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Continuous Signal Enhancement for Sensitive Aptamer Affinity Probe Electrophoresis Assay Using Electrokinetic Concentration

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Abstract

We describe an electrokinetic concentration-enhanced aptamer affinity probe electrophoresis assay to achieve highly sensitive and quantitative detection of protein targets in a microfluidic device. The key weaknesses of aptamer as a binding agent (weak binding strength/fast target dissociation) were counteracted by continuous injection of fresh sample while band-broadening phenomena were minimized due to self-focusing effects. With 30 minutes of continuous signal enhancement, we can detect 4.4 pM of human immunoglobulin E (IgE) and 9 pM of human immunodeficiency virus 1 reverse transcriptase (HIV-1 RT), which is among the lowest limit of detection (LOD) reported. IgE was detected in serum sample with LOD of 39 pM due to nonspecific interactions between aptamers and serum proteins. The method presented in this paper also has broad applicability to improve sensitivities of various other mobility shift assays.

For decades, antibody based immunoassays have been the method of choice for disease diagnosis that require highly specific and sensitive recognition elements. However, aptamers are recently emerging as an increasingly popular alternative to antibodies as affinity probes. Aptamers are single stranded oligonucleotides that have undergone multiple rounds of *in-vitro* selection to bind specifically to various molecular targets^{1, 2}, rivaling antibodies in terms of sensitivity and selectivity. Furthermore, they excel antibodies in other aspects such as low cost production by chemical synthesis, ability to survive harsh conditions, and labeling simplicity that ensures batch-to-batch uniformity³. Recently, high affinity aptamers have been generated for >800 human proteins that are potential biomarkers⁴. These are promising signs that aptamer biosensors will find widespread applications.

Several groups have demonstrated that capillary electrophoresis (CE) using aptamers as affinity probes can be used to detect specific target proteins such as IgE^{5–7}, thrombin^{5, 8}, ricin⁹, and HIV-1 reverse transcriptase (HIV-1 RT)^{6, 10, 11}. Unlike heterogeneous immunoassay methods such as Enzyme-Linked Immunosorbent Assay (ELISA) that require several hours and multiple washing steps, the homogeneous CE assay is performed in one step with only a short incubation time (≤ 30 minutes). Due to the ease of use and short assay time, CE methods are highly attractive for developing point-of-care biosensor platforms. However, CE assays are generally less sensitive than ELISA due to the ability of enzymes in ELISA to continuously convert a substrate to visible product over time. Furthermore, band dispersion and complex dissociation when using lower affinity (high K_d) aptamers as affinity probe in CE limits their applicability to detect low abundance biomolecules that could be important biomarkers.

Herein, we report an electrokinetic concentration-enhanced aptamer affinity probe electrophoresis assay to achieve highly sensitive and quantitative detection of low abundance biomarkers in a microfluidic device. This scheme features three simultaneous processes: 1) continuous injection, 2) focusing, and 3) separation of the free aptamers and aptamer-protein complexes. One of the significant disadvantages of aptamer affinity probe CE is that complex may dissociate during long migration times, leading to weak or even absence of signal¹². Decreasing the time spent on column, either by applying very high electric fields or utilization of hydrodynamic flow was often necessary to achieve reliable detection of the aptamer-protein complex¹². In this new scheme, we counteract dissociation of the aptamer-protein complex by continuous injection and accumulation of fresh sample from the inlet reservoir. Band broadening phenomena commonly encountered in CE are also minimized due to the self-focusing effect. When a continuous flux of sample from the equilibrium mixture in the reservoir is subjected to simultaneous focusing and separation the signal-to-noise ratio increases with time. A good signal enhancement scheme is the key to highly sensitive assays such as ELISA. The major contribution of this paper is the use of electrokinetic concentration to realize a continuous signal enhancement scheme applicable to homogeneous mobility-shift assay.

Various schemes that combine sample concentration and CE analysis have been reported previously, including sample sweeping⁸, preconcentration using a size-exclusion membrane⁷, transient isotachopheresis (t-ITP)¹³, and temperature gradient focusing (TGF)¹⁴. In the first two cases, preconcentration and separation are carried out sequentially, thus band broadening during separation reduces the sensitivity enhancement. The t-ITP method results in very high sensitivity improvement, but imposes certain restrictions on the sample and running buffer and concentration factor is limited by injected plug volume. Use of photomultiplier tubes (PMT) in conjunction with Laser Induced Fluorescence (LIF) further improves the sensitivity of the first three assays. In the TGF example, high concentration factors are obtained as sample is focused continuously throughout the 7.5min experiment. However, special temperature sensitive buffer is needed and higher limit of detection (LOD) is expected since detection is based on monitoring a small decrease in the large free aptamer peak. In all these examples, high voltages of $\geq 1\text{ kV}$ are required.

