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Sensitive Giant Magnetoresistive-based Immunoassay for Multiplex Mycotoxin Detection

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Abstract

Rapid and multiplexed measurement is vital in the detection of food-borne pathogens. While highly specific and sensitive, traditional immunochemical assays such as enzyme-linked immunosorbent assays (ELISAs) often require expensive read-out equipment (e.g. fluorescent labels) and lack the capability of multiplex 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 mycotoxins that functions on a sub-picomolar concentration level. Unlike fluorescent labels, magnetic nanotags (MNTs) can be detected with inexpensive giant magnetoresistive (GMR) sensors such as spin-valve sensors. In the system presented here, each spin-valve sensor has an active area of $90 \times 90 \mu\text{m}^2$, arranged in an 8×8 array. Sample is added to the antibody-immobilized sensor array prior to the addition of the biotinylated detection antibody. The sensor response is recorded in real time upon the addition of streptavidin-linked MNTs on the chip. Here we demonstrate the simultaneous detection of multiple mycotoxins (aflatoxins B₁, zearalenone and HT-2) and show that a detection limit of 50 pg/mL can be achieved.

Introduction

Mycotoxins, secondary metabolites of fungi, have received considerable attention over the past several years. Historically, mycotoxins have been a problem associated with the agricultural and food industries. Food lost due to fungal contamination not only causes substantial financial drain to the industries, it also poses significant health risks to humans and animals that consume contaminated feeds. Due to their resistance to temperature treatments within the range of conventional food-processing temperatures (Kabak 2009), mycotoxins have the tendency to remain in the human food chain in the form of the original toxins or their metabolites. Therefore

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it is of paramount importance for the industry to be able to identify the source of the problem at the earliest stage.

It has been documented that mycotoxins have a range of short-term detrimental effects on human health such as immune suppression, and they have also been linked to human hepatocellular carcinoma (Daly et al. 2000). No less than hundreds of fungal toxins have been identified thus far. However, a relatively small number are generally considered to play an important role in food safety (Shephard 2008). The most common types of fungal toxins that cause major health risks are produced by species with the genera *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* (van der Gaag et al. 2003).

Due to the widespread occurrence of fungal contamination in foodstuff and feeds, many efforts have been made towards the development of rapid and sensitive methods for mycotoxin detection. Traditionally, thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) have been employed for toxin detection. However, the tedious sample preparation and cleanup often led to inconsistent results and poor sensitivity (Daly et al. 2000).

Surface plasmon resonance (SPR), a technique that is frequently used to study molecular interactions, has been adapted for various sensing applications. It has been especially valuable in elucidating biospecific interaction analysis (Choi et al. 2009; Lee et al. 2006; Nabok et al. 2005; Shumaker-Parry et al. 2004; Wangkam et al. 2009). SPR continuously detects changes in the refractive index of the biorecognition layer on the sensor surface as a function of binding (Ferreira et al. 2009). The primary impact of SPR in this area is the ability to monitor the binding interactions of immuno-components in real-time. Another major advantage SPR has over other biosensing approaches is that the molecular interaction is monitored without the need for specialized and expensive labeling (Cunningham 1998; Hodnik and Anderluh 2009). The system has gained popularity in toxin detection with the commercialization of the SPR-based sensors by BIAcore (Hodnik and Anderluh 2009). Various research groups have employed the BIAcore system for applications such as inhibition immunoassays (Stubenrauch et al. 2009) and antibody affinity analysis (Reid et al. 2007). In their previous study, Schnerr et al. (2002) developed an inhibition immunoassay for the rapid quantification of the trichothecene mycotoxin deoxynivalenol using the BIAcore system. Despite its versatility, the complexity and the cost of the BIAcore instrumentation remain very high (Mullett et al. 1998).

Although SPR can detect a binding event of molecules as small as 200 Da, this requires highly sophisticated and expensive equipment (Skottrup et al. 2008). The low molecular weight of mycotoxins is often not enough to induce significant change in refractive index upon binding to the sensor surface. Consequently, an alternative assay strategy is required for mycotoxin detection using SPR. An extra step involving bioconjugation of target mycotoxin with high molecular weight carrier such as a bovine serum albumin (BSA) is often required to improve sensitivity (Vidal et al. 2009).

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 (Cunningham 1998). 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 (Skottrup et al. 2008). 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 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 (Meagher et al. 2008; Schulze et al. 2009; Skottrup et al. 2008; Warsinke 2009). In the present study we advance multiplex mycotoxin detection by integrating the classic sandwich-based immunoassay into a magnetic nanotag (MNT) detection platform. Here, we adapt MNT technology, which has previously been used to detect protein biomarkers in the >10 kDa range, to the detection of mycotoxins, whose much smaller size (<300 Da) and insolubility present unique challenges. Real-time measurements are conducted upon the addition of MNTs onto the spin-valve sensor surface immobilized with capture antibodies for mycotoxins (aflatoxin B₁, zearalenone and HT-2), mycotoxins, and detection antibodies. We examine the sensor's multiplexing capability and have demonstrated detection limits for mycotoxins in the range of pg/mL level. Our goal is to develop a sensitive and economical biosensing system for the rapid determination of relevant mycotoxins. We believe the assay system presented here has the capacity and potential to be developed into a cost-effective, point-of-use multiplexed mycotoxin test.

