High Sensitivity, Microfluidic Immunoassays utilizing DNA-conjugated Antibodies to Accelerate the Electrophoretic Separation of Antigen-Antibody Complexes.

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ABSTRACT

By combining the principles of liquid-phase binding and lab-on-a-chip microfluidics, we have developed a rapid and highly sensitive mobility-shift immunoassay system. In order to enhance assay speed and sensitivity, DNA-antibody conjugates were used to accelerate the immune-complex electrophoretic separation from unbound, fluorescent probes and to sharpen the complex peak. Sample concentration was also performed by isotachophoresis to enhance the detection signal and provide a narrow starting zone for capillary electrophoresis. The assay time was on the order of 15 minutes, and the assay was automated by using on-chip sample-antibody mixing, incubation, separation, and detection in a microfluidic device. Using this approach, a serum AFP assay was demonstrated with pM sensitivity, reproducibility in the range of 5-10% CV, and a 4-log linear dynamic range.

Keywords: microfluidics, lab-on-a-chip, immunoassay, AFP

ASSAY PRINCIPLE

The promise of microfluidic immunoassays in microchips has been the miniaturization, integration and automation of rapid immunoassays. The typical microfluidic assays have used fluorescent dye labeled antibodies in direct binding assays or labeled antigens in competitive binding formats[1]. Often electrophoretic or hydrodynamic separation is employed to measure the formation of antigen-antibody complexes. The problem with hydrodynamic separation is that the bead solid-phases[2] that are often used are inconvenient to load into microchannels and binding kinetics are slow. The electrophoretic mobility shift methods avoid this inconvenience and have faster binding kinetics because of liquid-phase binding and separation. One of the principal problems with the non-isotopic mobility shift methods has been the lack of assay sensitivity caused by the broader peak shape due to electrophoretic heterogeneities of antibodies and their immune-complexes. Another principal cause of poor assay sensitivity is the low concentration of sample analyte in a narrow injection zone and dispersion of this zone during separation.

We have used a two pronged approach to overcome the problem of low sensitivity. Firstly, a novel method was employed using DNA conjugated antibody to accelerate the electrophoretic mobility of the immune-complex and, thereby, reduce the separation time and dispersion during the zone electrophoresis step (Fig. 1). The relationship of the DNA size to immune-complex mobility was first established by Kawabata et al.[3] using the BioAnalyzer microchip system.

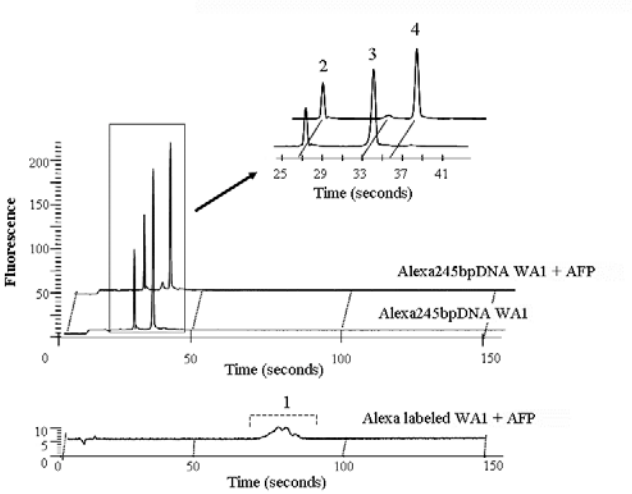

Figure 1. Improvement of the Electrophoretic Peak Shape and migration time by incorporating DNA. Alexa-labeled WA-2 Fab'-AFP complex showed a broad peak (1) and slow migration, but 245 bp DNA-WA-1 Fab'-AFP complex (4) migrated as a sharp peak, completely resolved from the 245 bp DNA-WA-1 Fab' peak (3) and free DNA peak (2).

Secondly, an in-line sample concentration step[4] was utilized to enhance detection sensitivity. An earlier on-chip method had used the field amplification stacking technique for sample concentration[5], but here we have used isotachophoresis to focus a larger volume of the sample to a narrow starting band for zone electrophoresis in a gel filled separation channel[6] (Fig. 2).

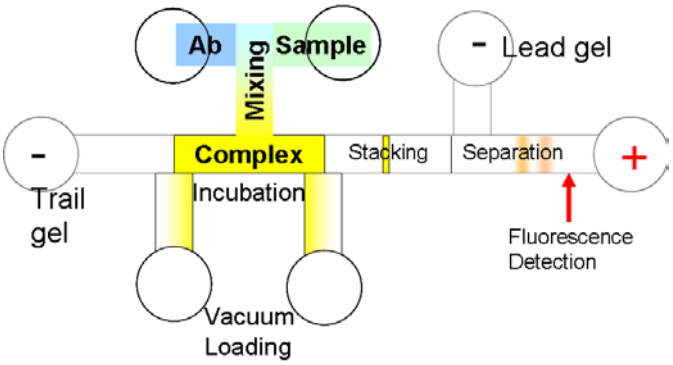

Figure 2. Mixing, Incubation, Stacking, Separation and Detection of Immune-complex On-chip.

