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Isotachophoresis with ionic spacer and two-stage separation for high sensitivity DNA hybridization assay†

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We present an on-chip electrophoretic assay for rapid and high sensitivity nucleic acid (NA) detection. The assay uses isotachophoresis (ITP) to enhance NA hybridization and an ionic spacer molecule to subsequently separate reaction products. In the first stage, the probe and target focus and mix rapidly in free solution under ITP. The reaction mixture then enters a region containing a sieving matrix, which allows the spacer ion to overtake and separate the slower probe–target complex from free, unhybridized probes. This results in the formation of two focused ITP peaks corresponding to probe and probe–target complex signals. For a 149 nt DNA target, we achieve a 220 fM limit of detection (LOD) within 10 min, with a 3.5 decade dynamic range. This LOD constitutes a 12× improvement over previous ITP-based hybridization assays. The technique offers an alternative to traditional DNA hybridization assays, and can be multiplexed and extended to detect other biomolecules.

Nucleic acid (NA) hybridization assays are important tools in a variety of diagnostic and biological applications, including disease detection, forensic sciences, genetic profiling, and food analysis.[1–3] However, hybridization assays for trace target concentrations suffer from slow second-order kinetics that result in very long assay times.[4] Isotachophoresis (ITP)-based hybridization is one approach that offers enhancement of reaction kinetics and integration with fluorescent detection. Recently, ITP has been shown to achieve up to 14 000-fold reaction speed-up in NA-based hybridization systems.[5] A current limitation to assay sensitivity is background signal inherent to unreacted fluorescent probes. To our knowledge, two methods have been reported to physically separate unreacted probes and complexes in ITP assays. This separation improves signal-to-noise ratio (SNR) by reducing fluorescence background. The first method used a gel polymer functionalized with DNA target molecules that bind to unreacted probe to remove excess reactant.[6] That technique demonstrated 2.8 pM sensitivity, which to our knowledge is the lowest limit of detection achieved using ITP-based DNA hybridization assays. However, sensitivity is limited by the remnant fluorescent reporter molecules following migration through the capture gel. The second method combined ITP with capillary electrophoresis (CE) to separate unreacted probe from the probe–target complex, and demonstrated 5 pM sensitivity.[7] The latter approach offers size-based separation, but the separated zones are subject to the dispersion effects of CE, thereby decreasing signal strength.

This brief communication presents a new method of improving the SNR of ITP DNA fluorescence hybridization assays: we use two separation regions in series and an ionic spacer to separate reaction products from reactants. The assay consists of three main stages: incubation of reactants under ITP focusing, separation of probes from probe–target complex, and detection of independently focused reaction products and fluorescent probes. Through the use of the ionic spacer, we maintain the products and reactants each in a discrete ITP zone while still providing a rapid (40 to 45 s) transition from incubation to the fully separated state. Maintaining products and reactants in ITP mode is advantageous as it gives the designer flexibility as to where to place a detector (since signal is preserved over time) and facilitates further downstream manipulations (such as fractionation) without incurring dispersion losses. Further, creating two focused ITP zones allows for an internal control based on the ratio of the integrated signal of the two zones, making this method particularly robust to variations in injection amounts. Using this technique, we demonstrate a limit of detection of 220 fM in less than 10 min, with 3.5 decade dynamic range.

ITP is an electrophoretic technique that relies on a heterogeneous buffer system comprising a high mobility leading electrolyte (LE) and low mobility trailing electrolyte (TE) to achieve focus target analytes.[8] Here, we achieve probe DNA and

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target DNA preconcentration of order 10 000× and higher. Our assay includes a spacer ion with intermediate mobility which forms a plateau region between the LE and TE, thereby creating two sharp interfaces between the LE and spacer and between the spacer and TE. Fig. 1a demonstrates the steps in our reaction-separation assay. First, we leverage ITP to focus the probe and target molecules and accelerate second-order hybridization kinetics (time t_1). The second and third stages of the assay, denoted respectively by t_2 and t_3, employ a linear sieving matrix to separate the reaction products. The channel initially contains two LE regions in series, as shown in Fig. 1b. LE1 includes no sieving matrix, while LE2 includes a sieving matrix. The sieving matrix primarily affects mobility of DNA molecules relative to small ions. In the LE1 region, spacer ions have an electrophoretic mobility lower than that of the probe, target, and probe–target complexes. This enables simultaneous rapid mixing and preconcentration of the probe and its target.[5] Upon entering LE2, the spacer ions overtake the now slower target and probe–target complex. The spacer has sufficient initial concentration to quickly form a plateau ITP region which separates excess probes from probes hybridized to target molecules. In this final stage, the excess probe molecules continue to focus between the LE and the spacer, while the probe–target complexes focus in a separate ITP zone between the spacer and the TE. This enables sensitive detection of the probe–target complexes in the absence of unhybridized fluorescent probe molecules.