Experimental

Reagents

Monoclonal antibodies for aflatoxin-B₁ (Anti-AFB₁, clone AT-B1) and zearalenone (Anti-Zearalenone, clone ZER-70) were purchased from Sigma-Aldrich (Si. Louis, MO). Monoclonal antibody for HT-2 (Anti-HT-2, clone C6B4) was purchased from Advanced ImmunoChemical Inc. (Long Beach, CA). Mycotoxins aflatoxin-B₁ (AFB₁), zearalenone and HT-2 were acquired from Sigma-Aldrich, each with a minimum purity of 97% or higher. Polyallylamine, used to form the base layer of biofunctionalization on the sensor surface, was acquired from Polysciences, Inc. (Warrington, PA). Immobilization of capture antibody onto the sensor surface was performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) chemistry (Thermo Fisher Scientific, Rockford, IL). The biotinylation of antibodies for the mycotoxins was completed using EZ-Link[®] Sulfo-NHS Biotinylation Kit purchased also from Thermo Fisher Scientific. The streptavidin-coated magnetic labels were produced by Miltenyi Biotec Inc. (Auburn, CA). The overall diameter of the magnetic nanotags, which are comprised of a cluster of Fe₂O₃ superparamagnetic particles within a dextran matrix, is approximately 50nm. MNT stock solution was used in the final step of labeling without dilution. Ridascreen[®] ELISA kits for AFB₁ and zearalenone were purchased from R-Biopharm AG (Darmstadt, Germany). PBS solutions with 0.05% Tween-20 (v/v) at pH 7.4 were prepared using standard method. All chemicals purchased were of reagent grades or better without further purification. Aqueous reagents were prepared using Nanopure water with >18MΩ cm⁻¹ resistance.

Instrumentation

Spectrophotometric measurements for biotinylation verification were carried out on a Victor² Multilabel Counter (Perkin Elmer, Waltham, MA). The platform is a home-made station consisting of a Helmholtz Coil, magnetic biochip adaptor interfaced with LabView program on a PC. The fabrication of the chips and the detailed descriptions of the electronics were described previously (Osterfeld et al. 2008; Xu et al. 2008). The chips were then assembled onto a ceramic 84-pin chip carrier (LCC08423, Spectrum Semiconductor Materials, San Jose, CA). Each chip was then cleaned with an oxygen plasma treatment (PDC-32G, Harrick Plasma, Ithaca, NY) prior to surface functionalization.

Chip Fabrication

Each sensor consists of 32 linear segments of $1.5 \times 100 \mu\text{m}$ connected in series with equal spacing that span over an area of approximately $100 \times 100 \mu\text{m}^2$. 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 (Osterfeld et al. 2008). Each chip, consisting an 8×8 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.

Surface preparation

The chip is subjected to oxygen plasma treatment to remove organic residues adsorbed onto the surface. A 1% (w/v) polyallylamine 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 $0.4\text{-}\mu\text{L}$ droplets onto the sensor surface at a concentration of $500 \mu\text{g/mL}$. 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.

MNT-based Multiplex Mycotoxins Assays

In this multiplex assay, we used a classic sandwich immunoassay approach. Capture antibodies were immobilized on different part of the GMR sensor surface. After the surface functionalization overnight, the chips were rinsed to remove the residual capture antibodies. A BSA blocking buffer (3% w/v BSA, 0.05 Tween-20 dissolved in PBS) was added into the reaction well for 1 h at room temperature to minimize the number of unbound active sites. Samples were prepared by dissolving and diluting all target mycotoxins into a single solution. Due to their extremely low solubility in water, the mycotoxins were first dissolved in pure methanol separately as a 10X stock. The final sample consists of all the target analytes involved with a total solvent content of 10% methanol + 90% PBS. The incubation period with the sample was 1 h at room temperature. After the removal of the analyte solution, biotinylated antibodies were added into the reaction reservoir and allowed to incubate for 1 h. Prior to the measurement, the excess detection antibodies were siphoned off and the reservoir was rinsed with PBS several times. Measurement began when the chip surface is free of solution, to ensure chip stability and to establish signal baseline. A slight shift of baseline was occasionally observed due to the wet/dry transitions and was largely negligible compared to the magnitude of the signals of interest. Signal usually stabilizes after 1 min. Once a signal baseline was established, $50 \mu\text{L}$ of undiluted streptavidin-coated MNT solution was added to the reaction well. The system remained unstirred for the duration of the measurement at room temperature. The measurements were terminated when the signal plateaued, usually in less than 20 min. Figure 1 depicts a schematic of the measurement upon the addition of MNTs.

