CONCENTRATION ENHANCED MOBILITY SHIFT ASSAY WITH APPLICATIONS TO APTAMER-BASED BIOMARKER DETECTION AND KINASE PROFILING

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ABSTRACT

We present the use of electrokinetic concentration to realize a continuous signal amplification scheme that increases the sensitivity of various homogeneous mobility shift assays. By simultaneously concentrating and separating reacted and unreacted species (with different mobilities) in this device, we can perform sensitive, quantitative and ratiometric measurement of target biomarkers. Using this platform, we improved the sensitivity of aptamer affinity probe capillary electrophoresis to achieve pM detection limit of IgE and HIV-RT in simple buffer and serum sample. As another application, we can perform multiplexed detect of kinase activity from lysate concentrations corresponding to lysing a single cell.

KEYWORDS: Preconcentration, Mobility Shift Assay, Aptamer, Kinase

INTRODUCTION

Due to the rapid assay and low sample requirement, miniaturized capillary electrophoresis (CE) devices are gaining popularity as tools to detect low abundance proteins that are important biomarkers. However, their usefulness and sensitivity are often limited by band dispersion and complex dissociation during the assay, as well as the lack of a signal amplification scheme. We have previously demonstrated microfabricated electrokinetic preconcentration devices that can continuously accumulate a charged biomolecule species at a specified location[1]. In this work, we show that these devices can also efficiently separate biomolecules for mobility-shift assays. Separation is achieved as biomolecules with different electrophoretic mobilities accumulate at different locations within the nonlinear electric field gradient set up by concentration polarization[2]. Using this scheme, the long-standing problems of band-broadening and complex dissociation in conventional CE are counteracted by the self-focusing effect. Electrokinetic concentration also provides a signal enhancement mechanism that improves sensitivity with time.

THEORY

Figure 1 shows the key operation of the poly(dimethylsiloxane) (PDMS) microfluidic electrokinetic concentration chip, which consists of five separate sample microchannels connected to two flanking side microchannels via a planar cation-selective Nafion thin film (Figure 1a). Under the voltage configuration shown in Figure 1b, ion depletion zones are created in the sample channels at the vicinity of the ion selective membrane due to concentration polarization phenomena. The conductivity gradient at the boundary of the ion depletion zone gives rise to a stable electric field gradient that can effectively focus negatively charged biomolecules at separate locations where electrophoretic velocity balances bulk flow velocity as illustrated in Figure 1c. Biomolecules with high electrophoretic mobilities (e.g. free aptamers, phosphorylated peptides) are concentrated at the low electric field region. On the other hand, lower mobility species (e.g. aptamer-protein complex, unphosphorylated peptides) concentrates nearer to the cation selective membrane where the electric field is higher. By simultaneously concentrating and separating reacted and unreacted species (with different mobilities) in this device, we can perform sensitive, quantitative and ratiometric measurement of target biomarkers (Figure 1d).

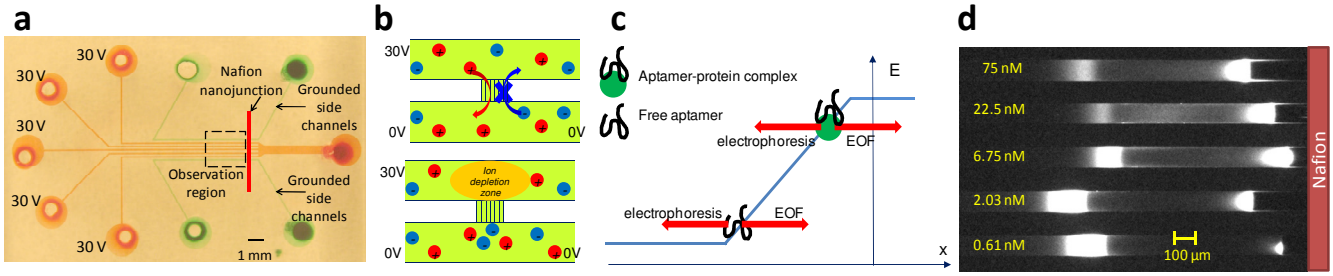

Figure 1: a) Optical image of multiplexd PDMS device with 200 µm wide surface patterned Nafion thin film on glass substrate. Sample channels and side channels are filled with red and green dyes respectively. Experimental images are taken at the observation region, b) Ion selective membrane creates a local ion depletion zone with high electric field upon applying a voltage, c) Free aptamers and aptamer-protein complex concentrate at different locations on the electric field profile due to their different electrophoretic mobility, d) Multiplexed electrokinetic concentration enhanced aptamer mobility shift assay for detecting IgE in buffer solution.

