Magnetic Nanotag-based Immunoassay for Multiplex Mycotoxin and Protein Detection

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ABSTRACT

Rapid, sensitive and multiplexed measurement is vital in molecular diagnostics such as cancer diagnosis and pathogens detection. By combining the superior specificity of immunoassays with the sensitivity and simplicity of magnetic detection, we have developed a novel multiplex magnetic nanotag-based detection platform for cancer biomarkers and mycotoxins at sub-picomolar concentration level. Magnetic biosensors use superparamagnetic nanoparticles as labels in the detection of biomolecules. Unlike fluorescent labels, magnetic nanotags (MNTs) can be detected with inexpensive giant magnetoresistive (GMR) sensors such as spin-valve sensors. Antibody-immobilized sensor array prior to the addition of the biotinylated detection antibody. The sensor response to the addition of streptavidin-linked MNTs is recorded in real time. Here we demonstrate the simultaneous detection of multiple mycotoxins (aflatoxins B_1, zearalenone and HT-2) and show that a detection limit of 50 pg/mL can be achieved.

Keywords: gmr sensor, multiplex mycotoxin detection, magnetic, nanotag, array

1 INTRODUCTION

A multitude of sensitive molecular interaction detection techniques in recent years has fueled the research interests for various biosensing applications. For instance, surface plasmon resonance (SPR) has been adapted for various applications to study molecular interactions. This technique has been especially valuable in elucidating biospecific interaction analysis [1-4]. Refractive index of the biorecognition layer on the sensor surface is continuously monitored as a function of binding [5]. The primary impact of SPR in this area is the ability to monitor the binding interactions of immuno-components in real-time. One major advantage of SPR over other biosensing approaches is the detection requires no specialized and expensive labeling [6,7].

However, molecules with low molecular weight, such as mycotoxins, often fail to induce change in refractive index significant upon binding to the sensor surface. Consequently, an extra step involving bioconjugation of target mycotoxin with high molecular weight carrier such as a bovine serum albumin (BSA) is required to improve sensitivity [8].

One of the most established laboratory-based biochemical assays for pathogen detection to date is ELISA, which is based on the detection of pathogen-specific surface epitopes using antibodies [6]. With its very high specificity and exceptional sensitivity, ELISA is often referred to as the gold standard of toxin detection. Nevertheless, current assays typically involve reporter molecules or labels conjugated to enzymes or fluorescent markers, which makes ELISA restricted to advanced laboratory settings with specialized read-out equipment [9]. Accurate and rapid read-out on site would provide vital efficiency in toxin detection, reducing potential risks of further unnecessary food borne pathogen contamination. However, implementing ELISA into a point-of-use test remains challenging due to the sheer complexity of the instrumentation involved.

The purpose of this project is to develop a simple, sensitive and versatile magnetic-based platform to detect complex sample matrices using a multiplexed protein array. Instead of conventional fluorescent or chemiluminescent/bioluminescent tags, the magnetic detection array uses magnetic nanoparticles, 10 – 16 nm in diameter, to tag the protein/antibodies, and detects the presence of a nanotag by the field it generates, using a very sensitive spin valve detector.

The current work was motivated by the growing interest in point-of-use applications in the food industry and point-of-care applications in biomedical diagnostics [9-11]. We chose a set of small molecules (mycotoxins, m.w. < 200Da) as our detection model, integrating the classic sandwich-

based immunoassay into a magnetic nanotag (MNT) detection platform. Real-time measurements are conducted upon the addition of MNTs onto the spin-valve sensor surface immobilized with capture antibodies for mycotoxins (aflatoxin B_1, zearalenone and HT-2), mycotoxins, and detection antibodies. Here we examine the sensor's multiplexing capability and have demonstrated detection limits for mycotoxins in the range of pg/mL level.

We believe the assay system presented here has the capacity and potential to be implemented as a cost-effective, point-of-use multiplexed mycotoxin test. Furthermore, this study shows that this is a versatile platform capable of detecting various types of agents, including small molecules.

