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Enhancing the speed of morpholino-DNA biosensor by electrokinetic concentration of DNA in a microfluidic chip

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ABSTRACT

Electrokinetic methods that conveniently concentrate charged analytes by orders of magnitude are highly attractive for nucleic acid assays where they can bypass the complexity and costs of enzyme-based amplification. The present study demonstrates an electrokinetic concentration device incorporating charge-neutral morpholino (MO) probes: as DNA analyte is concentrated in a microfluidic channel using ion concentration polarization (ICP) it is simultaneously hybridized to spots of complementary MO probes immobilized on the channel floor. This approach is uniquely favored by the match between the optimum buffer ionic strength of approximately 10 mM for both MO–DNA surface hybridization and electrokinetic concentration. The simple and easily scalable poly(dimethylsiloxane) (PDMS) microfluidic device was fabricated using soft lithography and contact printing of a conductive polymer, poly(3,4-ethylenedioxythiophene)-polystyrene sulfonate (PEDOT:PSS) as a cation-selective membrane material. Using the microfluidic concentrator, we could increase the concentration of DNA by three orders of magnitude in less than 5 min at an electric field of 75 V cm^{-1}. The 1000-fold increase in concentration of DNA led to an increase in the speed of MO–DNA hybridization by two orders of magnitude and enabled a detection sensitivity of ~1 nM within 15 min of concentration. Using the proposed microfluidic concentrator, we also demonstrated a rapid hybridization with a binary DNA mixture, containing a fully complementary and a non-complementary sequence to mimic molecular backgrounds present in real DNA samples.

1. Introduction

Perhaps the most important determinant of successful detection of an analyte is its concentration. In the case of nucleic acids, an effective, enzyme-free approach is to increase concentration through electrokinetic means (Kim et al., 2010). Although electrokinetic concentration works optimally at lower ionic strengths, such conditions suppress hybridization between the nucleic acid analyte and like charged DNA probes immobilized, for example, on a sensor surface (Fuchs et al., 2010; Gong and Levicky, 2008; Okahata et al., 1998). To overcome this shortcoming, the present study combines electrokinetic concentration of nucleic acid analyte with detection through hybridization to uncharged morpholino (MO) probes, which can readily hybridize with nucleic acids at favorably low ionic strengths. We envision this approach to ultimately provide a route toward concentration of DNA or RNA analyte without reliance on enzymatic amplification typically required by conventional assays.

MOs are synthetic nucleic acid analogs with a non-charged backbone of morpholine rings (Gong et al., 2010; Tercero et al., 2010, 2009). When substituted for DNA probes in surface hybridization applications, analyte detection becomes possible at low and moderate ionic strengths down to around 10 mM (Qiao et al., 2013), instead of the 0.1–1 M typically required with DNA probes (Gong and Levicky, 2008; Hassibi et al., 2009). Despite these advantages, MO-based assays nevertheless share the same limitations of conventional methods when it comes to analyte concentration, namely poor detection sensitivity and long hybridization durations at low concentrations (Janson and During, 2006; Wang and Smirnov, 2009). Poor detection sensitivity resulting from low target concentration is usually remedied by polymerase chain reaction (PCR) or other amplification prior to hybridization. However, amplification introduces sample processing that complicates assay workflow as well as can bias the sequence composition of the sample (Haddad et al., 2007; Ma et al., 2006; Pinard et al., 2006). Therefore, strong motivation exists for

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developing alternate approaches for enhancing analyte concentration.

To enhance the MO–DNA hybridization assay speed and sensitivity, we propose integration of an ion concentration polarization (ICP)-based microfluidic concentrator with a MO microarray. The ICP concentrator collects target biomolecules from a ~μL sample volume and concentrates them into a ~nL plug in the vicinity of the capture probes (Kim et al., 2010; Ko et al., 2011; Wang and Han, 2008). ICP concentration not only reduces the analyte diffusion length but also increases the hybridization rate as a result of the locally enhanced concentration of biomolecules. Hence ICP-enhanced surface hybridization assays are expected to provide much faster detection than conventional assays without ICP enhancement.

