

RAPID SOUTHERN-BLOT-TYPE ASSAYS USING BIDIRECTIONAL ISOTACHOPHORESIS

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ABSTRACT

We present a novel method for coupling rapid isotachophoresis (ITP)-based DNA hybridization with post-reaction electrophoretic separation via bidirectional ITP. Our method leverages physicochemical changes associated with the interaction of anionic and cationic ITP shock waves in bidirectional ITP to rapidly and automatically trigger CZE from ITP. We demonstrate the speed and sensitivity of our assay by detecting 5 pM, 39 nt ssDNA target within 3 min, using a molecular beacon probe. We also demonstrate the feasibility of our assay as a rapid alternative to southern-blotting by simultaneously detecting 39 and 90 nt ssDNA targets.

KEYWORDS

DNA hybridization, isotachophoresis, capillary zone electrophoresis, multiplexed detection

INTRODUCTION

DNA hybridization is essential to a variety of fields, including mutation analysis, single nucleotide polymorphism (SNP) genotyping and pathogen detection. However, slow, second-order hybridization kinetics at low concentrations of DNA samples often results in long analysis times, limiting assay speed and applicability. One way of accelerating DNA hybridization is through isotachophoresis (ITP), wherein nucleic acid fragments preconcentrate, mix and react within a small (order 10 pL) reaction volume inside a microchannel.[1,2] Persat *et al.*[1] applied ITP-hybridization to the profiling of micro-RNA using a molecular beacon (MB) probe; a DNA probe whose fluorescence increases upon hybridization to its target. Bercovici *et al.*[2] developed and experimentally validated an analytical model for the physicochemical process that governs ITP-hybridization.

Here, we present a method of extending the functionality of ITP-based hybridization by integrating it with post-hybridization capillary zone electrophoresis (CZE). Electrophoretic separation of products of the ITP hybridization reaction provides a method of removing the background signals associated with unreacted labeled probes and enables multiplexed detection of multiple length DNA fragments. We note that CZE has been used previously for removal of background signal from the products of hybridization reaction between a synthetic oligonucleotide and a fluorescently labeled probe.[3] However, such studies involved slow (over 40 min), offline/off-chip hybridization of relatively high (order μ M) concentration probe and target. Also, previous studies on CZE detection of DNA hybridization reaction products involved manual loading of reaction products into an electrophoresis setup.

In the current work, we use bidirectional ITP to couple ITP based DNA hybridization and CZE separation of hybridization products. Bidirectional ITP[4] is itself a newly developed process where we set up two ITP interfaces between cations and anions respectively from opposite ends of a microchannel such that these ion-concentration shock waves approach each other and interact. This interaction triggers a rapid transition from ITP to CZE. Our bidirectional ITP based technique enables sensitive sequence-specific detection of multiple length DNA fragments, and provides a rapid and automated alternative to Southern blotting.[5]

THEORY

Isotachophoresis (ITP) is an electrophoresis technique which focuses analytes between zones of high effective mobility leading electrolyte (LE) ions and low effective mobility trailing electrolyte (TE) ions if the mobilities of LE and TE ions bracket those of analytes.[6] In the current work, we use bidirectional ITP, which involves simultaneous anionic and cationic ITP processes in a single channel. Therefore, our experiments require two oppositely charged pairs of LE and TE ions. Here we term these ions LE⁺, TE⁺, LE⁻, and TE⁻. LE and TE denote the leading and trailing electrolyte ions, respectively, and + and - correspond to cations and anions, respectively. Figure 1 shows a schematic of our technique. We first fill the microfluidic channel with a mixture of LE⁻ and LE⁺ ions. We fill the right (anodic) reservoir with the LE⁻/TE⁺ mixture, and similarly the left (cathodic) reservoir with a mixture of LE⁺, TE⁻, ssDNA target and the molecular beacon probe. When electric field is applied along the channel (Fig. 1b), the target and the probe mix, focus, and react at the interface of LE⁻ and TE⁻ zones while propagating rightwards. Simultaneously, a cationic ITP shock forms between LE⁺ and TE⁺ zones near the right reservoir, and propagates leftwards. Anionic ITP preconcentration of target and probe results in rapid hybridization and corresponding increase in fluorescence signal. Later, when the anionic and the cationic ITP shocks interact (Fig. 1c), LE⁺ is replaced with TE⁺ as the counter-ion for anionic ITP. This counter-ion exchange decreases the local pH of anionic ITP zones to a value at which effective mobility of LE⁻ becomes significantly lower than the mobilities of all DNA fragments. Thus, shock wave interaction causes target, probe, and target-probe hybrid to migrate into the LE⁻ zone, triggering electrophoretic separation of the hybridization products and unreacted species. The separation removes background signal associated with unreacted MB probes, and makes possible hybridizations between a probe and multiple targets.

