LEVERAGING PEPTIDE NUCLEIC ACID PROBES AND ISOTACHOPHORESIS FOR ON-CHIP HIGH SENSITIVITY DETECTION OF DNA

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ABSTRACT

We present a novel assay which couples isotachophoresis (ITP) focusing with peptide nucleic acid (PNA) probes for sequence-specific detection of nucleic acids on a microfluidic chip. Our method is a simple, single-step assay which does not require amplification. Utilizing the neutral backbone of PNA, our method is based on the design of low electrophoretic mobility PNA probes, which do not focus under ITP unless bound to their target sequence. Thus, background noise associated with free probes can be entirely eliminated, while maintaining a simple single-step assay requiring no amplification steps. We expect the method to be useful for a wide range of applications where rapid profiling of DNA or RNA sequences is required. We demonstrate the feasibility and sensitivity of the method by detecting 100 pM of ssDNA targets, ranging from 17 to 78 nucleotides , within 1 minute.

KEYWORDS: Isotachophoresis, Peptide nucleic acid, DNA detection.

INTRODUCTION

Sensitive, sequence specific DNA detection plays a crucial role in various biosensing applications including medical diagnostics, medical and biological research, food and water safety and forensics. The use of amplification techniques such as polymerase chain reaction (PCR) is a common approach for improvement of sensitivity. However, PCR reactions suffer from an inherent amplification bias, require significant sample preparation, and a well-controlled environment. This results in a growing need for simpler and faster alternatives to amplification. An amplification-free nucleic acids detection method able to reduce the typical assay time is of special importance in faster disease diagnosis and treatment.

ITP is an electrophoretic technique offering a potential alternative for rapid and sensitive detection of nucleic acids. Bercovici *et al.* demonstrated the use of ITP for accelerating nucleic acid hybridization reactions.[1] Using molecular beacons as probes, this technique was utilized by Persat *et al.* and Bercovici *et al.*[2,3], for sequence specific detection of miRNA and bacterial 16S rRNA, respectively. However, the limit of detection (LOD) in these applications was linked to poor signal to noise ratio (SNR) due to high background fluorescence of free (unhybridized) probes in the ITP interface area. Recently, several complementary methods for reducing the background signal have been developed. These include excess probe capturing by gels,[4] a subsequent CZE separation step,[5] or use of a mobility spacer and sieving matrix.[6] While these techniques have been able to markedly improve the signal to noise ratio (SNR), they require multiple steps and are significantly more complex to implement. We here present a significantly faster (~1 min) and simpler one-step assay, using a single straight channel, which relies on PNA probes, and entirely eliminates background fluorescence of unbound probes.

PRINCIPLE OF THE ASSAY

Isotachophoresis (ITP) is an electrophoretic separation and preconcentration technique. In peak mode ITP, analytes of interest are focused at the interface between a high electrophoretic mobility leading electrolyte (LE) and a low mobility trailing electrolyte (TE). The sample is typically mixed with the TE and an initial interface between the LE and TE is established. In order to focus, sample ions must have an intermediate electrophoretic mobility between those of the LE and TE. When an electric field is applied, such ions over-speed the slow trailing ions and accumulate at the migrating LE-TE interface, creating a highly concentrated zone.[7]

In this assay, we couple ITP-based focusing of nucleic acids with PNA-based probes. PNA is an artificial DNA analogue in which the natural negatively charged deoxyribose phosphate backbone has been replaced by a synthetic neutral pseudo peptide backbone. The four natural nucleobases are retained on the backbone at equal spacing to the DNA bases. This results in a weakly charged, chemically and biologically stable molecule, capable of sequence specific binding to DNA and RNA, offering higher stability and hybridization rates compared to standard DNA probes.[8]

Figure 1 presents a schematic illustration of the assay. We inject the sample and an excess amount of PNA probes into the TE reservoir of an anionic ITP setup, allowing probes to rapidly bind to any matching target sequences present. Once an electric field is applied, the negatively charged PNA-DNA hybrids electromigrate and focus at the ITP interface, resulting in a fluorescent signal. Since the free (unhybridized) PNA probes have an electrophoretic mobility lower than that of the TE, excess (unbound) PNA probes remain in the reservoir. Hence, a fluorescent signal is obtained only in the presence of the target sequences "carrying" the otherwise neutral PNA probes to the interface. This allows a highly sensitive, direct detection of target nucleic acids while completely eliminating background noise associated with unbound probes.