EXPERIMENTS AND RESULTS

Aptamer Based Biomarker Detection

We first demonstrate this scheme to improve the sensitivity of aptamer affinity probe CE (APCE)[3]. Aptamers are low cost and robust oligonucleotide capture agents that could potentially replace antibodies, but their sensitivities are often hampered by their relatively high dissociation constant. Since fluorescent DNA aptamers undergo significant mobility shifts upon binding to target proteins, our method can be used to quantify target biomarkers in a sample by measuring the relative intensities of the separated free aptamer and aptamer-protein complex bands. With this scheme, we showed enhanced detection sensitivity for IgE and HIV-1 RT using APCE. The limits of detection (LOD) for IgE are 4.4 pM in simple buffer and 39 pM in 10% serum, while LOD for HIV-1 RT is 9 pM in buffer (Figure 2,3). As the assay is gravitational flow driven, uses low voltages (30V), and does not require multiple processing steps, it is well-suited towards low-cost point-of-care analysis.

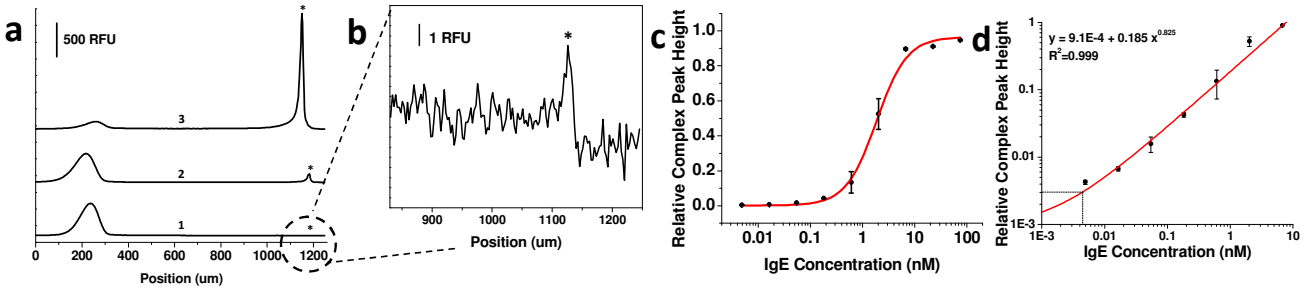

Figure 2: a) Electropherogram for optimized electrokinetic concentration and separation of IgE aptamer (5nM) and different concentrations of IgE: (1) 4.92pM, (2) 0.6 nM, and (3) 6.75 nM IgE. The complex band is labeled with an asterisk, b) Inset demonstrates detection of 5 pM IgE in buffer, c) Dose-response curve of anti-IgE aptamer with IgE spiked in buffer, error bars represent standard error from duplicate experiments, d) Linear relationship in the log-log plot is obtained at low concentrations of IgE. The measured LOD is 4.4 pM IgE.

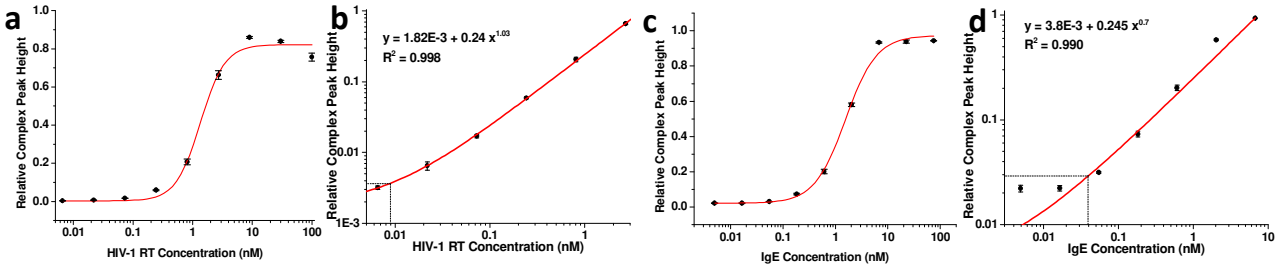

Figure 3: a) Dose response curve of anti-HIV-1RT aptamer with HIV-RT spiked in buffer, error bars represent standard error from duplicate experiments, b) Linear relationship in the log-log plot is obtained at low concentrations of HIV-1RT, measured LOD is 9 pM HIV-1 RT c) Dose response curve of anti-IgE aptamer with IgE spiked in 10% serum, error bars represent standard error from duplicate experiments, b) Log-log plot at low concentrations of IgE in serum showing effects of nonspecific binding on sensitivity. The measured LOD is 39 pM IgE.

Single Cell Kinase Profiling

Next, we show that this platform can be used to measure various cellular kinase activities *in vitro*. Kinases are important proteins that regulate many cell signaling pathways, whose activities may be altered in disease states such as cancers. As a fluorescent peptide substrate undergoes mobility shift upon phosphorylation by its target kinase, our technique can measure kinase activity in a sample by measuring the relative intensities of the separated unphosphorylated and phosphorylated peptide. We are able to reliably measure activities from low concentrations (<2ng/mL) of recombinant kinases (Akt, MK2 and PKA) due to the signal-enhancing capability of this technique (Figure 4a-c). Finally, we show that our method is able to detect changes in Akt and PKA activity in HepG2 cell lysates in response to insulin and forskolin stimulation respectively (Figure 4e,f). The assay is sensitive enough to detect kinase activity from bulk cell lysate concentrations equivalent to lysing a single cell. Moreover, multiplexed detection of several kinases in a single run would be possible by designing peptide susbtrates with widely different mobilities. Spatial separation between different fluorescent peptide substrates can be independently tuned by formation of plateau regions in the electric field profile due to isotachophoretic stacking of species with intermediate mobilities (Figure 5a), through a process known as depletion zone isotachophoresis[2]. Figure 5b shows multiplexed detection of the kinases MK2 (substrate: MK2tide) and PKA (substrate: Kemptide) from the same sample by concentration enhanced mobility-shift assay. Nonfluorescent synthetic peptides designed to have specific mobilities are used as discrete spacers to increase the separation resolution between the fluorescent bands.