Our group has previously reported on nanofluidic electrokinetic concentration devices that can continuously collect negatively charged molecules in a given sample into a much smaller volume, thereby increasing local concentration significantly¹⁵. This electrokinetic concentration effect has been used to enhance protein binding kinetics to surface-bound antibodies¹⁶, increase the sensitivity of homogeneous enzyme assays^{17, 18}, as well as improve the sensitivity of ELISA by more than an order of magnitude¹⁹. However, simultaneous concentration and separation of biomolecules based on mobilities have not been demonstrated due to the predicted low separation resolutions at high electric field gradients in these devices. The assay described in this paper exploits the fact that low molecular weight aptamers with high charge density undergo a significant mobility shift upon binding to a larger target protein with small net charge^{6, 7}. Therefore, bound and unbound aptamers could be baseline-separated even under high electric field gradient conditions. Moreover, binding of the aptamers to the proteins makes the complex negatively charged⁶, which facilitates the electrokinetic concentration of neutral or basic proteins in buffers at physiological pH.

In this paper, we demonstrate electrokinetic concentration-enhanced aptamer affinity probe electrophoresis assays for two different disease biomarkers, namely human Immunoglobulin E (IgE) and Human Immunodeficiency Virus 1 Reverse Transcriptase (HIV-1 RT). IgE is the least abundant class of antibodies produced in human, and plays an important role in generating allergic response as well as defending against parasites^{20, 21}. Some recent studies

have suggested the use of serum IgE as a predictive biomarker for diseases such as asthma and peanut allergy^{21, 22}. On the other hand, HIV-1 RT is a key diagnostic and therapeutic target of HIV-1^{23, 24}. Many aptamer based sensor have been used to detect IgE with different LOD, these include methods based on fluorescence enhancement (57 pM)^{25, 26}, carbon nanotube field effect transistors (250 pM)²⁷, surface plasmon resonance (18.5 pM)²⁸, CE (46 pM)⁵⁻⁷ and aptamer microarray using labeled IgE (10 pM)^{29, 30}. Meanwhile, for detection of HIV-1 RT, the methods reported are predominantly based on CE (100 pM)^{6, 10, 11}, temperature gradient focusing (84 pM), transient isotachopheresis (<1pM) and CE followed by PCR (30 fM). It is worth noting that the LOD reported is dependent on detection instruments and the affinity of the particular aptamers, and that coupling separation with amplification step often leads to dramatic increase in sensitivity.

Using our platform, we obtained LOD of 4.4 pM and 9 pM for human IgE and HIV-RT respectively in simple buffer after 30 minutes preconcentration, compared to LOD of 46 pM⁵ and 100 pM⁶ obtained with conventional CE methods. These are the lowest assay LOD reported in the literature for aptamer affinity probe capillary electrophoresis in spite of the inferior detector used for our assays (arc lamp and CCD) versus LIF and PMT for CE. To demonstrate the applicability of this assay to complex sample analysis, we performed the assay in 10-fold diluted donkey serum. Initial experiments showed significant nonspecific interaction between DNA aptamers and serum proteins. However, we found that addition of nonspecific and nonfluorescent oligonucleotides largely suppresses the matrix interference, thus enabling us to detect IgE in 10% donkey serum with a LOD of 39 pM.

Principle of the assay

Figure 1 shows the key operation of the poly(dimethylsiloxane) (PDMS) microfluidic electrokinetic concentration chip. Under the voltage configuration shown in Figure 1a, 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 1b. Free aptamers, which have very high electrophoretic mobilities due to the highly negative-charged backbone of the oligonucleotide, are concentrated at the low electric field region. On the other hand, the aptamer-protein complex has a lower mobility due to its larger mass; therefore it concentrates nearer to the cation selective membrane where the electric field is higher. Conventional aptamer affinity probe CE operate in a nonequilibrium condition, since there are no targets in the run buffer that allow for rebinding of aptamers that have dissociated from their initial target during separation. A unique advantage of this platform is that the free target protein molecules are able to travel downstream beyond the concentrated aptamer-protein complex band (an even higher electric field is needed to stop the low mobility free protein). Therefore, aptamers that have dissociated from their target in the complex band can quickly rebind with free proteins in the run buffer and regenerate the complex, akin to the Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (ECEEM)³¹ where a plug of equilibrium aptamer-target mixture is injected and separated in a capillary prefilled with target. This is an important advantage which allows even aptamers with relatively high K_d 's to be used in this platform with good sensitivity. Using this device, we realized a multiplexed microfluidic platform where homogeneous aptamer affinity probe electrophoresis assays can be performed with low voltages (30V) and gravitation-induced flow without the need of periphery equipments (syringe pumps, temperature blocks) or multiple buffers.

