ON-CHIP ISOTACHOPHORESIS AND FUNCTIONALIZED HYDROGEL CAPTURE FOR SENSITIVE MICRO-RNA DETECTION

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ABSTRACT

We integrate on-chip isotachophoresis (ITP) and photopatterned functionalized hydrogels to perform rapid high-sensitivity detection of nucleic acids. We use ITP to enhance hybridization kinetics between target microRNAs and reporter oligos and remove excess reporters with a functionalized polyacrylamide capture gel. We achieve over 4000-fold background signal reduction, ~1 pM sensitivity, 4 orders of magnitude dynamic range, selectivity for mature microRNAs, and running time under 10 min.

KEYWORDS

isotachophoresis, functionalized hydrogels, photopatterning, hybridization, microRNA

INTRODUCTION

Analysis of low-abundance nucleic acid (NA) biomarkers for clinical applications requires the use of bioanalytical techniques able to rapidly detect and accurately quantify small amounts of NAs. We use an on-chip electrokinetic focusing technique called isotachophoresis (ITP) to enhance reaction kinetics in hybridization-based analytical assays. We here present a new ITP-based hybridization assay that integrates a photopatterned functionalized hydrogel for high-sensitivity NA detection. We demonstrate this technique using an important class of emerging biomarkers called microRNAs. The work described here was very recently published as Garcia-Schwarz *et al.*; we here provide a summary of this work and additional information and data published as supplementary information.[1]

microRNAs are short (~22 nt) non-coding molecules identified as cancer biomarkers with important diagnostic and prognostic value.[2-6] Absolute quantification of microRNA species is extremely challenging as microRNAs are low in abundance,[7] their concentrations can vary by three orders of magnitude,[8] and active (mature) microRNAs are not easily distinguished from longer (~70 nt) inactive precursors that contain the entire active sequence.[5,6] Current approaches for microRNA detection rely on qPCR, which is sensitive to contamination, offers poor quantification limited to roughly 2-fold changes in concentration, requires validated internal controls for absolute quantification, and is not easily automated for use in clinical settings.[5,6,9-11] Other microRNA profiling such as northern blotting and microarrays require either large amounts of sample (~10 µg total RNA) or very long incubation times (~1 day).[5,6,12] While we developed a technique using ITP and molecular beacons for nucleic acid profiling,[13,14] the dynamic range and sensitivity of molecular beacons-based assays is limited by significant background signal associated with fluorescence quenching inefficiencies.[5]

The assay presented here addresses these limitations by integrating ITP-based rapid hybridization and a functionalized hydrogel stationary phase (Fig. 1A). This combination allows for separation of hybridization and signal transduction phases. We first focus together microRNAs and fluorescently-labeled reporters using ITP. To perform ITP we choose leading (LE) and trailing electrolytes (TE) having respectively higher and lower mobility than the NAs being focused. ITP readily achieves 10^4-fold preconcentration of NAs, and therefore dramatically enhances the second-order hybridization reaction between complementary sequences.[15]

After ITP-aided hybridization the focused sample enters a photopatterned hydrogel region designed to remove excess reporters. This capture gel is functionalized with immobile DNA molecules with sequence complementary to fluorescent reporters. Reporters already hybridized to target microRNAs continue to migrate due to slow off-rates, while unreacted reporters remain exposed and bind to the functionalized hydrogel matrix (Fig. 1A,B). We have used quantitative fluorescence measurements to show that this capture strategy results in over 4000-fold reduction in background signal, thus conferring high sensitivity and large-dynamic range detection to our technique.
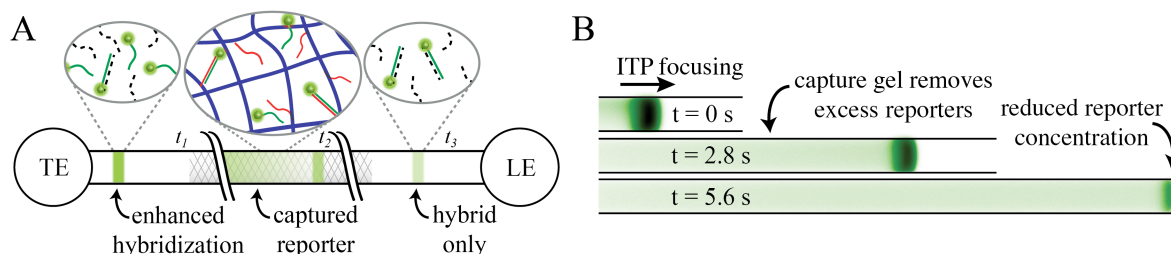