2 EXPERIMENTAL

As test articles, we chose 3 common mycotoxins: aflatoxin B1, zearalenone and HT-2 produced from the fungal species Aspergillus (AFB_1) and Fusarium (Zearalenone and HT-2). The antibodies specific for the chosen toxins have been well characterized and high quality antibodies are commercially available. The schematic representation of the setup is shown in Figure 1. i) Positive probes are immobilized with capture antibodies of interest. ii) Analytes are mixed into a single pool for incubation; and iii) finally biotinylated detection antibodies are added. iv) The binding of streptavidin-coated MNTs with detection antibodies and the detection of magnetic signal in real time. Under the external magnetic field applied through the Helmholtz coil, the superparamagnetic nanoparticles become magnetized. Their presence at the close proximity of the GMR sensor surface alters the local magnetic field, which induces change in resistance of the sensor. After establishing a baseline resistance (Fig. 1a), the MNT was dispensed into the reaction well (t = 0). The binding event of MNT to the biotinylated detection antibodies took place immediately upon contact and was recorded in real time (Fig. 1b). The available binding sites for the MNTs are a function of analyte added before the incubation of biotinylated antibodies. Therefore saturation level of the MNT binding curve is taken as a direct correlation of analyte concentration. Typically signal saturates (Fig. 1c) in 15 min or less when few MNT binding sites are available; and the absolute signal values at t = 10 min are used for data analysis and comparison purposes.

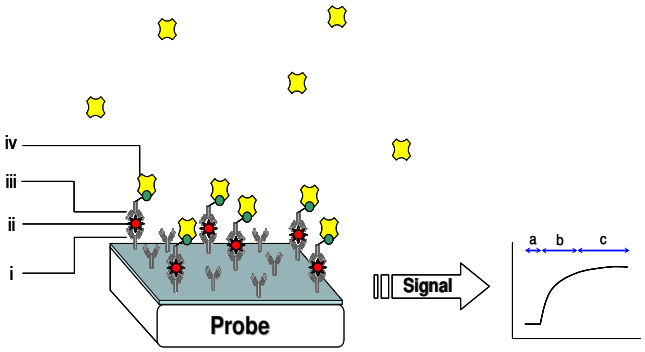

Figure 1. Schematic illustration of the magnetic nanotag-based immunoassay for mycotoxins and typical MNT binding curve.

2.1 Chip Fabrication

Each sensor consists of 32 linear segments of 1.5 x 100 μm connected in series with equal spacing that span over an area of approximately 100 x 100 μm^2. Ion milling process was used to pattern individual sensors with a spin valve film with a layer sequence similar to that of hard disk drives read heads [12]. Each chip, consisting an 8 x 8 sensor array, was further passivated with a tri-layer oxide (SiO_2 10 / Si_3O_4 10 / SiO_2 10nm) during the final fabrication process.

2.2 Surface Preparation

The chip is subjected to oxygen plasma treatment to remove organic residues adsorbed onto the surface. A 1% (w/v) polyallyamine solution dissolved in deionized water was added onto the chip surface for 5 min, followed by a heat treatment at 120°C for 1 h. A solution of 10% (w/v) each of EDC and NHS was then added to the sensor surface for 45 min at room temperature. The chip was rinsed further with deionized water after the incubation with EDC/NHS. In the final immobilization step, capture antibodies for various mycotoxins were delivered manually in a form of 0.4-μL droplets onto the sensor surface at a concentration of 500 μg/mL. As opposed to antibodies, control sensors were immobilized with a high concentration (10% w/v) BSA dissolved in PBS. Finally, the chips were incubated at 4°C at 95% relative humidity for at least 24 hours.

3 RESULTS AND DISCUSSION

Specificity. A chip that was functionalized with anti-AFB_1, anti-zearalenone, anti-HT-2 and BSA was incubated with a sample solution containing only AFB_1 (10 ng/mL). The resulting curves are shown in Figure 2. The data shows that the binding of MNT occurred immediately on the sensors that have been immobilized with anti-AFB_1. The average signal saturates at approximately 12μV after 12 min. Meanwhile the other negative control sensors, anti-

zearalenone, anti-HT-2 and BSA, which were not expected to show interaction with AFB_1, gave a negligible signal. The result verifies the specificity of the antibodies and shows that the system does not suffer electronic cross-talk problems.