ASSAY PROTOCOL

A serum α-fetoprotein (AFP) assay was developed as a model assay using a mobility-shift microfluidic immunoassay format. A pair of monoclonal mouse antibodies were used for the assay, WA-1 Fab' was conjugated to a 5'-amine-labeled 250 bp DNA fragment using the hetero-bifunctional cross-linker, EMCS and the other antibody, WA-2, was unconjugated IgG. Alexa-fluor 647 dye was used as the fluorescent label used to label either the DNA on the antibody conjugate or the IgG antibody. These antibodies were diluted into leading gel, containing 0.9% p-DMA in 75 mM Tris-HCl buffer, pH 8.0. Additional protein, detergent, and polymeric blockers were added to prevent non-specific binding[7]. The human serum samples was diluted 1/10 into the leading buffer. The immune-complexes were formed by mixing the antibody reagent and diluted sample 1:1 either in a tube off-chip or by pipetting into the sample and conjugate wells and mixing on-chip. The on-chip reagent mixing enabled the study of antibody-antigen binding kinetics. The chip was primed with a trailing gel containing 0.9% p-DMA, 125 mM HEPES, and 75 mM Tris, pH 7.5, in the channel adjacent to the complex and cathode well. The other channels including the anode well were primed with either leading gel or sample and conjugate (see Fig. 2). A computer script driven high voltage and vacuum controller, initiated the assay by pressure loading the sample and antibody into the chip, and after a defined period of loading and incubation (28 sec to 2 min), the electrical field was applied to the chip through electrodes positioned in the anode and cathode wells. An auto-switching circuitry was utilized to hand-off the voltage from the trailing gel cathode to the leading gel cathode after the stacked sample plug passes the side-arm intersection. This voltage hand-off corresponds to the switch from isotachophoresis stacking to capillary electrophoresis (CE) separation. Epi-fluorescence detection optics were used to detect the Alexa-fluor 647-labeled immune-complex as it passed the detection window excited by a red diode laser. The chip design used for these experiments had four independent parallel assay channel networks, making it possible to test four samples in the same run (Fig. 3).

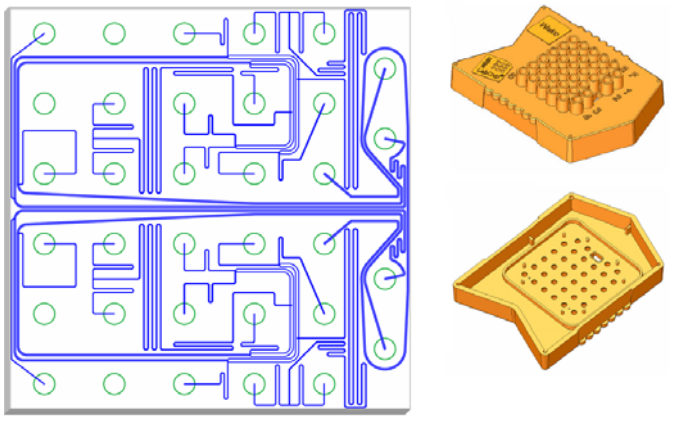

Figure 3. 4-Parallel channel Networks for running Microfluidic Mobility-Shift Immunoassays. The chip substrate dimension was 23.04 x 23.04 x 1.45mm.

RESULTS

1 BOUND/FREE SEPARATIONS

We have found that the increased charge to mass ratio of the immune-complex, when DNA-conjugate antibody was used, enhanced the stacking of the immune-complex as well as the speed of CE separation. In experiments using a similar chip and protocol, the complex was stacked to about 200 times the starting concentration using serum samples that are complex mixtures of proteins, lipids and various solutes and salts. For comparison, Field Amplification Stacking gave about 20 times the starting concentration in earlier studies[5]. The electrophoregram in Figure 4 shows that four replicate assays gave highly reproducible results and excellent resolution of the immune-complex from internal DNA standards spiked into the sample diluent. The internal standards were used to align the electrophoregrams for the identification and quantitation of complex peaks.

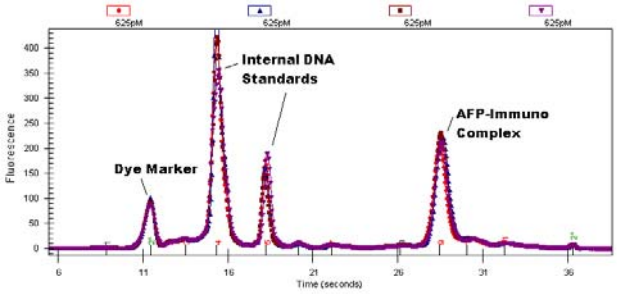

Figure 4. Electrophoregram of AFP Immune-complex run on 4-Channel Chip. Four replicate samples of pooled human serum spiked with 625 pM AFP are run in the four channels on the Mobility-Shift Immunoassay chip, and aligned using the DNA Internal Standards.

