrays afforded high-quality results with LPA matrixes for about 10−14 runs, after which time they were recoated using the procedure outlined in the Experimental Section. Unlike polyDuramide-coated arrays, commercially available covalently LPA-coated capillary arrays designed for DNA sequencing applications showed much less robust performance for SSCP/HA analysis. Following five to seven successful CAE-SSCP/HA runs in these covalently coated capillaries, the electropherograms started to show broad, irreproducible, and unresolved peaks, especially for SSCP conformers, pointing to a fast irreversible deterioration or fouling of the protective LPA coating. Notably, the same LPA-coated arrays continued to perform adequately for DNA sequencing separations (M13 standard), suggesting that either the nature of SSCP conformers (i.e., increased hydrophobicity of ssDNA vs dsDNA) or the SSCP running conditions (i.e., 1× TBE buffer with glycerol for SSCP vs 1X TTE buffer with 7M urea for sequencing) place different requirements on the protective coating. For comparison, we also performed tandem CE-SSCP/HA in bare fused-silica capillary arrays dynamically coated with long-chained PDMA ($M_w$ 3−4 MDa), another self-coating polymer prepared in our laboratory, but again we were not able to obtain reproducible, sharp, and resolved SSCP/HA peaks. We attributed this to detrimental interactions of the borate ions present in the 1× TBE buffer with the PDMA coating and confirmed it by registering high electroosmotic flow in a PDMA-coated capillary in the presence of borate ions (unpublished results). These results support some earlier studies that demonstrated the importance of the protective coating and its interactions with the DNA separation matrix for high-resolution CE-HA.[32] At this stage, it was also discovered that the matrix composed of 6% of the long-chained LPA2.4M was too viscous to be loaded into the capillaries, and so it was excluded from the study.

**Analysis of SSCP/HA Electropherograms.** Typical tandem SSCP/HA electropherograms obtained in this study are presented in Figure 1. In the figure, different single-stranded and double-stranded DNA species are distinguished, and peaks are labeled (a−g). Electropherograms can be broadly divided into three clearly separate regions, which are listed here in the order of increasing migration times: (1) primers, which elute at ∼20−30 min (see Supporting Information), (2) homoduplex (peaks labeled (a)) and heteroduplex dsDNA (e), and (3) ssDNA conformers (b, c, f, g) and primer−ssDNA constructs (d). Facile differentiation between all of these DNA species was made possible by the two-color labeling and LIF detection scheme. Specifically, the homo- and heteroduplexes each demonstrate two perfectly overlapping peaks of red (reverse strand, JOE) and blue (forward strand, FAM) colors as expected from two comigrating DNA strands. Single-strand conformers exhibit two overlapping peaks of different intensities, the smaller of which results from a minor overlap of the emission spectra for the two dyes picked up by the detector. Peaks representing primer−ssDNA constructs are clustered around the peaks for the ssDNA conformers and are composed of two perfectly overlapping peaks of the two LIF colors. An unambiguous assignment of the peaks resulting from annealed primer−ssDNA species was confirmed by an experiment in which