Results and Discussion

As test articles, we chose 3 common mycotoxins: aflatoxin B_1 , 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 (Holtzappple et al. 1996; Hsu and Chu 1994; Yuan et al. 1997) and high quality antibodies are commercially available. Figure 1 also shows a typical MNT binding curve from the assay with a single probe. 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. The moment when MNT solution is added to the reaction reservoir is defined as $t = 0$. The binding event of MNT to the biotinylated detection antibodies took place immediately upon contact and was recorded in real time (Fig. 1b). It is important to note that the signal reflects the streptavidin-avidin binding kinetics, rather than antibody-toxin binding kinetics. 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.

Specificity

A chip that was functionalized with anti-AFB₁, anti-zearalenone, anti-HT-2 and BSA was incubated with a sample solution containing only AFB₁ (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-AFB₁. The average signal saturates at approximately 12 μ V after 12 min. Meanwhile the other negative control sensors, anti-zearalenone, anti-HT-2 and BSA, which were not expected to show interaction with AFB₁, gave a negligible signal. The result verifies the specificity of the antibodies and shows that the system does not suffer electronic cross-talk problems.

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 AFB₁, 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. 3a). At 33.3 ng/mL, the positive sensors all displayed typical binding kinetics and three distinctive signal intensities upon the addition of MNTs. At very low toxin concentrations (333 pg/mL, Fig. 3b), the signal averages were observably lower than the previous experiment. Indeed, the signal average from HT-2 became indistinguishable from that of the negative control (BSA), suggesting that the detection limit for HT-2 during this multiplex measurement had been reached. 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.

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 AFB₁ 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.

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

AFB₁, ranging from 50 ng/mL to 50 pg/mL. As the results indicate, the concentration of AFB₁ 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 AFB₁ and zearalenone. At the lowest concentration tested (50 pg/mL), AFB₁ yields an average signal of $1.86 \pm 0.59 \mu V$. 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 (Osterfeld et al. 2008). 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 AFB₁, 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 AFB₁ and zearalenone detection are shown in Figure 5.

We have also tested the analytes at concentrations above 50 ng/mL. For higher concentrations (data not shown), the sensors did not show significant increase in signal although it has been demonstrated previously that our platform can detect magnetic signal intensity up to 100 μV (Osterfeld et al. 2008). We speculate that this is the result of relatively low surface immobilization density after the sensor functionalization step. The drop in number of available binding sites for the target analytes inevitably leads to quicker saturation of antibody-toxin binding, thus limiting the upper range of detection. Theoretically then, the upper range of concentration can be increased with more optimized and efficient surface immobilization, further expanding the dynamic range of the assay.

Conclusion

Mycotoxin contamination in food industry carries not only economic burden but also associated health concerns. For this reason, it would be extremely beneficial to have a point-of-use detection assay that provides sensitive, rapid, and inexpensive determination of the presence or absence of mycotoxins. By combining GMR sensing with the classic sandwich immunoassay, we have prototyped a new method that can ultimately realize industry and public health goals.

Given the diversity of mycotoxins, multiplex detection remains one of the biggest challenges for researchers. 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. Thus far, our system has shown a LOD comparable to or better than conventional ELISA for AFB₁ and zearalenone, with a dynamic range of over 4 orders of magnitude. Moreover, we believe that the dynamic range can easily be expanded with further exploration of surface functionalization.

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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Figure 1.

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

a) Signal baseline after the rinsing and the subsequent removal PBS solution; b) binding kinetics upon the addition of MNTs, defined as $t = 0$; and c) signal saturation achieved when the MNTs have saturated the available binding sites.

Figure 2.

Cross-reactivity study of anti-AFB₁, anti-zearalenone and anti-HT-2. Sensors in an 8 × 8 array on the chip were immobilized with the 3 antibodies and BSA (4 – 9 sensors for each functionalization). During the analyte incubation, only 100 ng/mL AFB₁ was added, whereas all 3 detection antibodies were present prior to the addition of MNTs. Only the sensors modified with anti-AFB₁ (6 sensors) recorded magnetic signal.

Figure 3a

Figure 3b

Figure 3. Multiplex detection for AFB₁, zearalenone and HT-2 at a) 33 ng/mL concentration and b) 330 pg/mL.

Figure 4. Magnetic signal of AFB₁ detection as a function of concentrations, ranging from 50 pg/mL to 50ng/mL, spanning 4 orders of magnitude.

Figure 5. Comparison between standard curves from ELISA kit ($\circ$ and $\square$) and the immuno-magnetic detection ($\bullet$ and $\blacksquare$) for AFB₁ and zearalenone detection. Results show a dynamic range for the platform of at least 4 orders of magnitude with respectable linear range with the proposed system.

Table 1

Average magnetic signal saturation of AFB₁ and zearalenone detection.

Mycotoxin	Signal saturation (μV), after baseline correction			
	50 ng/mL	5 ng/mL	500 pg/mL	50 pg/mL
Aflatoxin B ₁	11.10 $\pm$ 1.74	6.61 $\pm$ 0.57	3.50 $\pm$ 0.74	1.86 $\pm$ 0.59
Zearalenone	21.40 $\pm$ 0.95	12.88 $\pm$ 0.59	7.20 $\pm$ 2.01	5.16 $\pm$ 2.18