EXPERIMENTAL SECTION

Reagents and Chemicals

Unless stated otherwise, all chemicals used in the experiments were purchased from Sigma (St. Louis, MO). Human myeloma IgE was purchased from Athens Research and Technology, Inc. (Athens, GA). Recombinant HIV-1 reverse transcriptase (HIV-1 RT) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Oligonucleotides were synthesized and fluorescently labeled by Integrated DNA Technologies, Inc. (Coralville, IA). IgE-binding aptamer (5'-GGG GCA CGT TTA TCC GTC CCT CCT AGT GGC GTG CCC C-3') was labeled with 6-carboxyfluorescein (FAM) during synthesis at the 5' end using ethylene glycol linker⁵. HIV-1 RT-binding aptamer (5'-AT CCG CCT GAT TAG CGA TAC TCA GAA GGA TAA ACT GTC CAG AAC TTG GA-3') was labeled directly at the 5' end with FAM⁶. Nonfluorescent nonspecific oligonucleotides (5'-TGG TCT TGT GTG GCT GTG GCT ATG TCT GAT CTT AAT CCA CGA AGT CAC C-3')⁶ were also obtained from the same source. Donkey serum was purchased from Innovative Research (Novi, MI). All solutions were made with deionized water (18.2MΩ) by Fluid Solutions (Lowell, MA).

Microchip Fabrication

The microchip was fabricated using poly(dimethylsiloxane) PDMS (Sylgard 184, Dow Corning Inc., Midland, MI) irreversibly bonded on a glass slide. Microchannels were molded in PDMS by replica molding technique¹⁶. To obtain the positive master mold, the desired design was photolithographically patterned onto a silicon wafer using positive photoresist. Next, the wafer was etched to a depth of 6 μm via a reactive ion etching (RIE) process. The silicon master was further treated with trichlorosilane (T2492, UCT Specialties, Bristol, PA) in a vacuum desiccator overnight to prevent adhesion to PDMS.

We fabricated the ion-selective nanoporous structures by using the microflow patterning technique to obtain a thin strip of Nafion film on a standard glass slide^{17, 18}. A 50 μm deep and 200 μm wide PDMS microchannel was used to define the flow path of the Nafion solution (20 wt% solution in lower aliphatic alcohol/H₂O mix, Sigma Aldrich, St. Louis, MO). The PDMS chip with microchannels was irreversibly bonded on top of the glass slide by standard plasma bonding.

Figure 1c showed the top view of the actual PDMS device used in the experiments. 0.1–10 μL pipette tips (USA Scientific, Ocala, FL) were cut at the tip end with a razor blade and inserted into the punched PDMS holes to act as fluid reservoirs. There were five separate inlets connecting to one outlet, allowing five samples to be preconcentrated simultaneously. Two side channels flanked the inlet channels to provide symmetrical electrical ground. The ion-selective nanojunction was fabricated at the center of the device to concentrate sample molecules by electrokinetic trapping when voltages are applied. The channels were filled with dyed solution for visualization purpose.

Microchip Operation

Before the experiment, the PDMS device channels were passivated with 1% BSA for 10 minutes to reduce nonspecific binding of the sample to channel walls. After that, the channels were flushed with DI water 3 times and filled with buffer solution (10mM Tris-HCl, pH 7.4) until the samples were ready to be loaded. Sample was prepared by mixing 5 nM of fluorescently labeled aptamer with different concentrations of analyte in buffer solution (10mM Tris-HCl, 1mM MgCl₂, 200μg/mL BSA, pH 7.4 (IgE)/pH 8(HIV-1 RT)).

After 30 minutes incubation at room temperature, 30 μ L of sample was loaded into each of the five inlet reservoirs and drawn into the microchannel by applying a brief suction at the outlet reservoir. The liquid height difference between the inlet reservoir and the empty outlet reservoir caused a well-controlled gravitational flow of sample solution from inlet to outlet, without any need for external pump.

Electrodes were inserted into the inlet and buffer reservoirs on the chip and connected to a power supply (Stanford Research Systems, Sunnyvale, CA). To initiate the concentration-enhanced affinity probe electrophoresis assay, we applied 30 V at the inlet reservoirs while grounding the side channels. An ionic concentration gradient was induced near the ion-selective membrane by concentration polarization effect. Meanwhile, charged sample molecules are continuously separated and stacked at the location where its electrophoretic velocity equals the bulk flow velocity. Within the experimental duration of 30 minutes, the fluorescent intensity of the stacked molecules increases linearly with time while background noise remained constant, resulting in a high signal-to-noise ratio. To study the reproducibility of the assay, we repeated the experiment in the same device after removing the contents in the inlet reservoirs and replacing them with new samples.

Measurement Instrument and Image Analysis

An inverted epifluorescence microscope IX 71 (Olympus, Center Valley, PA) equipped with a cooled CCD camera (SensiCam, Cooke Corp., Romulus, MI) was used for fluorescence imaging. A mechanical shutter which only opens for 100 ms every 5 s when images are taken was used to prevent photobleaching of the fluorescent molecules. The images were analyzed using the NIH ImageJ software. Flat-field correction was performed by dividing a reference image of the device taken before each experiment. Concentrations of bound and unbound aptamers were assumed to be directly proportional to the focused peak height as demonstrated in previous work¹⁴. Complex peak heights are normalized by the sum of complex peak height and free aptamer peak height. Dose response curves were fitted using a four-parameter logistic model. Origin 7 software (OriginLab Corp., Northampton, MA) was used for curve fitting.