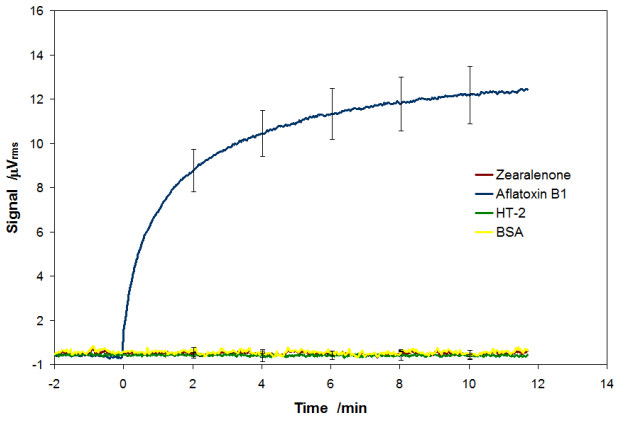

Figure 2. Cross-reactivity study of anti-AFB_1, anti-zearalenone and anti-HT-2.

Multiplex detection. To demonstrate the multiplex detection capability of the system, we performed a series of experiments using mixtures of mycotoxin analytes. The chips were functionalized as above with 3 different antibodies, and also BSA as negative control. In the first experiment, the analyte solution contained 33.3 ng/mL each of AFB_1, zearalenone and HT-2 toxins. The average signals for the mycotoxins were data generated from at least 4 - 9 sensors each on the sensor chip (Fig. 3). At 333 pg/mL, the positive sensors all displayed typical binding kinetics and three distinctive signal intensities upon the addition of MNTs. From these results, it is evident that our magnetic immunoassay platform is capable for multiplexed detection with the proper choice of antibody-analyte pairings. In addition, despite identical concentrations the differences in signal intensity from the mycotoxins used in this study are a strong indication that the antibodies have very different binding affinities towards their respective toxins. Therefore, it is possible for the detection limit of this assay to be further optimized and enhanced with antibodies of extremely high binding affinity.

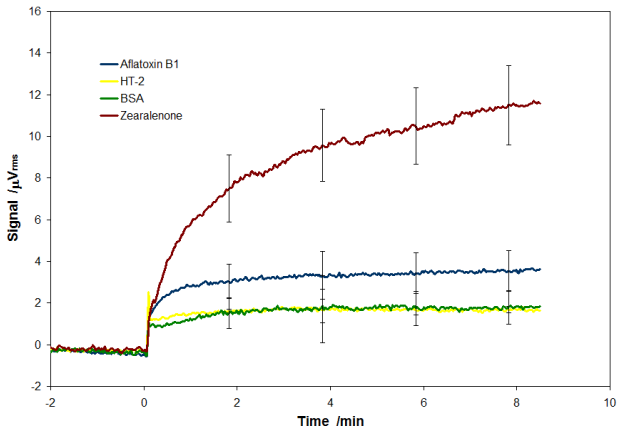

Figure 3. Multiplex detection for AFB_1, zearalenone and HT-2 at 330 pg/mL.

Limits of detection. Having already reached the limit of detection for HT-2, we undertook a more rigorous investigation of the detection limit of the MNT-based immunoassay using AFB_1 and zearalenone as our model analytes. We chose these analytes partly because their signal strength allows a more complete determination, and partly because commercial ELISAs are readily available for these two toxins, allowing direct comparison. Under the conditions of the experiments, signal saturation is usually achieved within 10 minutes. Therefore, for comparison purposes, the absolute signal gain was measured and reported at t = 10 min, unless otherwise stated, after subtracting the background generated from the negative controls.