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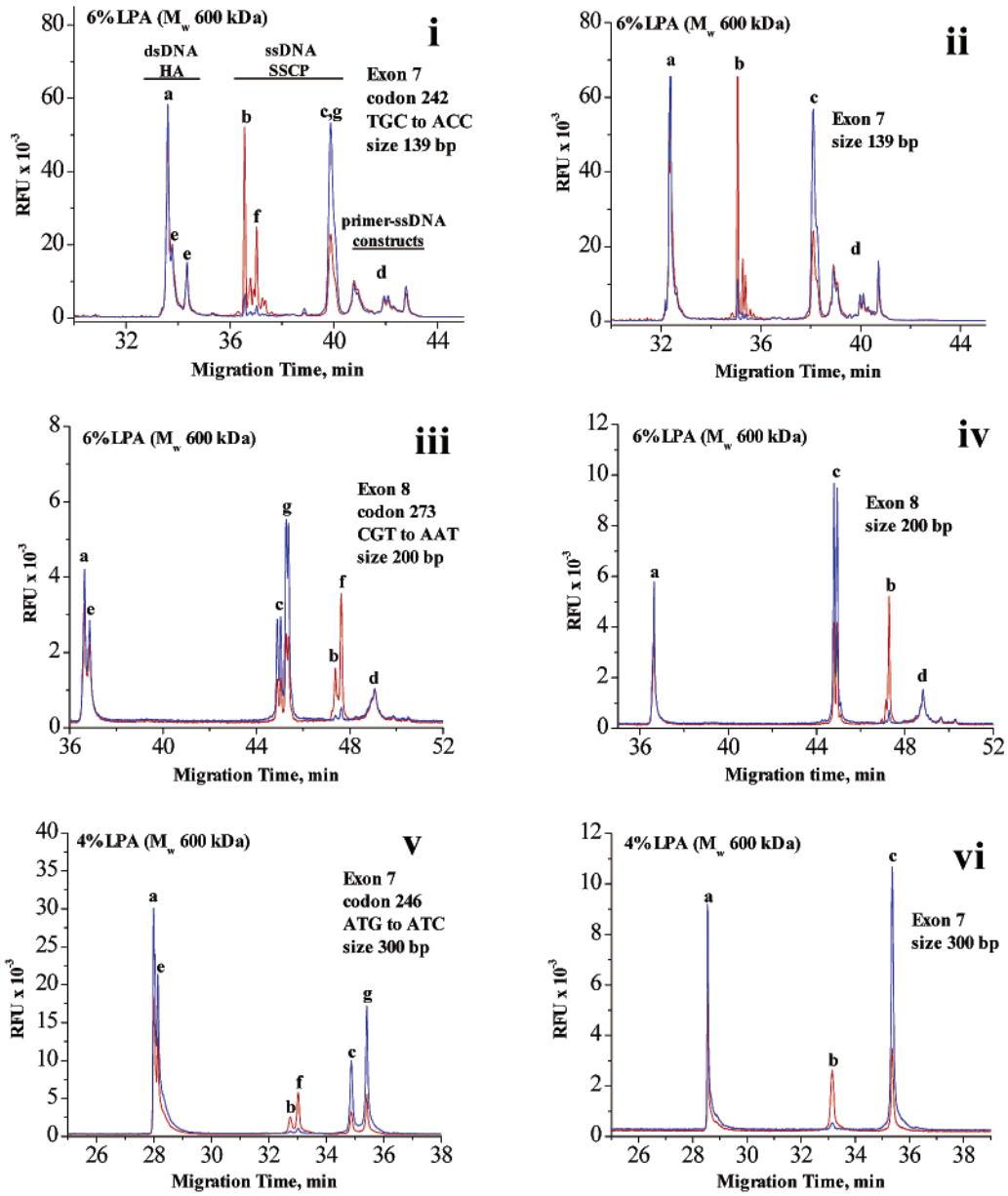


Figure 1. Precise identification of CAE-SSCP/HA peaks associated with genetic screening of p53 gene samples labeled with FAM on the forward strand (blue) and with JOE on the reverse-strand (red): (a) homoduplex; (b) wild-type reverse-strand SSCP; (c) wild-type forward-strand SSCP; (d) primer-ssDNA constructs; (e) heteroduplex; (f) mutant reverse-strand SSCP; (g) mutant forward-strand SSCP.

removal of the primers led to the disappearance of primer−ssDNA constructs, and their addition resulted in their reappearance, as evidenced by CE.[27]

**Interpretation of Electropherograms.** During early stages of this study, we attempted to evaluate the LPA matrix performance for CE-SSCP/HA by simply comparing the time of separation between the wild-type and mutant DNA peaks, a fairly common approach adopted in other studies that have looked at the effects of polymer concentration on CE-SSCP performance.[19−21] However, as the study progressed, we deemed this methodology inadequate, because more concentrated matrixes based upon long-chain LPA resulted in longer DNA migration times and more widely separated and broader peaks than those obtained in less concentrated matrixes and in those of lower LPA $M_w$. At this point, we decided that resolution is a much better gauge for the performance of the different LPAs. The migration times of the wild-type DNA ($t_{wt}$) and mutant DNA ($t_{mut}$) were determined from the electropherograms, and resolution ($R$) was calculated according to the following classical equation

$$R = 2(t_{wt} - t_{mut})/(w_{wt} - w_{mut})$$

where $w_{wt}$ and $w_{mut}$ are wild-type and mutant DNA peak widths, respectively.

Since peak widths could vary from capillary to capillary and from run to run by as much as 50%, they were modeled in the