RESULTS AND DISCUSSION

Optimization of assay

We first determine the optimal conditions that promote stable aptamer-protein complex formation in free solution. The presence of divalent cations such as Mg^{2+} has been reported to be necessary for certain aptamer-protein complex formation³². Without Mg^{2+} , no aptamer-IgE complex is formed while the aptamer-HIVRT complex band is only weakly fluorescent. Addition of 1mM of $MgCl_2$ greatly improves the interaction between the species. We have also found that complex stability is a sensitive function of buffer pH. Best results were obtained in 10mM Tris-HCl buffer at pH 7.4 and pH 8.0 for IgE and HIV-1 RT assays respectively.

In initial experiments, aptamer and target proteins (IgE and HIV-1 RT) were simultaneously concentrated and separated in bare PDMS-glass devices. We observed no complex bands until high concentrations (> 10 nM) of target proteins are added. Precoating the microchannels with 1% BSA for 10 minutes enabled us to clearly visualize the complex band corresponding to 750 pM of IgE (Figure 2a), suggesting that prevention of nonspecific adsorption of proteins to the microchannel surface is important to increase sensitivity. Precoating microchannel with 5% BSA did not lead to additional improvement in sensitivity. Interestingly, as shown in Figure 2, adding BSA into the sample increases sensitivity of the assay. Adding 50 μ g/mL of BSA led to a clear complex band

corresponding to 75 pM of IgE, while addition of 100 $\mu\text{g/mL}$ of BSA to the sample enabled detection of 7.5 pM of IgE. No further sensitivity improvement was obtained when more than 200 $\mu\text{g/mL}$ of BSA was added to the sample. Similar trends were observed with the HIV-1 RT assays. This observation is thought to be due to BSA stabilizing the aptamer-protein complex³³. Based on previous reports, it has been suggested that the presence of BSA in solution helps maintain the correct aptamer and target protein conformation for optimal binding³³. Presence of BSA in the solvent could also maintain the ratio of hydrophobic and hydrophilic regions on the target protein, thus preventing it from denaturation³⁴. Both the IgE and HIV-1 RT specific aptamers did not interact with BSA, as the negative controls containing BSA but no target proteins did not form a visible complex band. In all our subsequent experiments, the microchannel surfaces were passivated with 1% BSA for 10 minutes and 200 $\mu\text{g/mL}$ of BSA were added to the samples to obtain the best sensitivities.

We also observed that the separation distance between the bound and free aptamers increased with the addition of high concentrations of BSA into the sample. We believe that this is due to an isotachopheresis-like effect where preconcentration of an intermediate mobility species (BSA) results in a broadening electric-field plateau that separates the bound and unbound aptamer bands³⁵. This suggests a method whereby the separation resolution between two species can be independently tuned by adding a spacer molecule with intermediate mobility in the sample.

Detection of IgE and HIV-1 RT from buffer

We first demonstrate electrokinetic concentration-enhanced affinity probe electrophoresis assay of IgE using a specific aptamer for this protein. Upon binding to IgE, the mobility of a free aptamer ($-2.81 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) is expected to shift to $-0.58 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ ⁶. Figure 3a,b shows the representative results for electrokinetic concentration-enhanced affinity probe electrophoresis assay for human IgE using anti- IgE aptamer as affinity probe. Experiments were performed in optimized buffer conditions (10mM Tris-HCl, pH 7.4, 1mM MgCl_2 , 200 $\mu\text{g/mL}$ BSA) with constant aptamer concentration (5nM) and varying concentrations of human IgE protein (5pM to 75nM). During the 30 minutes experiment, the fluorescent intensity of the bands increased linearly with time and achieved concentration factors of >1000. The aptamer and complex bands were well-resolved (resolution~3.9). The position of the aptamer-protein band was also remarkably stable; moving less than 200 μm after it reached an equilibrium position at around 2 minutes. Slight variations in the band locations are due to differences in gravitation-induced flow, but the ratiometric assay results are relatively insensitive to the exact band locations. As expected, with increasing target protein concentration, the free aptamer peak decreased and the complex peak increased. Figure 3d shows the aptamer-protein complex peak due to 4.92 pM of IgE. For comparison, no complex band was observed in the negative control experiment.

The full dynamic range of the assay is shown in Figure 3e. The dose response curve was fitted using the four-parameter logistic model. The average and standard deviation of the zero dose response is calculated by performing two separate experiments where the sample contains no IgE, and taking the peak ratios as described in the methods section. Figure 3f shows a linear relationship in the log-log plot obtained at low IgE concentrations (5 pM – 7 nM). The LOD for IgE, calculated to be the analyte concentration needed to produce a signal three standard deviations above the zero dose response, is 4.4 pM. This is the lowest LOD reported to date for detection of IgE using aptamers. Interestingly, the apparent K_d (1.85 nM) is found to be more than an order of magnitude lower than the reported dissociation constant for this aptamer (64 nM)⁵. One possible explanation for this binding enhancement is that aptamer-protein rebinding events in the electrokinetic concentration zone more than offset the effect of dissociation during the experiment. Given that aptamers generally have

higher K_d compared to antibodies, this scheme could significantly increase the sensitivity and utility of aptamer based assay.