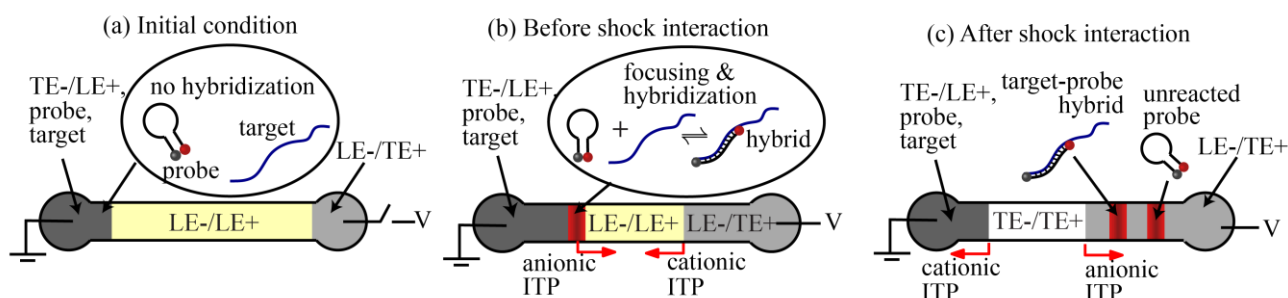


Figure 1: Schematic illustrating preconcentration and hybridization of DNA followed by electrophoretic separation using bidirectional ITP. (a) The microchannel is initially filled with LE-/LE+ mixture. The left (cathodic) reservoir is filled with a mixture of TE-, LE+, ssDNA target, and probe. The right (anodic) reservoir is filled with the LE-/TE+ mixture. (b) When voltage is applied, anionic and cationic ITP interfaces form, respectively, at the cathodic and anodic ends of the channel and propagate towards each other. Probe and target preconcentrate and hybridize in anionic ITP. (c) Within about 2 minutes of ITP-driven hybridization, the cationic ITP interface interacts with focused hybridization products, and triggers CZE separation. The resulting electropherogram shows peaks corresponding to the fluorescent unreacted probe and the target-probe hybrid.

MATERIALS AND METHODS

We demonstrated our method using a Cy5 labeled 39 nt molecular beacon probe and two synthetic unlabelled ssDNA targets (39 and 90 nt). For all experiments, we used the following electrolyte concentrations: 150 mM sodium bicarbonate for the LE+/LE- mixture; 10 mM sodium hydroxide and 34 mM Hepes for the LE+/TE- mixture; and 100 mM pyridine and 50 mM hydrochloric acid for the TE+/LE- mixture. To the LE+/LE- mixture, we added 1% w/w of hydroxyl ethyl cellulose (HEC) to serve as a sieving matrix for DNA separations. We also added 1% w/w polyvinylpyrrolidone (PVP) to all electrolyte solutions to suppress electroosmotic flow. We performed all experiments on an inverted epifluorescent microscope (IX70, Olympus, Hauppauge, NY) equipped with a Cy5 filter-cube (Cy5-4040A, Semrock, Rochester, NY). For all experiments, 1 kV was applied from a high voltage sourcemeter (model 2410, Keithley Instruments, Cleveland, OH, USA). We recorded fluorescence intensity using either a CCD camera (Figure 2) or a photomultiplier tube module (Figure 3).

EXPERIMENTS

Figure 2 shows representative spatiotemporal plots of the experimentally measured fluorescence intensity in the channel (scalar) versus distance along the channel (abscissa) and time (ordinate). Prior to the interaction of anionic and cationic ITP shocks ($t < 10$ s), the ssDNA target and MB probe mix, focus, and hybridize in a narrow anionic ITP zone traveling at constant velocity (Figure 2a). At about $t = 10$ s, focused ssDNA target and MB probe interact with a counter-migrating cationic ITP front, and within less than 1 s previously focused analytes begin to separate in CZE mode. Figure 2b shows separation of the unreacted probe and the target-probe hybrid at later times. The separated zones diffuse and move further apart over time as in CZE.

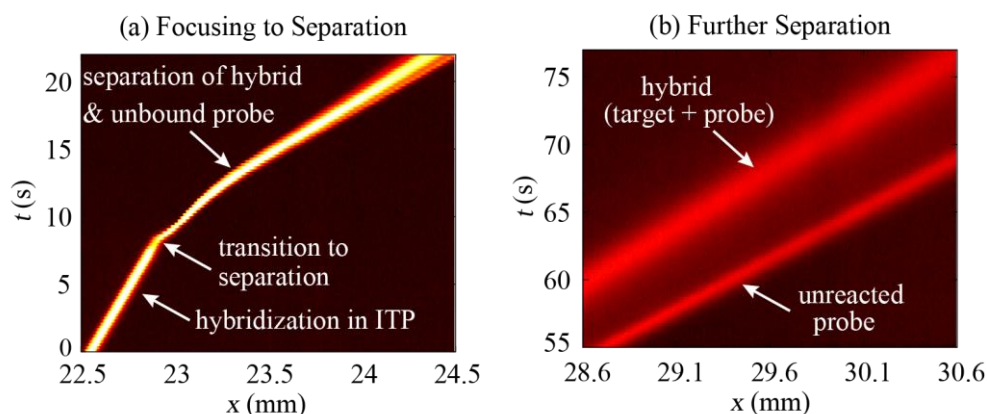


Figure 2: Visualization of ITP-based DNA hybridization and post-hybridization separation of reaction products. Spatiotemporal plots showing the intensity of fluorescent probe in channel versus the distance along the channel axis, x , and time, t . (a) Initially ($t < 10$ s) ssDNA target and molecular beacon probe mix, focus, and hybridize in anionic ITP. At $x = 23$ mm and $t = 10$ s, a counter-migrating cationic ITP interface interacts with the focused analyte zones, triggering CZE separation. (b) shows resolved peaks of fluorescent hybrid and unreacted probe in the CZE mode. Axial distance was measured from the left well, and time was measured from the start of data recording.