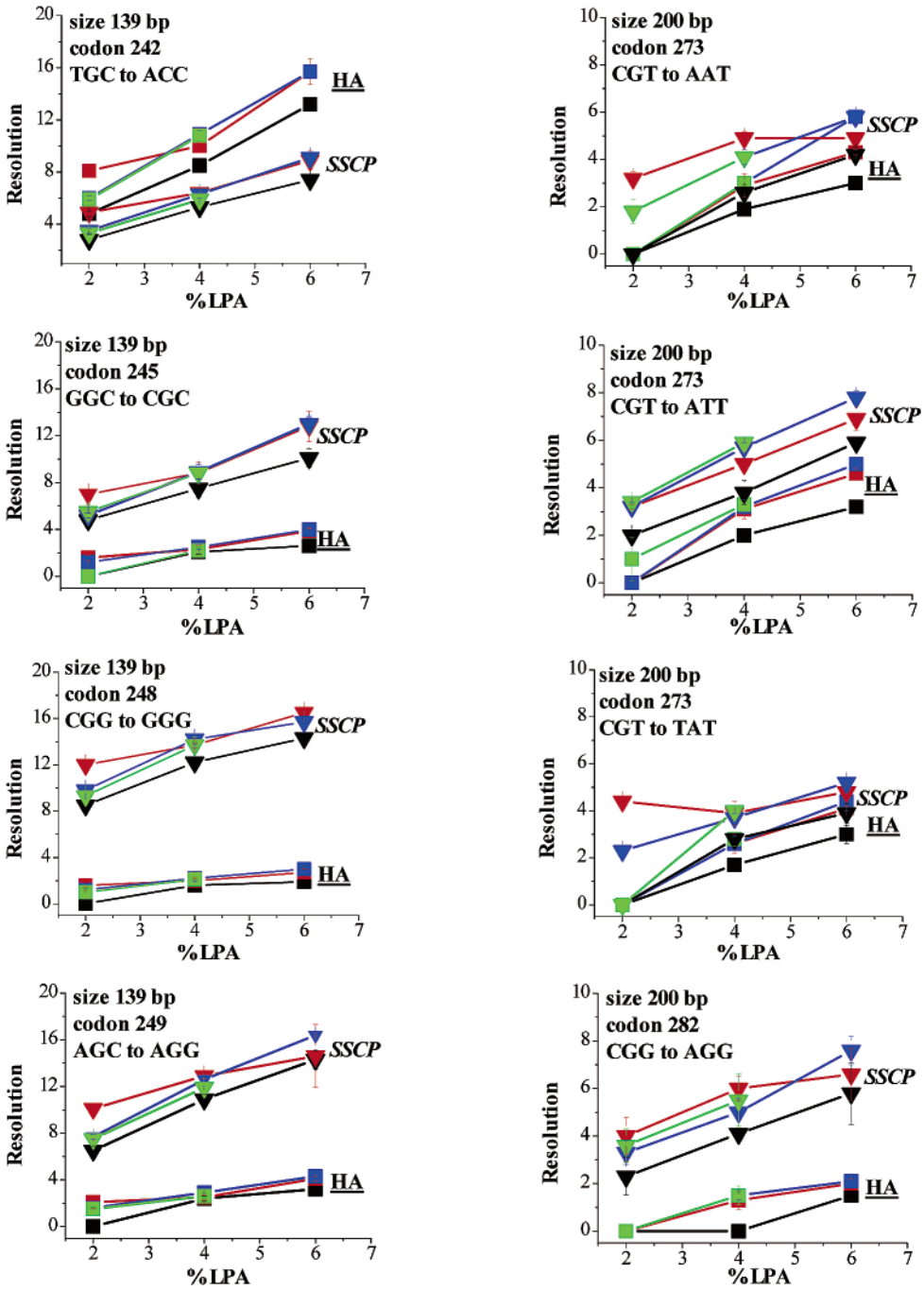


Figure 2. Combined impact of LPA weight-average molar mass and matrix concentration on the resolution of CAE-SSCP and CAE-HA for eight different DNA samples of the p53 gene, for separations performed in LPA200K (black), LPA600K (red), LPA1M (blue), and LPA 2.4M (green). Squares represent HA data, and inverted triangles represent SSCP data.

following fashion. Three mutant DNAs that possessed the most distinct and clear peak patterns were identified from the SSCP/HA electropherograms, and the peak widths recorded in all 12 matrixes were modeled in ORIGIN (Gaussian fit) and measured. The data were plotted as peak width versus peak migration time, and the plot showed an excellent linear fit ($w_{wt,\ mut} = 0.00131 t_{wt,\ mut}$), which was used for all the peak width estimations used in resolution calculations (Figure 34S, Supporting Information).

**LPA Optimization for Sensitive CAE-SSCP/HA Mutation Detection.** Plots of $R$ versus LPA concentration (%) were constructed for all of the separation matrixes, and each includes the data for both SSCP and HA (Figure 2). If a mutant species was resolved from the wild-type in both forward and reverse SSCP strands and also in both heteroduplexes, only one heteroduplex and one SSCP strand were taken into account and used for $R$ calculations. Each data point in these plots represents an individual analysis that was repeated at least 3 times; typical standard deviation of mobilities in these analyses was 3%. It is evident from the plots that resolution increases with increased LPA concentration in an almost linear fashion. We found that extremely short-chained LPA200K provided relatively poor resolution of SSCP/HA species at any concentration. LPA600K and LPA1M both

offered good resolution of CE-SSCP and CE-HA analysis for short DNA fragments (139 and 200 bp), while longer LPA2.4M polymer did not offer further improvements and also slowed the analyses and hindered capillary loading due to its higher matrix viscosity. With the exception of two DNA samples of p53 exon 7 that contained 2-bp substitutions (Figure 2), resolution of mutant species from wild-type was always higher for SSCP than HA.