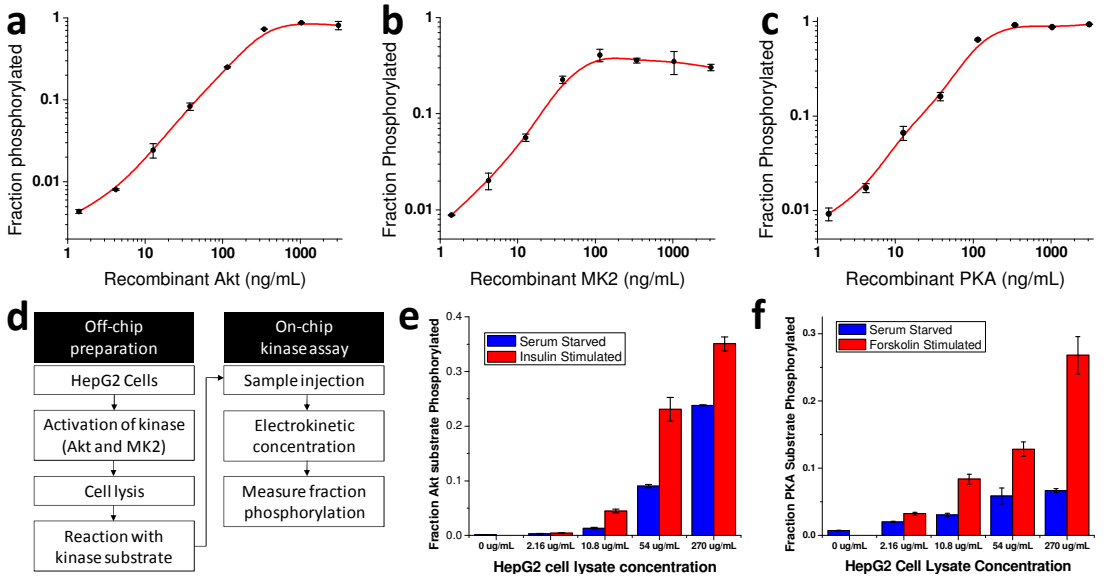

Figure 4: a-c) Dose response curve of recombinant Akt, MK2 and PKA kinase activities, d) Experimental schemes with off-chip preparation and on-chip concentration-enhanced mobility shift kinase assay, e) Akt activity profile in insulin treated and serum starved HepG2 cell lysate, f) PKA activity profile in forskolin treated and serum starved HepG2 cell lysate,

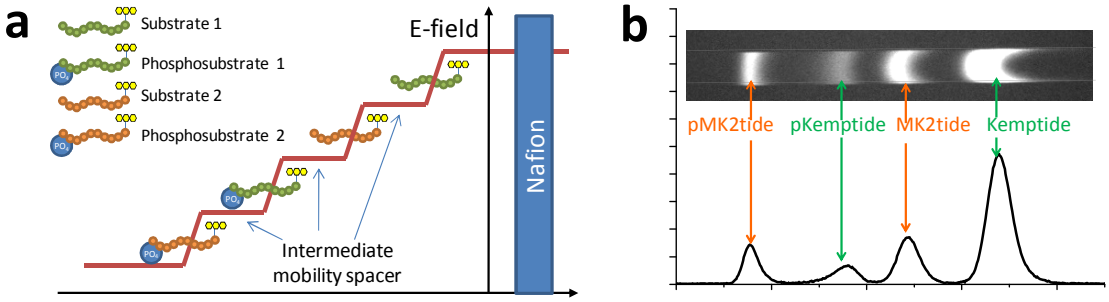

Figure 5: a) Simultaneous concentration and separation of multiple peptide substrates. Separation resolution is enhanced by formation of plateau regions in the electric field profile due to isotachophoretic stacking of intermediate mobility peptide spacers, b) Multiplexed detection of MK2 (substrate: MK2tide) and PKA (substrate: Kemptide) in a single run by concentration enhanced mobility-shift assay.

CONCLUSION

In conclusion, we demonstrated the use of electrokinetic concentration to realize a continuous signal amplification scheme that increases the sensitivity of various homogeneous mobility shift assays. Minaturized capillary electrophoresis devices are one of the first microfluidic systems that gained popular acceptance, and remains a mainstay in lab-on-chip platform. While many detection principles scales down unfavorably due to reduction of optical path lengths, the method here could boost the sensitivity of lab-on-chip system to be comparable, if not better, than conventional macroscale techniques.

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