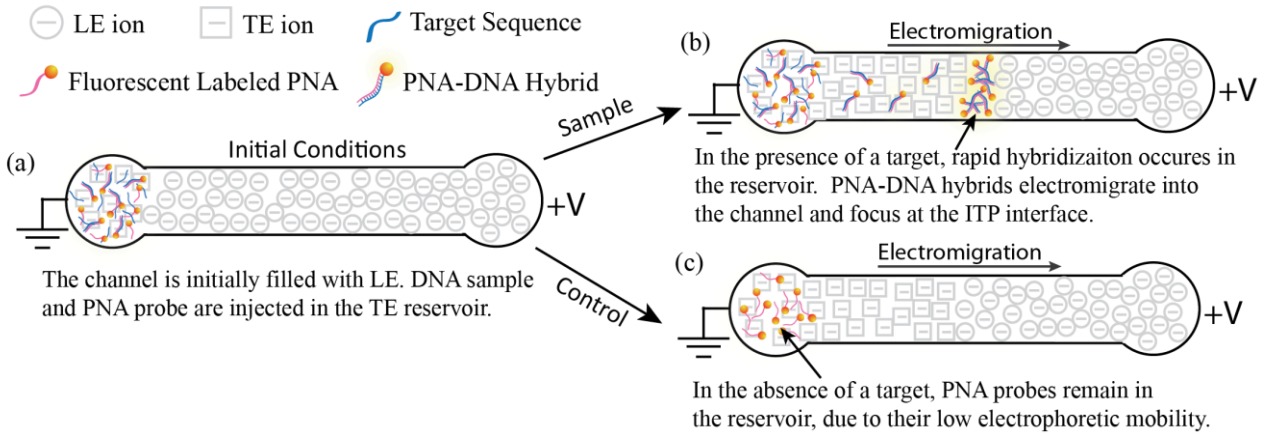

Figure 1: Schematic illustration of the assay. (a) A microfluidic channel connecting two reservoirs is initially filled with LE. The left reservoir is filled with a mixture of TE, DNA sample and PNA probes. (b) When an electric field is applied across the channel, the negatively charged DNA and matching PNA-DNA hybrids electromigrate into the channel and focus at the ITP interface. Unbound PNA probes, which are weakly charged, remain in the reservoir. (c) In a control case, no targets are available to carry the probes into the channel, and all the probes remain in the reservoir.

EXPERIMENTAL

We demonstrated our method using a 17 nt TAMRA labeled PNA probe, TAMRA-OO-ATTCGTTGGAAACGGGA, (Bio-Synthesis, Lewisville, Texas, USA), and three unlabeled DNA targets of different lengths: 17, 35 and 78 nt (Sigma-Aldrich, St. Louis, MO, USA). For all experiments we used an LE composed of 100 mM HCl and 200 mM bistris and TE composed of 10 mM MES and 20 mM bistris. To the LE we also added 1% polyvinylpyrrolidone (PVP) for suppression of electroosmotic flow (EOF).

We mixed 1μM of fluorescently labeled PNA probes with varying concentrations of the sample in the TE reservoir and applied a voltage of 300 V across the channel using a high voltage sourcemeter (2410, Keithley Instruments, Cleveland, OH, USA).

We performed all experiments using an inverted epifluorescent microscope (Eclipse Ti-U, Nikon Instruments, Melville, NY), equipped with a TRITC filter-cube (49004, Chroma, Bellows Falls, Vermont, USA), a 20x objective (CFI Plan Fluor, NA=0.5, Nikon Instruments, Melville, NY), on a commercially available borosilicate microfluidic chip (NS95x, Caliper Life Sciences, Mountain View, CA). The images were captured using a CCD camera (Clara E, Andor Technology, Belfast, NIR, UK) and processed using MATLAB (R2010b, Mathworks, Natick, MA). All images were captured at a distance of 7 mm from the TE reservoir.