Currently, several strategies are available to provide sample preconcentration in liquids, including isotachophoresis (Bercovici et al., 2012; Eid et al., 2013; Garcia-Schwarz and Santiago, 2012), field-amplified sample stacking (Bur and Chien, 1991), isoelectric focusing (O'Neill et al., 2006), electrokinetic trapping (Astorga-Wells and Swerdlow, 2003; Wang et al., 2005), micellar electrokinetic sweeping (Quirino and Terabe, 1998), chromatographic trapping (Yu et al., 2001), temperature-gradient focusing (Ross and Locascio, 2002), and membrane preconcentration (Rohr et al., 2001; Yu et al., 2001). Many of these techniques are originally developed for capillary electrophoresis, and require special buffer arrangements and/or reagents. Among these, we consider a coupling of the ICP-based electrokinetic concentration and the MO diagnostic platform as uniquely synergistic since both deliver optimal performance at moderate ionic strength (~10 mM) (Ko et al., 2012; Qiao et al., 2013). Additionally, ICP concentrators are straightforward in design and fabrication consisting of a single PDMS microchannel with an integrated Nafion ion-selective membrane (B. Kim et al., 2013a, M. Kim et al., 2013b; Ko et al., 2012). Based on this microfluidic concentrator concept, we have developed an ICP concentrator featuring, for the first time, a conductive polymer, poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) as a cation-selective membrane. To this end, PEDOT:PSS was printed on a glass substrate using a fluid microplotter (Cheun et al., 2006), instead of using the micro-flow patterning technique which is not reproducible (B. Kim et al., 2013a, M. Kim et al., 2013b), leads to variable membrane thicknesses (Lee et al., 2008), and allows for nanogaps along the membrane edge due to an incomplete PDMS bonding that may dominate the nanofluidic ICP phenomena (B. Kim et al., 2013a, M. Kim et al., 2013b). Microplotter dispensing of PEDOT:PSS is a flexible technique that allows for the deposition of discrete (spots), continuous (lines or arcs) or even three dimensional (multiple layers) features (Larson et al., 2004). PEDOT:PSS can also be processed using cleanroom fabrication techniques (Charlot et al., 2012). In particular, it can be deposited on a wafer substrate by spin coating and then patterned by UV lithography in conjunction with reactive ion etching. Such a fabrication capability of PEDOT:PSS polymer is highly advantageous since it allows building of a high-density array of concentrators on a large wafer scale.

In the present study, we built a single concentrator device by printing PEDOT:PSS next to a spotted array of capture MO oligomers and then reversibly sealing the slide using a PDMS microchannel. After characterizing the DNA concentration enhancement at different electric field strengths as a function of the concentration time, we compared the rates of MO/DNA surface hybridization without and with concentration. We also interrogated hybridization specificity using DNA target sequences having different numbers of nucleotide mismatches, from a fully non-complementary sequence down to a single-nucleotide mismatch. Lastly, we quantified the increase of hybridization speed in the presence of a fully non-complementary sequence added to a solution of fully complementary DNA under electrokinetic concentration. This study demonstrates potential applications of ICP-based microfluidic concentrators for improving the performance of solid phase DNA and RNA analysis.

2. Materials and methods

2.1. Materials

The unlabeled MO probe sequence PM1 and fluorescein isothiocyanate (FITC, λ_{exc}=494 nm and λ_{em}=518 nm) labeled PM2 in Table 1 were purchased from Gene Tools LLC. The FITC-labeled morpholino was used to verify the printing quality of MO probes on glass slides. The 20mer probes were modified with an amino group at the 5' end to allow for surface attachment to aldehyde groups on Superaldehyde 2 microarray slides from Arrayit. Cyanine 5 (Cy5, λ_{exc}=649 nm and λ_{em}=670 nm) and cyanine 3 (Cy3, λ_{exc}=550 nm and λ_{em}=570 nm) labeled DNA targets were purchased from Integrated DNA Technologies. For MO–DNA hybridization experiments, we used 25 nucleotides long oligonucleotides having different numbers (N) of nucleotide mismatches, as listed in Table 1: fully complementary target (N=0) TD1; single-nucleotide mismatch (N=1) strand TD2; 28% mismatch target (N=7) TD3; 48% mismatch target (N=12) TD4; and non-complementary target (N=25) TD5. These DNA targets were labeled at the 3'-end with Cy5 to enable fluorescence detection. Cy3-labeled non-complementary DNA target TD6 was combined with fully complementary TD1 in a 1:1 ratio to investigate ICP-enhanced surface hybridization in the presence of a non-complementary background. All hybridization solutions were prepared in 0.1 × PBS at pH 7.1 with no other additives. For storage, as received probes and targets listed in Table 1 were diluted with deionized (DI) water of 18 MΩ cm resistivity to a concentration of 200 μmol L^{-1} before storing at −20 °C. Conductive polymer PEDOT:PSS 2.2–2.6% in H_2O (high conductivity grade) was obtained from Sigma-Aldrich. Phosphate buffer saline (PBS) solution at 0.1 × pH 7.1 was prepared by diluting 10 × PBS purchased from Gibco. Sodium phosphate buffer (PB) of desired pH was prepared by combining monobasic sodium phosphate and dibasic sodium

Table 1
Morpholino probes and DNA target sequences.

Sequence	Abbreviation	Comments
5′ NH_2-GTA GCT AAT GAT GTG GCA TCG GTT 3′	PM1	MO probe
5′ NH_2-GTA GCT AAT GAT GTG GCA TCG GTT-FITC 3′	PM2	MO immobilization control
5′ CAA CCG ATG CCA CAT CAT TAG CTA C-Cy5 3′	TD1	Complementary DNA target
5′ CAA CCG ATG CCA **T**AT CAT TAG CTA C-Cy5 3′	TD2	2% mismatch (N=1) DNA target
5′ CAA C**AT** ATG C**TC** CA**G** CAT T**CT** CTA C-Cy5 3′	TD3	28% mismatch (N=7) DNA target
5′ **TC**A C**AT** AT**A** C**TC** CA**G** CAT T**CT** C**CC** C-Cy5 3′	TD4	48% mismatch (N=12) DNA target
5′ TGG AAT GCA TTG AGC AGC CGT AGC T-Cy5 3′	TD5	Non-complementary DNA target (N=25)
5′ TGG AAT GCA TTG AGC AGC CGT AGC T-Cy3 3′	TD6	Non-complementary DNA target Cy3 tagged