All experiments were performed on a 12 μm deep Crown glass NS-260 chip from Caliper Life Sciences (Mountain View, CA). We chose 100 mM chloride as our LE anion and 20 mM HEPES anion for our initial TE buffer mixture. The LE and TE buffers contained respectively 200 and 40 mM Tris cation, resulting in predicted pH of 8.1 in the LE zone and 8.6 in the TE zone. The spacer for all experiments was MOPS, which has an effective free-solution mobility of 18.5×10^{-9} m^2 V^{-1} s^{-1}, higher than the 15.5×10^{-9} m^2 V^{-1} s^{-1} effective mobility of HEPES in the adjusted TE zone. We chose the linear polymer hydroxyethyl cellulose (HEC) as our sieving matrix. At overly high HEC concentrations, the reproducibility of the experiments was compromised by the high viscosity of the HEC solution. We found that an HEC concentration of 1.8% (w/v) offered an effective compromise between fast resolution of the peaks and repeatability. In all buffers we included 1% (w/v) polyvinylpyrrolidone (PVP) for further electroosmotic flow suppression.[9] Interestingly, we have found that PVP does act as a sieving matrix at high concentrations (above 4%) but not significantly at 1%. We also included 5 mM magnesium chloride in the LE to improve DNA hybridization kinetics.[10] Finally, we use 4 M urea in the LE1 region to improve selectivity and reaction completion by denaturing secondary structure. We note that we have optimized the assay to optimally separate 149 nt long DNA, but the chemistry and sieving matrix concentration can be optimized for targets of varying lengths. Denatured DNA have well-studied mobilities,[11] which facilitates such modifications of the assay.

We performed experiments to visualize the reaction and separation processes using a charged coupled device (CCD) camera (Coolsnap, Roper Scientific, Trenton, NJ), as shown in Fig. 2. To demonstrate experimentally the three stages of our assay, we constructed a spatiotemporal plot showing the signal intensity as a function of axial channel dimension (abscissa) and time (ordinance) (Fig. 2a). We also show individual snapshots of the separation process at various times (Fig. 2b). Initially, the analytes migrate together in a single peak in free solution (time t_1). Upon entering the sieving matrix, the two