2 BINDING REACTION KINETICS

The on-chip reagent mixing enabled the study of antibody binding kinetics by automating the mixing and incubation interval, followed by a rapid bound/free separation and quantitation, requiring 60 seconds. In Figure 5, the binding kinetics at 20° C is presented. The binding reaction followed the pseudo 1st order rate equation, $A/Ao = 1\text{-}e^{-T/\tau}$, and the τ value was 99 sec, corresponding to 64% maximum binding. By this measure, >95% binding will be achieved in 5 minutes at 20° C. By increasing the antibody reagent concentrations, the kinetics can be accelerated and the binding time reduced to 1-2 minute.

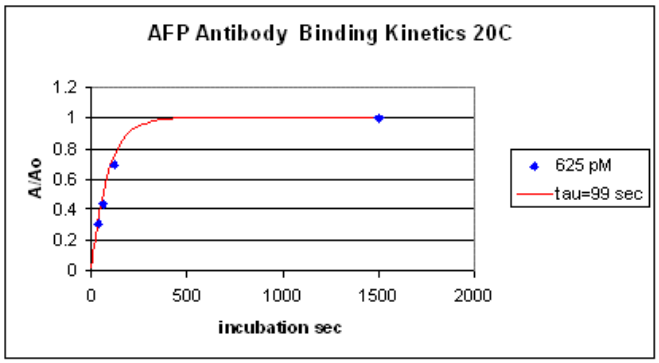

Figure 5. The Antibody Binding Kinetics. 625 pM AFP, 20 nM DNA conjugated Fab' and 20 nM Alexa labeled antibody were mixed on-chip at 20 C, and incubation for 33, 60, and 120 sec prior to electrophoretic separation. Flow velocity in the incubation channel was used to vary time. Maximum binding (Ao) was measured after a 20 min incubation of Sample and Antibody reagent in chip well. The chip temperature was maintained by heating of the chip caddy in a cooled chamber. The peak area (A) for the immune-complex peak was integrated, and the results are plotted as the ratio of A/Ao. τ is calculated from the pseudo 1st order rate equation.

3 AFP ASSAY PERFORMANCE

The concentration response curve for the AFP assay, as shown in Figure 6, demonstrated that the assay was linear for 4 logs of dynamic range, since the Limit of Detection (LOD) was <10 pM AFP as defined by 3 x SD of the peak noise in the region of the AFP complex peak. Since Normal human serum contains about 10 pM AFP, 2% BSA was used as a test sample for LOD tests, and 2 pM was measured as the detection limit in this sample matrix (data not shown).

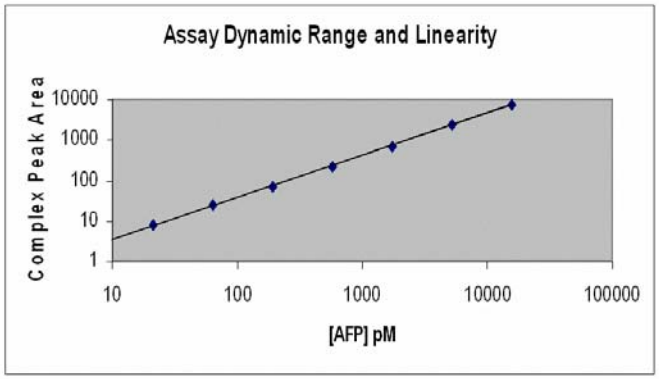

Figure 6. Concentration Response Curve for AFP Spiked into Pooled Human Serum.

The reproducibility of the assay system was characterized by the CV of Within Day measurements that ranged from 3 to 10 % for spiked serum samples containing 7.8, 250 and 5,780 pM AFP incubated with antibody off-chip, in tubes. Between Days reproducibility was characterized by CV's of 2 to 6% for variation of the mean values for 3 days of Within Day testing.

AFP pM	Within Day CV, N=8	Within Day CV, N=8	Within Day CV, N=8	Between Days* CV, N=3
0	11.9%	8.8%	8.3%	6.2%
7.8	5.2%	10.1%	6.6%	5.9%
210	4.9%	6.9%	3.1%	4.4%
5780	4.7%	2.7%	3.2%	1.9%

*Between Day CV was calculated from mean values of Within Day data.

CONCLUSIONS

This study demonstrated that it is possible to develop a rapid and sensitive mobility-shift immunoassay on a microfluidic assay format, as illustrated here using monoclonal antibodies coupled with DNA. The assay was applicable to the measurement of AFP in human serum samples, and this novel approach should have broad applications whenever cost effective, automated, and rapid quantitative immunoassays are required.

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