Similar experiments were performed using Human Immunodeficiency Virus 1 Reverse Transcriptase (HIV-1 RT) and an aptamer against this protein to demonstrate that this method is general and can be applied to multiple analytes. Upon binding to IgE, the mobility of a free aptamer ($-2.81 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) is expected to shift to $-0.50 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ ^{5,6}. The results (Figure 4) showed a LOD of 9 pM, which is also among the lowest LOD reported to date for this aptamer-protein pair.

Detection of IgE from serum sample

To demonstrate the applicability of this assay to complex sample analysis, we performed the IgE assay in a buffer that consists of diluted donkey serum. Initial experiments showed significant interactions of serum components with the aptamers. Figure 5 showed a representative IgE assay performed in 1% donkey serum. We observed fluorescent precipitation in the sample solution. Moreover, there is formation of an extra fluorescent band in between the free aptamer and aptamer-protein band. Interestingly, the extra band is well defined and baseline-separated from the other two bands. This suggests that the aptamer is interacting with a particular species in serum, such as DNA binding proteins found in mammalian serum³⁶. Although this interference did not interfere with the formation and determination of the free aptamer and the aptamer-protein complex (they are all baseline separated), the fluorescent precipitation in the sample solution caused large spikes in the electrophoregram.

It has been reported that serum matrix interference on aptamer affinity probe capillary electrophoresis can be suppressed by addition of nonspecific oligonucleotides that bind competitively to the interfering serum proteins⁶. We found that addition of 10 μM of a nonspecific and nonfluorescent 49-mer oligonucleotide⁶ eliminated the extra band and fluorescent precipitation in a sample solution containing 10% donkey serum.

Figure 6 shows the experimental results for IgE assay in 10% donkey serum after 1 minute preconcentration, with addition of 10 μM of nonspecific oligonucleotide to suppress matrix interference. Due to the high total protein concentration in the sample, preconcentration leads to a rapid broadening of electric field plateau between the free aptamer and the aptamer-protein complex as discussed in the previous section. We can only perform experiments for 2 minutes before the separation distance exceeds the microscope field of view. Due to the shorter preconcentration time, there is less sensitivity enhancement. We obtained a LOD of 39 pM for IgE assay in 10% donkey serum.

The LOD using this scheme is ultimately restricted by the specificity, rather than the affinity (K_d) of the aptamers against the target protein. Experiments in the serum sample showed that two bands were observed even in the case of the negative control (Figure 6b), which indicated that nonspecific binding was not completely eliminated by addition of nonspecific oligonucleotides. On the other hand, experiments in simple buffer showed that signal-to-noise ratio increased with time. We can obtain better sensitivities at the expense of longer assay time. The key advantage of this technique is a continuous influx of sample that counteracts the effects of dissociation and a self-focusing ability that minimizes band dispersion, so even aptamers with relatively high dissociation constant can be used in this assay.

CONCLUSION

In conclusion, this paper demonstrates the use of electrokinetic concentration to realize a continuous signal amplification scheme that increases the sensitivity of homogeneous mobility shift assay. Aptamers are attractive alternatives to antibodies for point-of-care diagnostic purposes due to their stability, low cost, and homogeneity. Our aptamer based affinity probe electrophoresis assay in a lab-on-chip device could detect 4.4 pM and 9 pM of IgE and HIV-RT in simple buffers, and detect 39 pM of IgE in 10% serum sample. These are among the lowest LOD obtained for aptamer affinity probe capillary electrophoresis experiments. Furthermore, this method has an advantage over many other assays since it is rapid, uses low voltage, consumes very little sample, can be multiplexed, and is very user-friendly (no multiple processing steps required).

Miniaturized capillary electrophoresis devices are one of the first microfluidic systems that gained popular acceptance, and remains a mainstay in lab-on-chip platforms. The method presented in this paper has broad applicability to improve the sensitivity of various capillary electrophoresis assays, such as those involving protein-protein interactions and enzymatic reactions. We plan to pursue these topics and apply them to clinically relevant samples in the future.