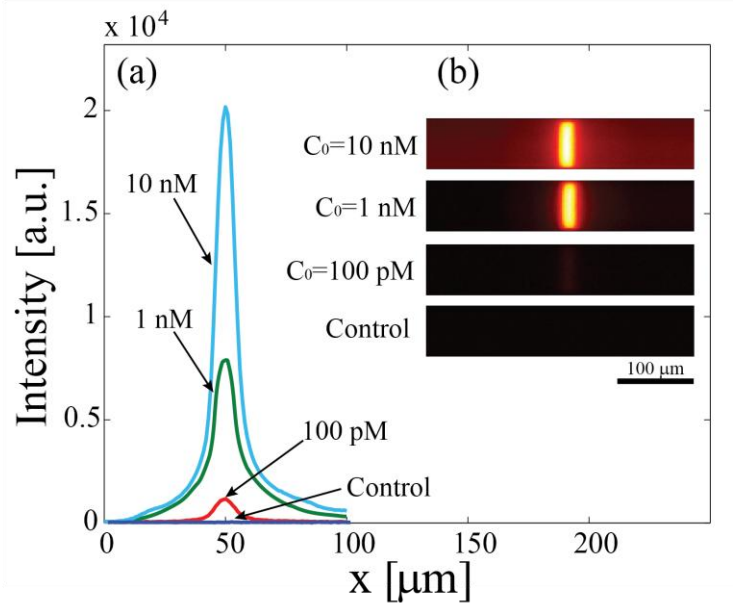

Figure 2: Experimental demonstration of quantitative DNA detection using fluorescently labeled PNA probes. (a) Area averaged intensity profiles of the fluorescent signal registered 7 mm from the TE reservoir. (b) Raw intensity images corresponding to each concentration. We injected into the TE a fixed concentration of 8 μM PNA probes, and varied DNA target concentration between 100 pM and 10nM. In the control case no targets were added to the reservoir. Despite the high, 8 μm, probe concentration the control case shows no signal, indicating that free probes do not focus at the ITP interface. LE is 100 mM HCl and 200 mM bistris. TE is 10 mM MES, and 20 mM bistris. A voltage of 300V was applied across the 35 mm channel.

RESULTS AND DISCUSSION

Figure 2 presents experimental results demonstrating the feasibility of the assay and its ability to quantify the amount of initial target concentration. We note that despite an extremely high initial probe concentration of 8 μM, the control case registers no signal. Figure 3 demonstrates the applicability of the assay for detection of DNA targets of varying lengths. Importantly, We show the assay is able to detect sequences as short as 17 nt. This is in contrast to sieving-matrix based separations, which are limited to detection of much longer sequences. The electrophoretic mobility of the hybrid increases with the length of the target, leading to higher signal for longer targets. For targets longer than several hundred nucleotides, we expect the signal will be independent with target length , as the mobility of free DNA reaches a constant value.[9]

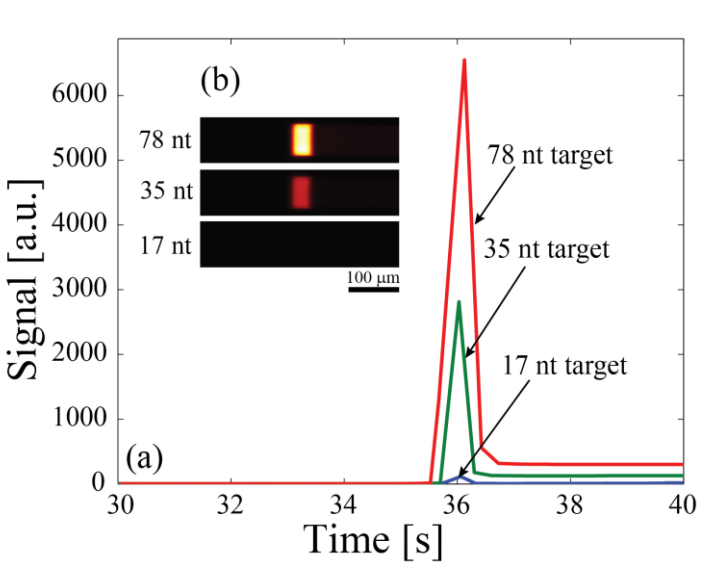

Figure 3: Experimental results demonstrating the applicability of the assay for detection of DNA targets between 17 and 78 nt in length. (a) Raw fluorescence intensity images corresponding to each target. (b) We recorded a series of images at a rate of 3 frames per second, and used the temporal data to illustrate detection using a fixed point detector. As the length of the target increases, so does the electrophoretic mobility of the hybrid [9]. This results in a higher influx into the ITP interface and thus higher signals for longer targets. Importantly, even targets as short as 17 nt result in sufficiently high mobility complexes which focus under ITP. LE is 100 mM HCl and 200 mM bistris. TE is 10 mM MES and 20 mM bistris. Target concentration is 100 nM, and 300V were applied across the 35 mm channel.

CONCLUSION

We demonstrated a rapid and high sensitivity method for amplification-free detection of nucleic acids on a microfluidic chip using PNA-based probes and ITP. Our method offers a simple, single-step assay for detection of nucleic acids as short as 17 nt. We currently report a limit of detection (LOD) of 100 pM, and believe significant improvement can be obtained using a higher sensitivity optical system. To the best of our knowledge, this is the first report of using the electrophoretic mobility properties of PNA for diagnostic purposes.

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