Figure 1. *(A) Schematic of microRNA detection assay. ITP accelerates target-reporter hybridization upstream of functionalized capture gel. The ITP zone then migrates into a DNA-functionalized gel region with immobilized probes complementary to reporters. The gel removes un-hybridized reporters while hybridized reporters remain focused and can be detected downstream. (B) Experimental images demonstrating ITP gel capture. ITP-focused reporters migrate through a hydrogel functionalized with probes complementary to reporters. After the ITP zone sweeps by, we observe a low-level fluorescent signal left behind in the gel corresponding to immobilized reporters.*

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EXPERIMENTS

We performed experiments with a custom point-confocal microscope setup (Fig. 2A). We prepared LE pre-polymer solution containing 100 mM HCl, 200 mM tris, 2.5 mM $MgCl_2$, 4%T acrylamide/bisacrylamide monomer (19:1), and 0.13% (w/v) VA-086 photoinitiator. We patterned the microfluidic channel by filling with pre-polymer solution with and without acrydite-modified DNA capture oligos followed by polymerization by flooding the chip UV light. Following polymerization, we replace contents of the North well with TE containing 10 mM HEPES, 20 mM tris, 90% formamide, and 2 nM reporter oligo combined with varying concentrations of target microRNAs. We then performed ITP by applying constant current (1 µA) between the LE and TE reservoirs and detected the ITP peak following the hybridization and capture regions (Fig. 2B).
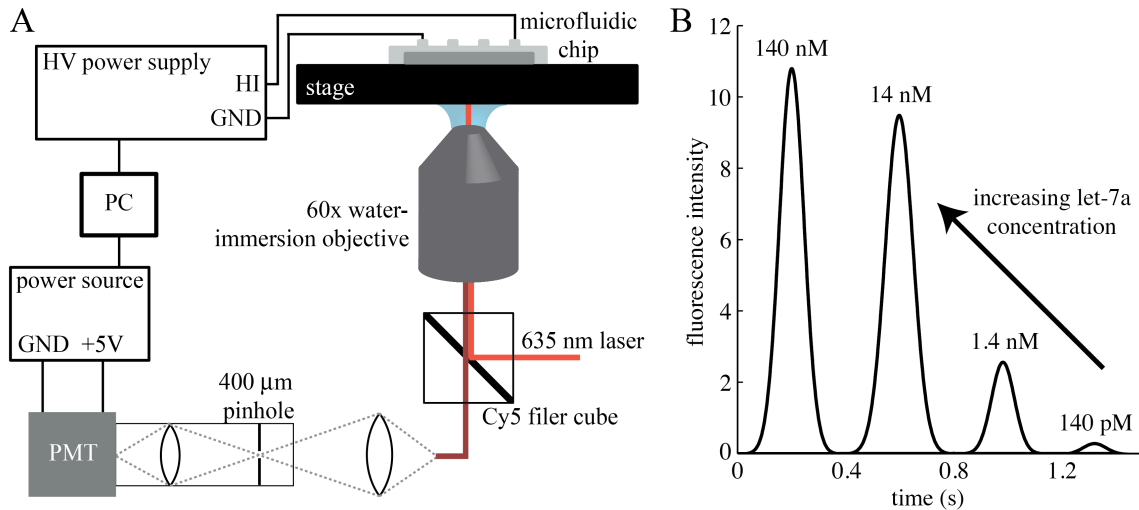

Figure 2. *(A) Schematic depiction of the experimental setup used to visualize the fluorescent signal from end-labeled reporters. The custom point-confocal microscope setup is constructed around an inverted epifluorescent microscope with 60X water-immersion objective. We use a 635 nm diode laser for illumination and a photo-multiplier tube (PMT) for detection. We use a 400 µm pinhole to filter out-of-plane light. (B) Gaussian best fits to the measured fluorescence signal with varying amounts of let-7a target. Below ~140 nM signal scales with target concentration.*

RESULTS & DISCUSSION

We performed titration experiments in order to quantify the dynamic range and limit of detection (LOD) of our assay. These experiments demonstrated 4 orders of magnitude dynamic range with absolutely no change in assay conditions (Fig. 3A). Under these conditions, and without further optimization (e.g., temperature, denaturant concentration), Fig. 3B demonstrates an assay limit of detection of 2.8 pM ($P = 0.0005$). While in this study we do not address high-stringency microRNA detection, we measured the resulting signal from a near-complete mismatch microRNA (miR-15a). We found that 140 pM let-7a yields a fluorescence intensity of ~80 times higher than the same amount of miR-15a. In addition, the mismatch signal was not statistically distinguishable from the negative control signal.