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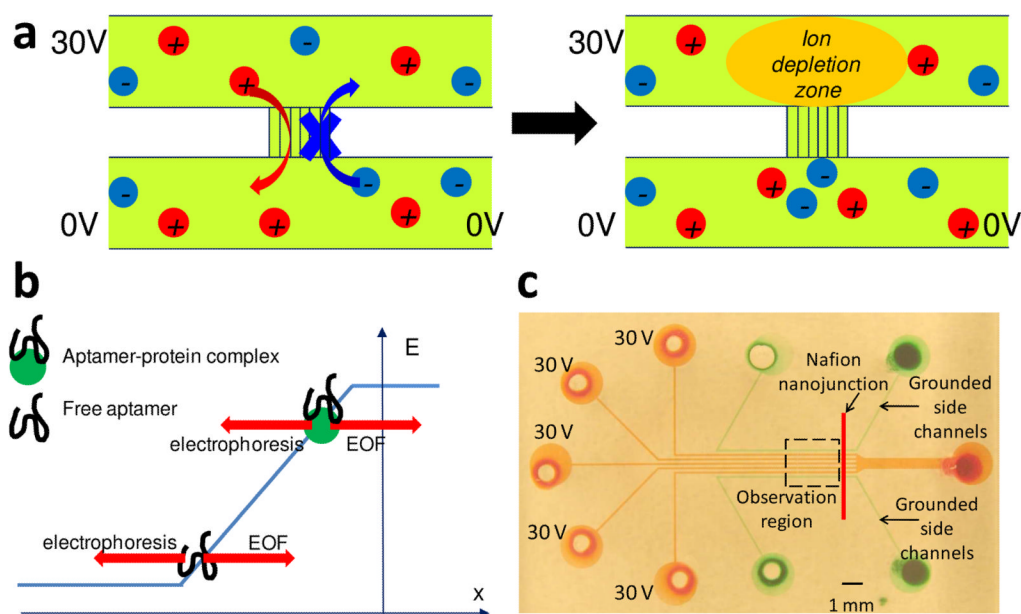


Figure 1.

a) Ion selective membrane creates a local ion depletion zone with high electric field upon applying a voltage, b) Free aptamers and aptamer-protein complex concentrate at different locations on the electric field profile due to their different electrophoretic mobility, c) Optical image of multiplexed 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.

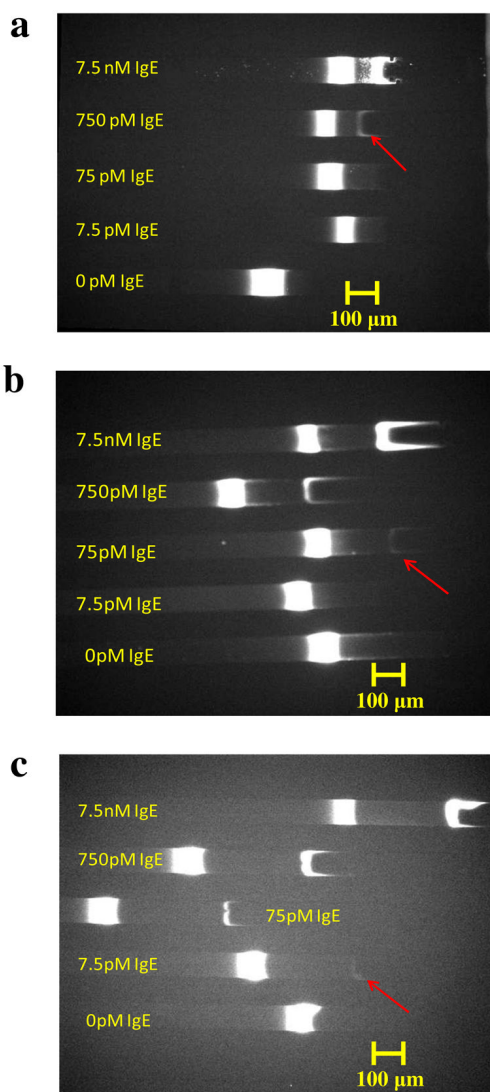


Figure 2. Addition of BSA improved detection of IgE in buffer using concentration-enhanced aptamer affinity probe electrophoresis assay. a) no BSA in sample (cannot detect 75 pM IgE), b) 50 µg/mL BSA in sample (can detect 75 pM IgE), c) 100 µg/mL BSA in sample (can detect 7.5 pM IgE).