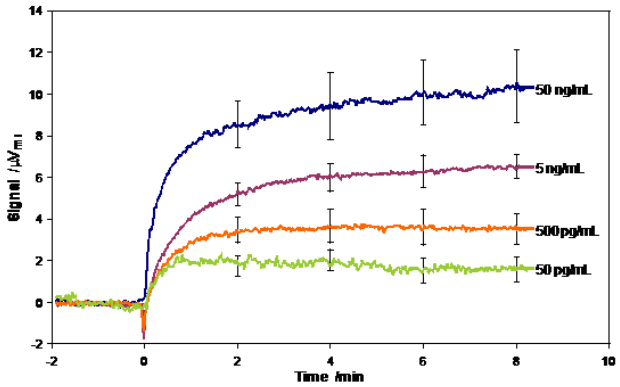

Figure 4. Magnetic signal of AFB_1 detection as a function of concentrations.

We systematically studied the signal dependence on toxin concentration in order to determine the detection limit of this platform. Figure 4 shows the signal-concentration relationship for AFB_1, ranging from 50 ng/mL to 50 pg/mL. As the results indicate, the concentration of AFB_1 exhibits a positive correlation with the magnetic signal. In this case, the data points at t = 8 min were chosen and tabulated

(Table 1) for AFB_1 and zearalenone. At the lowest concentration tested (50 pg/mL), AFB_1 yields an average signal of 1.86 ± 0.59 μV. The average signal approximately doubles for every 10-fold increase in analyte concentration, which is in excellent agreement with the previous study with this detection platform [12]. For zearalenone, a similar trend was observed with increasing analyte concentration. The results demonstrate that our magnetic immunoassay system can easily achieve a dynamic range of at least 4 orders of magnitude, exceeding the dynamic range (3 orders of magnitude) of the commercial ELISA kits we have tested. Furthermore, we have compared the limit of detection (LOD) of our system to the ELISA kits. Our system attained the same LOD for zearalenone at 50 pg/mL. For AFB_1, our system achieved an even lower LOD than the ELISA kit tested (0.05 vs 1 ng/mL). The standard curves for the ELISA kit and the MNT-based detection of AFB_1 and zearalenone detection are shown in Figure 5.

Mycotoxin	Signal saturation (μV), after baseline correction			
	50 ng/mL	5 ng/mL	500 pg/mL	50 pg/mL
Aflatoxin B_1	11.10 ± 1.74	6.61 ± 0.57	3.50 ± 0.74	1.86 ± 0.59
Zearalenone	21.40 ± 0.95	12.88 ± 0.59	7.20 ± 2.01	5.16 ± 2.18

Table 1. Average magnetic signal saturation of AFB_1 and zearalenone detection.

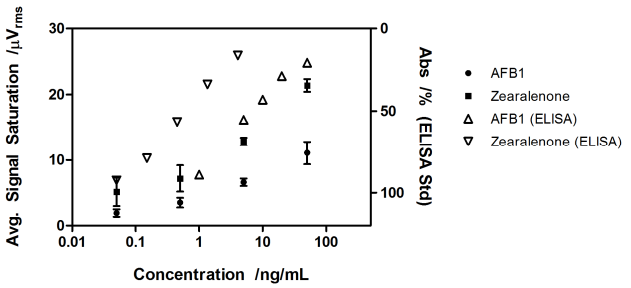

Figure 5. Comparison between standard curves from ELISA kit (○ and □) and the immuno-magnetic detection (● and ■) for AFB_1 and zearalenone detection.

4 CONCLUSION

By combining GMR sensing with the classic sandwich immunoassay, we have prototyped a new method that is capable of detecting the molecular interactions between the immobilized antibodies and target small molecules.

In this study, we have developed an innovative, ultra-sensitive magnetic nanoparticle immunoassay for mycotoxin detection that provides real-time quantitative results for multiple analytes. We successfully demonstrated that more than one mycotoxin can be readily detected with our MNT-based detection system. While the overall sensitivity of the system pivots on the specificity, selectivity, and binding affinity of the chosen antibodies, it is obvious that with the right antibody-analyte pairings, this platform can differentiate multiple mycotoxins in the same run. Our system has shown a LOD comparable to or better than conventional ELISA for AFB_1 and zearalenone, with a dynamic range of over 4 orders of magnitude.

With its ultra-high sensitivity, multiplexing capability and the simplicity of its detection scheme, the immuno-MNT assay is an excellent candidate for the adaptation to point-of-use testing not only for mycotoxin detection but also many proteomic applications.

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