We model the hybridization process using a three-species reaction model. This model assumes that reactions occur in a homogeneous bulk, and we include a fitting parameter, F, which represents the rate of accumulation of reporters and microRNAs in the ITP zone. Under these assumptions, the governing equations are:

$$\frac{dc_H}{dt} = k_{on}c_R c_T - k_{off}c_H \;, \quad \frac{dc_T}{dt} = -k_{on}c_R c_T + k_{off}c_H + Fc_{T,0} \;, \quad \frac{dc_R}{dt} = -k_{on}c_R c_T + k_{off}c_H + Fc_{R,0}$$

Here c_i is the species concentration where the index i stands for hybrid (H), target (T), and reporter (R) species. The parameters k_{on} and k_{off} are the on- and off-rates, respectively, describing the NA hybridization kinetics (here we assume $k_{on} = 10^6$ $M^{-1}s^{-1}$ and $k_{off} = 10^{-15}$ s^{-1}). As demonstrated in Fig. 3A, our approximate model shows excellent agreement with titration experiments.

We address selectivity for mature microRNA *versus* precursor microRNA by incorporating a second functionalized hydrogel used to remove precursors from the focused zone. This region targets the loop sequence of precursor microRNAs (not present in the mature sequence). We demonstrate in Fig. 3A that the presence of precursor does not affect accurate absolute quantification of mature microRNAs.

CONCLUSIONS

We have introduced an on-chip electrokinetic microRNA detection assay with picomolar sensitivity, selectivity for mature microRNAs over inactive precursors, 4 orders of magnitude dynamic range, and the capability to analyze < 300 fg of target microRNA in under 10 min. Our assay presents a unique and highly-quantitative framework generally applicable to detection of biomolecules and reactants.
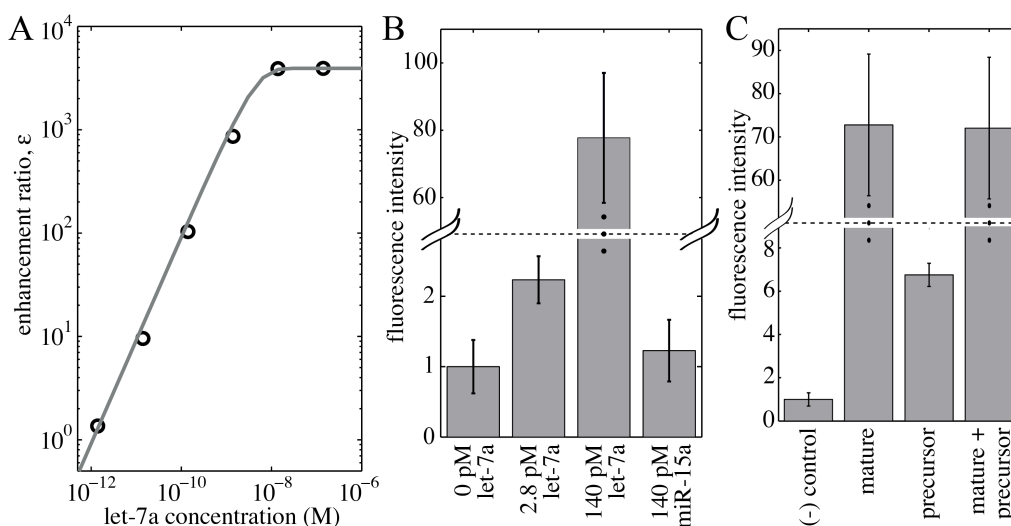
Figure 3. *(A) Titration curve spanning five orders of magnitude, from 1.4 pM to 140 nM of let-7a microRNA. The assay exhibits a quantitative dynamic range of approximately 4 orders of magnitude (with no changes in assay conditions). Shown with data (solid line) is a plot of results from our model with a single, global scalar fitting parameter. (B) Limit of detection study showing mean signal intensity for negative control, 2.8 pM let-7a, 140 pM let-7a, and 140 pM of mismatch species (miR-15a). We successfully distinguish between negative control and 2.8 pM let-7a (P = 0.0005). (C) Demonstration of selectivity for mature microRNAs. The enhanced hybridization region contains an immobilized oligo targeting the loop sequence of the let-7a precursor. The plot shows mean integrated signal intensity for negative control and 140 pM let-7a, pre-let-7a, and each of let-7a and pre-let7a. The pure precursor signal is slightly higher than the control signal, and we attribute this to the difficulty in synthesizing long RNA oligos of high purity. We estimate the purity of our pre-let-7a sample is only approximately 31%.*

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