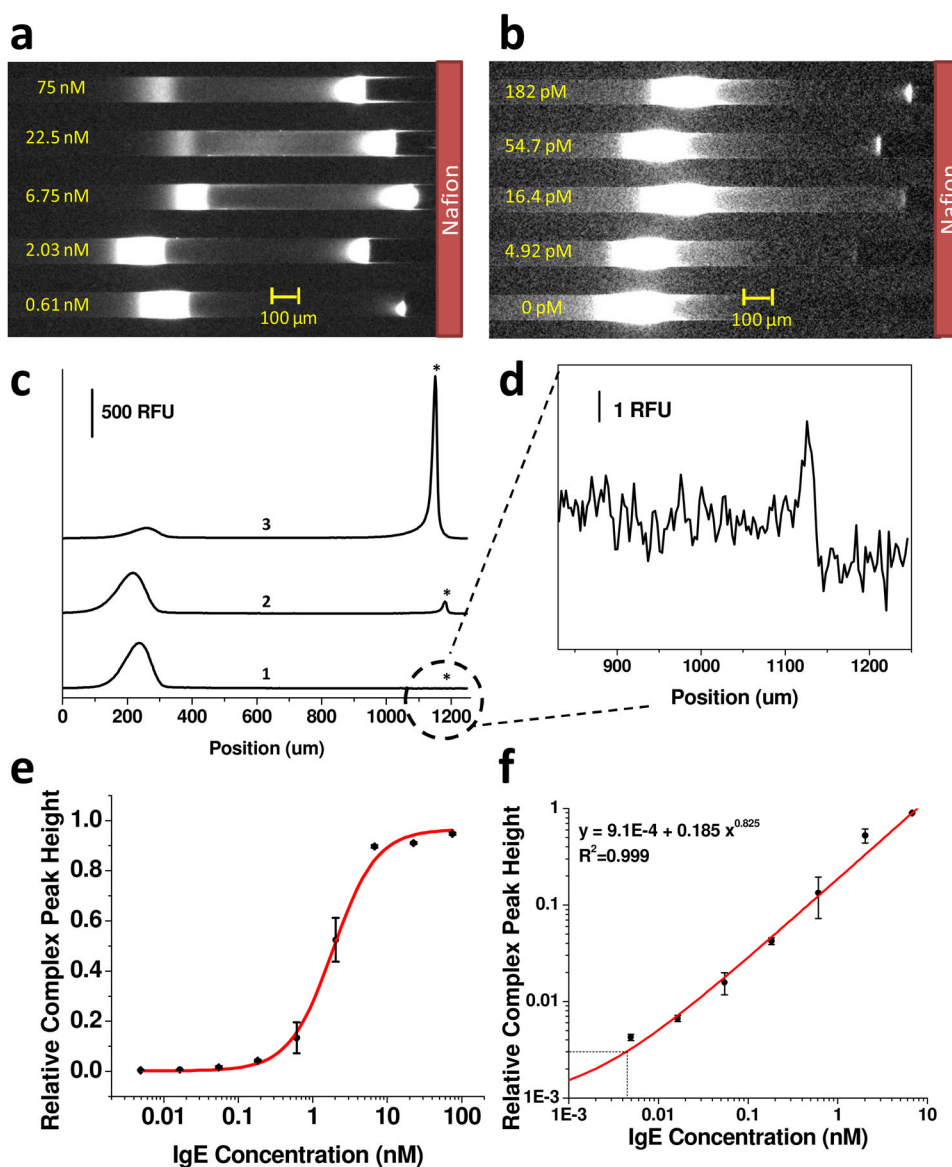


Figure 3.

a,b) Experimental results for multiplexed concentration enhanced aptamer affinity probe electrophoresis assay for detecting IgE in buffer solution after 30 minutes of concentration, c) Electropherogram for optimized electrokinetic concentration and separation of IgE aptamer (5 nM) and different concentrations of IgE: (1) 4.92 pM, (2) 0.6 nM, and (3) 6.75 nM IgE. The complex band is labeled with an asterisk, d) Inset demonstrates detection of 4.92 pM IgE in buffer, e) Dose-response curve of anti-IgE aptamer with IgE spiked in buffer, error bars represent standard error from duplicate experiments, f) Linear relationship in the log-log plot is obtained at low concentrations of IgE. The measured LOD is 4.4 pM IgE.

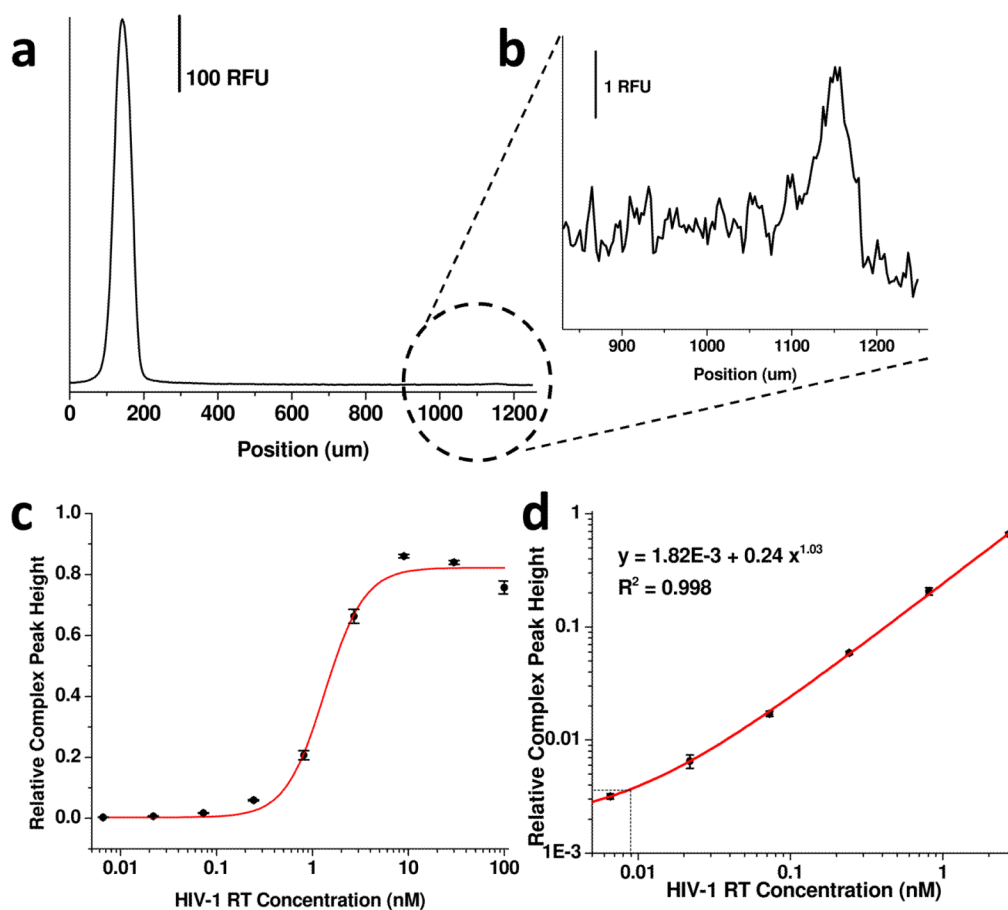


Figure 4.