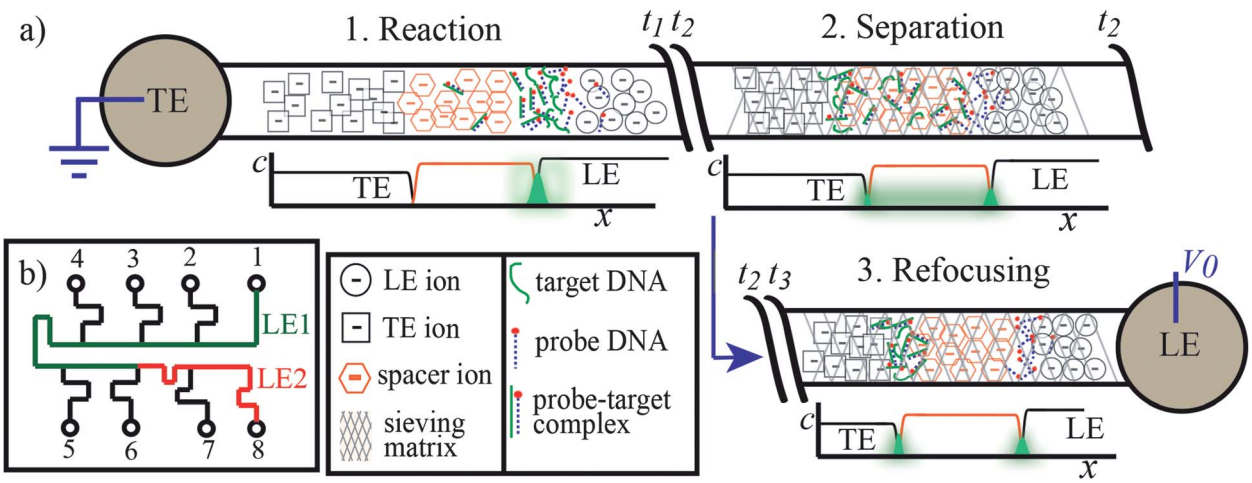

Fig. 1 (a) Schematic representation of the ITP-spacer assay, showing the three stages of the assay: (1) reaction between the short (27 nt) DNA probe and the complementary (149 nt) target in free-solution conditions. In this stage, spacer molecules migrate at a mobility lower than that of the target DNA. (2) Upon entering the sieving matrix region (1.8% HEC), the ionic spacer molecules gradually overspeed the now slower target molecules and probe–target hybrids. (3) Following approximately 40 s of separation, the reaction products are fully separated and refocused among the two ITP interfaces. Excess probe molecules focus between the LE and spacer, whereas the probe–target hybrids focus between the spacer and the TE. (b) Schematic of the Crown glass chip layout used for the assay. We initially load the microchannels with LE1 (no sieving matrix) and LE2 (1.8% HEC). We apply voltage between reservoir wells 1 (TE) and 8 (LE). The region containing LE1, which spans 5 cm in length, allows for simultaneous mixing and preconcentration of the reactants in ITP mode. In the region containing LE2, spanning 3 cm in length, the reactant products separate and refocus.

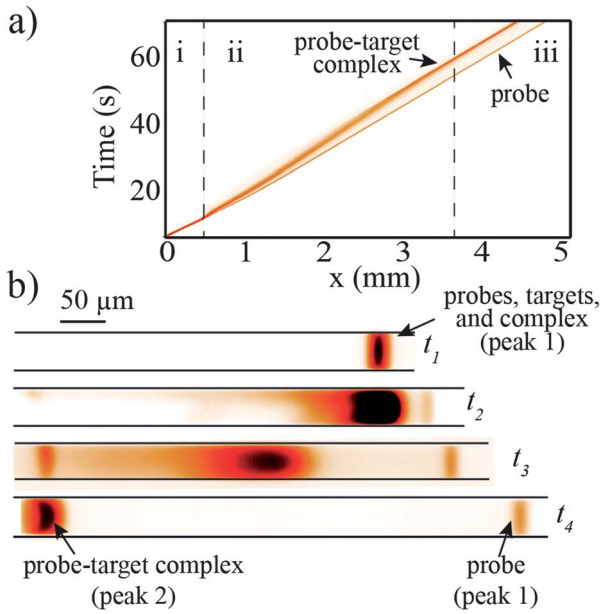

Fig. 2 Experimental visualizations of reaction and separation regions of the ITP-spacer assay. (a) Spatiotemporal plot demonstrating the three stages of the assay: (i) the two reactants co-focus between the LE and spacer, resulting in a single peak, (ii) upon entering the separation region, the spacer ions overtake the probe–target complex, and (iii) reaction products are fully separated by the spacer region and refocus at separate ITP interfaces. (b) Images of the separation process at four times. Image intensities are scaled individually to optimize contrast. Times t_1 are the mixing/reaction stage, t_2 and t_3 show the separation, and t_4 shows the steady state after refocusing.

peaks begin to separate, with a comet-shaped cloud migrating from the first ITP zone toward a second, newly formed ITP zone which trails the first by about 0.6 mm (t_2 and t_3) Eventually, the spacer ions overtake the slower probe–target complexes completely, and the reaction products are fully separated and focused in ITP mode (time t_4). The separation process takes approximately 40 s for a spacer concentration of 0.5 mM MOPS (included in the TE).

The mobility of the target–probe complex within the sieving matrix can be approximated by measuring the relative velocity of the target–probe complex peak relative to the ITP interface velocity, as follows:

$$\Delta V = V_{\text{peak1}} - \mu_{\text{complex}} E_{\text{spacer}} = \frac{L}{t} \quad (1)$$

where V_{peak1} is the velocity of the front peak containing unreacted probe, μ_{complex} is the mobility of the longer target, E_{spacer} is the electric field in the spacer region, L is the length of the spacer zone, and t is the time for transition from the first peak (right peak in Fig. 2b) to the second peak (left peak). V_{peak1}, L, and t are determined experimentally. We estimate E_{spacer} using the electrophoresis solver SPRESSO software.[12] For the simulation, we assume that the mobility of small ions is unaffected by the presence of a sieving matrix (see the ESI† for further discussion). We solve eqn (1) for μ_{complex} in the separation region and obtain a mobility estimate of 15.6×10^{-9} m^2 V^{-1} s^{-1}, higher than that of our TE anion and lower than that of the spacer.