In Figure 3, we present experimental results for multiplexed detection of 39 and 90 nt targets. Here, post-hybridization CZE makes separation not only between the unreacted probes and hybrids, but also between the two hybrids bound with different length targets. The negative control (Fig. 3a) shows a single peak corresponding to probe in the absence of target. When two untagged target fragments are added, the electropherogram shows two additional peaks, indicating successful detection of each target (Fig. 3c-d).

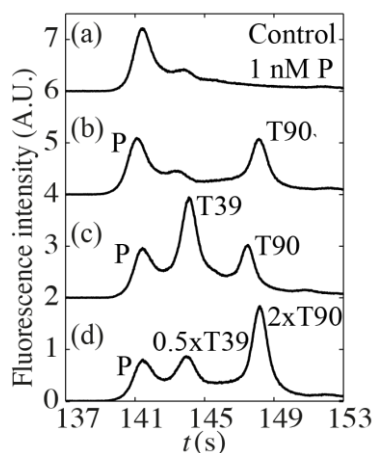


Figure 3: Multiplexed sequence-specific detection of 39 nt and 90 nt DNA targets, both having a 27 nt sequence complementary to the molecular beacon probe. Plots show fluorescence intensity of unreacted probe (P) and target-probe hybrids (T39 and T90) during CZE separation. For all experiments, probe concentration was fixed at 1 nM. (a) Negative control signal of 1 nM probe. (b) When 200 pM of 90 nt target is mixed with the probe, the second peak corresponding to the 90 nt target-probe hybrid appears. (c) Addition of 39 nt and 90 nt target, respectively, at 100 and 200 pM concentrations, yields the three peaks. (d) We identified the peaks by observing variations in peak intensity upon changing the initial concentration of 39 nt and 90 nt targets to 50 and 400 pM respectively. For these experiments, we first performed unidirectional anionic ITP for 40 s to ensure enough time for probes and target to hybridize, and subsequently initiated bidirectional ITP. The abscissa shows the total analysis time, including anionic and bidirectional ITP steps. For all cases, we applied 1 kV voltage. Electrolyte chemistry is provided in the Materials and Methods section.

Lastly, we demonstrated rapid and high sensitivity sequence-specific detection of 39 nt target. Figure 4 shows magnitudes of the target-probe hybrid peak (after subtracting the background signal from unreacted probe) for target concentrations ranging from 5 to 30 pM and a fixed 200 pM concentration of MB probe. Because the probe concentration is significantly higher than the target concentrations in our experiments, DNA hybridization obeys pseudo-first order kinetics. As a result, magnitude of target-probe hybrid peak increases linearly with the target concentration. The total assay time including hybridization and separation was less than 3 min for all experiments. As shown in Figure 4, limit of detection for our assay is 5 pM, which is a 20-fold improvement over the previous ITP-hybridization assays. [1-2]

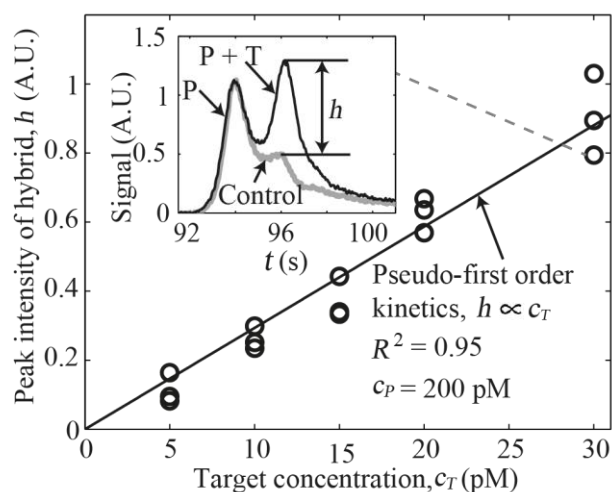


Figure 4: Titration curve obtained from fluorescent intensity measurements of target-probe hybrid while changing the initial target concentration from 5 to 30 pM. Probe concentration was fixed at 200 pM. A linear fit (passing through the origin) to the experimental data yields R^2 value of 0.95. Inset shows a representative signal measured during CZE step for the case of 30 pM initial target concentration. The signal (black line) shows two peaks corresponding to the unreacted probe (P) and the target-probe hybrid (P+T). To extract the peak intensity of hybrid zone, h , we first scaled and time-shifted the negative control signal (grey line) to match the peak intensity of unreacted probe peak in the signal. We then extracted the difference between the peak heights of signal and adjusted-negative control as the measure of peak intensity of target-probe hybrid.

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