a,b) Electropherogram demonstrating detection of 6.5 pM HIV-1 RT in buffer, c) Dose response curve of anti-HIV-1RT aptamer with HIV-RT 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 HIV-1RT, measured LOD is 9 pM HIV-1 RT

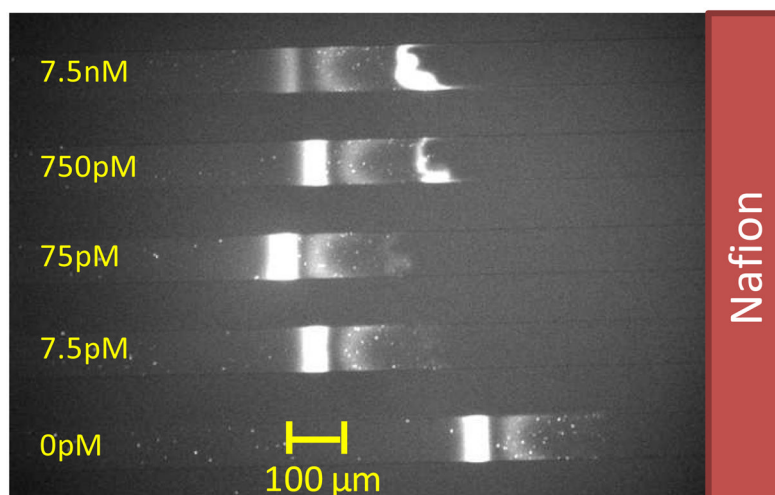
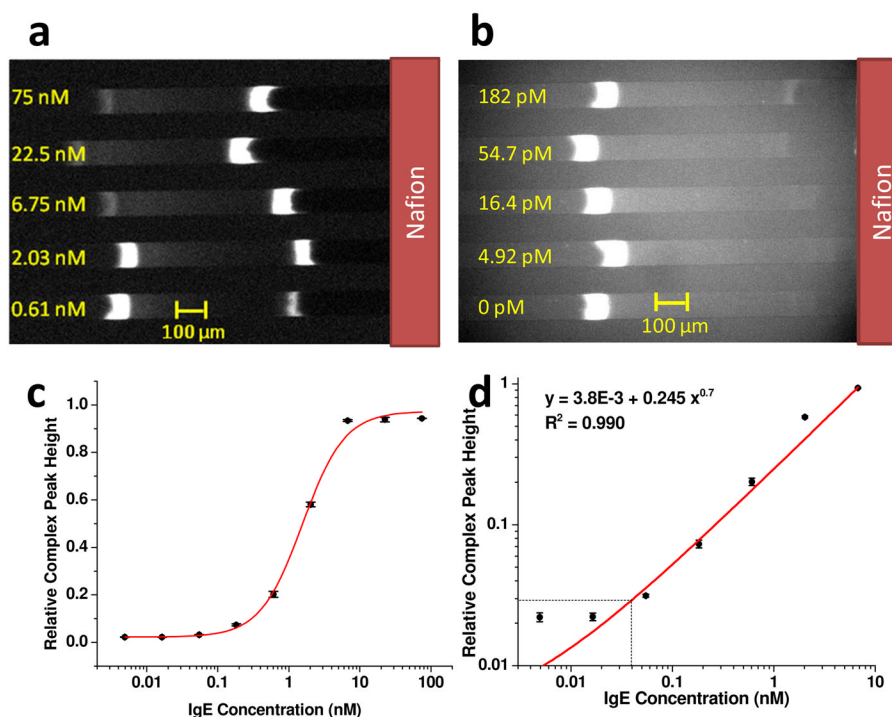


Figure 5. Concentration enhanced aptamer affinity probe electrophoresis assay for detecting IgE in sample containing 1% donkey serum. Aptamer binding to serum components gave rise to a third fluorescent band in between the free aptamer and aptamer-target protein band. Fluorescent precipitation is also observed in solution,

**Figure 6.**

a,b) Experimental results for multiplexed concentration enhanced aptamer affinity probe electrophoresis assay for detecting IgE in 10% donkey serum, c) Dose response curve of anti-IgE aptamer with IgE spiked in 10% serum, error bars represent standard error from duplicate experiments, d) Log-log plot at low concentrations of IgE in serum showing effects of nonspecific binding on sensitivity. The measured LOD is 39 pM IgE.