We chose to demonstrate and quantify the performance of this assay using synthetic DNA as a well-characterized model target. The experimental setup is detailed in the ESI† section of Garcia-Schwarz and Santiago.[6] The probe was a 27 nt DNA with a sequence complementary to bacterial 16S ribosomal RNA (rRNA). 16S rRNA is closely associated with urinary tract infection (UTI).[13] Our target is a synthetic 149 nt DNA containing the 16S rRNA sequence complementary to the shorter probe. Fig. 3a shows the titration curve we constructed to quantify the dynamic range of the assay. We held the probe concentration constant at 100 pM, and varied target concentration over approximately 5 orders of magnitude, from 220 fM to 73.6 nM. For each run, we divide the signal associated with the second peak (attributed to the probe–target complex) by the total fluorescence signal in the two ITP peaks. We therefore normalize our measurement, and can account for variations in injection concentrations. Assay sensitivity is limited by fluorescence remaining in the second peak for a negative control run. We hypothesize this background signal is due to probe impurity. We therefore estimate hybridized product amount, N_{hyb}, by subtracting the background signal from the measured signal:

$$N_{\text{hyb}} = \frac{f_{\text{peak2}}}{f_{\text{total}}} - \frac{f_{\text{c,peak2}}}{f_{\text{c,total}}} \quad (2)$$

here f_{peak2} and $f_{\text{c,peak2}}$ denote the fluorescent signal associated with the second peak (peak 2 in Fig. 2b) in the data and control runs, respectively. f_{total} and $f_{\text{c,total}}$ denote the total fluorescent signal associated with both peaks (peak 1 plus peak 2) in the

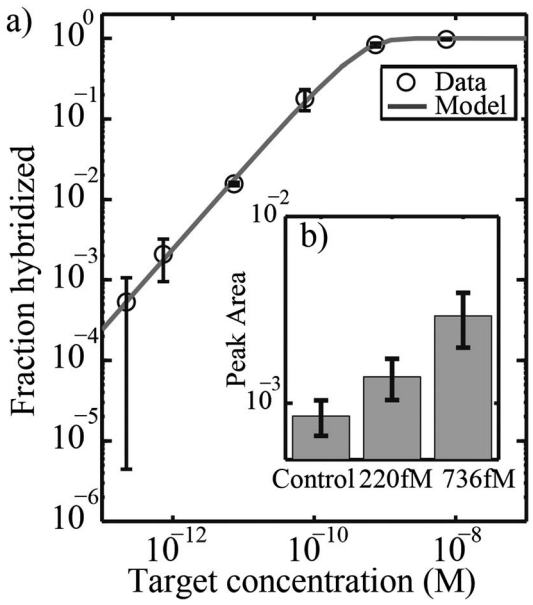

Fig. 3 Experimental data demonstrating the sensitivity and dynamic range of the ITP-spacer assay for detection of a 149 nt DNA target. All error bars correspond to 95% confidence on the mean. (a) Titration curve of target concentrations ranging from 220 fM to 7.36 nM, with probe concentration fixed at 100 pM. The assay has a linear dynamic range (R^2 = 0.99) of nearly four orders of magnitude. Along with the experimental data, we show results from a numerical reaction model with a single global fitting parameter. (b) Limit of detection study showing the computed mean peak area for the lowest three target concentration values: $c_T = 0$ (negative control), 220 fM, and 736 fM.

data and control runs, respectively. Based on the models of Bercovici–Han *et al.*[5] and Garcia-Schwarz and Santiago,[6] we expect this fraction to increase linearly with increasing target concentration. The assay has a 3.5 decade dynamic range, on the same order as that presented by Garcia-Schwarz, and higher than other ITP-hybridization assays.[14] Shown together with the experimental data is a plot of ITP hybridization reaction model developed by Garcia-Schwarz (a simplification of the more comprehensive Bercovici–Han model) using kinetic on-rate as a fitting free parameter.

We found an LOD of 220 fM, with a *p*-value of 0.03 (Fig. 3b). This result constitutes 100× lower detection limit than ITP assays using molecular beacons (MB), and 12× improvement over the most sensitive NA hybridization assay with ITP thus far.

We estimate that we are detecting approximately 0.1 fg of target DNA, which corresponds to 1300 target molecules. We hypothesize that further improvement in LOD can be obtained given probes of higher purity.

In summary, we introduced a rapid (~10 min), highly sensitive assay for sequence specific quantitation of a DNA target. The assay leverages ITP-enhanced hybridization and an ionic spacer ion and sieving matrix for background fluorescence removal. Our assay includes an initial mixing and reaction stage, and a subsequent separation stage. This results in two separate, focused ITP zones: the first zone containing unreacted probes and the second zone containing probe–target complexes. We demonstrated 220 fM LOD with a 149 nt target, a 12× improvement over previous ITP-based hybridization assays, and 100× improvement over ITP-MB assays. We hypothesize this LOD can be improved with purer probes and longer channel lengths to allow more time for the hybridization reaction. Further, our technique has the advantage of producing two peaks, each focused in ITP mode, which allows for easy downstream manipulation and automation. We intend to extend this technique to detect other biomolecules (such as proteins) for use in biological and clinical applications.

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