Attempts at analyzing longer DNA fragments from exon 7 (300 bp) of the p53 gene proved to be less successful. Tandem CAE-SSCP/HA of one mutant DNA sample gave higher resolution in LPA1M than in LPA200K and even LPA600K. Additionally, in the LPA1M matrix resolution did not necessarily depend linearly on the concentration of LPA (not shown), as in the case of shorter DNA fragments, and was higher in 4% LPA solutions than in 6% LPA solutions. These trends, however, have not yet been confirmed because SSCP/HA analysis of the rest of the limited number of the long DNA fragments we have studied so far yielded inconsistent results. We are continuing to evaluate these phenomena.

Although the highest resolution of SSCP/HA conformers was achieved in 6% LPA1M (Figure 2), its routine use for tandem CE-SSCP/HA was compromised by the increased number of failed runs and excessive CE noise, presumably as a result of high viscosity of this polymer solution. Therefore, 6% LPA600K, which provides comparable resolution, but does not have the shortcomings associated with LPA1M, was selected as the optimized DNA separation matrix. The optimized DNA separation matrix was utilized to screen 32 mutant DNA samples of p53 exons 7 and 8 that include both single-base and double-base substitutions. CAE-SSCP was able to detect 30 of 32 mutations (93% sensitivity), and CAE-HA was able to detect 24 of 32 mutations (75% sensitivity) (Table 2). Since only 6% LPA600K allows for highly sensitive genetic screening, while other polymer compositions demonstrated much lower sensitivity due to poor resolution, excessive noise, or a large number of failed CE runs, it is likely that a higher than usual individual sensitivity of mutation detection by CE-SSCP or CE-HA is due to the powerful separation capability of the optimally synthesized polymer. Significantly, *tandem* CAE-SSCP/HA was able to identify all 32 mutations (100% sensitivity), and this perfect sensitivity of mutation detection is attributed to the complimentary abilities of CE-SSCP and CE-HA. Generally, the resolutions and sensitivity were higher for the smaller p53 exon 7 fragments than for the larger exon 8 samples, which can only be nonspecifically attributed to differences in either DNA sequence or size. Moreover, three p53 exon 7 mutant DNA specimens were successfully screened for mutations by SSCP performed on a plastic microchip during proof-of-concept experiments in our laboratory (data not shown). The microchannels (length 18 cm) were filled with the optimized polymer matrix (LPA600K, 6%), and the separations were achieved in less than 13 min. Further tandem SSCP/HA studies in plastic and glass microfluidic chips are ongoing in our laboratory.

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## CONCLUSIONS

We have conducted the first systematic investigation of the combined impact of the average molar mass of LPAs and their concentration in the DNA separation matrix on the resolution of tandem capillary array electrophoresis single-strand conformation polymorphism/heteroduplex analysis, coupled with complete and precise assignment of all of the peaks in the electropherograms. We conclude that moderately short LPA (~600 kDa) dissolved at ~6% in 1× TBE serves as an optimal DNA separation matrix for the analysis of short DNA fragments (<200 bp) of the p53 gene and can afford 100% sensitivity of mutation detection by tandem SSCP/HA. Unlike *tandem* SSCP/HA, SSCP and HA applied separately to the same set of p53 gene samples afforded only 93 and 75% sensitivity of mutation detection, respectively, and at this level would not be applicable for clinical patient genetic screening, for which a sensitivity of at least 97% is desirable.

It was shown that the sensitivity of CAE-SSCP/HA strongly depends on a simultaneous optimization of both the protective capillary wall coating and the DNA separation matrix and that polyDuramide, a novel self-coating polymer, is essential for dynamic capillary coating employed in CAE-SSCP/HA. Neither a covalent LPA coating nor a dynamic PDMA coating gave reproducible SSCP/HA results with efficient DNA peaks.

We are presently applying these optimized tandem CE-SSCP/HA protocols to the mutation analysis of the PTEN and $\beta$-catenin genes derived from human prostate tumors, to test their broader generality. We are also working to transfer CAE-SSCP/HA to a microchip electrophoresis format, a task for which these optimized LPA-based matrixes are particularly well suited given their good separation ability, moderately low viscosities, and high shear thinning rate.[33] Preliminary results suggest that analogous mutation detection separations can be achieved on plastic microchips in less than 13 min, using optimized LPA as the DNA separation matrix.

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## SUPPORTING INFORMATION AVAILABLE

Plot showing peak width vs peak migration time, tandem CE-SSCP/HA electropherograms illustrating mutation detection in all 32 screened DNA mutant specimens. This material is available free of charge via the Internet at http://pubs.acs.org.

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