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Sensitive competitive immunoassay of multiple mycotoxins with non-fouling antigen microarray

Weihua Hu [a,b,1], Xin Li [c,1], Guangli He [a,b], Zhaowei Zhang [c], Xinting Zheng [a,b], Peiwu Li [c,*], Chang Ming Li [a,b,**]

[a] Institute for Clean Energy & Advanced Materials, Southwest University, Chongqing 400715, PR China
[b] Chongqing Key Laboratory for Advanced Materials and Technologies of Clean Energies, Chongqing 400715, PR China
[c] Key Laboratory of Biology and Genetic Improvement of Oil Crops, Key Laboratory of Detection for Mycotoxins, Ministry of Agriculture, Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, PR China

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ABSTRACT

Various mycotoxins with strong carcinogenesis and toxicity are fatal threats in food safety, and require highly sensitive and high-throughput detections greatly. Herein a fluorescent competitive immunoassay microarray based on a non-fouling polymer brush, poly[(ethylene glycol) methacrylate-*co*-glycidyl methacrylate] (POEGMA-*co*-GMA) is explored to sensitively detect multiple mycotoxins with aflatoxin B1 (AFB1), ochratoxin A (OTA) and zearalenone (ZEN) as template targets. Due to uniformly large protein loading and high resistance to nonspecific protein absorption of the POEGMA-*co*-GMA brush, the optimal microarray exhibits wide dynamic ranges of three orders of magnitudes and low detection limits of 4, 4 and 3 pg mL^{-1}, respectively, which is much better than that obtained with an epoxy-functionalized antigen microarray, and is comparable or even better than the conventional ELISA method. This work offers a powerful high-throughput tool to fast screening of toxins in food quality and environmental monitoring.

1. Introduction

Mycotoxins are a series of secondary metabolites produced by fungi (mainly *Aspergillus* and *Fusarium*) and are known as main and fatal contaminants in food, feed and environment. (Lee et al., 1980; Visconti et al., 2005; Frenich et al., 2009; Li et al., 2009a, 2009b; Zhang et al., 2011) Most mycotoxins are extremely toxic, carcinogenic, teratogenic and mutagenic compounds. For example, aflatoxin B1 (AFB1) is listed as a Group 1 carcinogen by the International Agency for Research in Cancer as it may inhibit the synthesis of RNA and interfere the inductive of specific enzymes, thus being considered as a causative agent in human hepatic and extrahepatic carcinogenesis. (Daly et al., 2000) Ochratoxin A (OTA) is classified as possibly carcinogenic one in human (Group 2B) and may lead to many diseases such as hepatic intumescences, renal intumescences and enteritis. (Pfohl-Leszkowicz and Manderville, 2007) Zearalenone (ZEN) could induce an estrogenic effect to damage reproduction of the mammal. (Reddy et al., 2010) Moreover, as thermally stable molecules, mycotoxins could remain in the food production and marketing chain in a form of their original toxins or metabolites to fatally threat the health of human and animals. Therefore it is very important to sensitively high-throughput detect various mycotoxins in food, feeds and related productions for the health of human being worldwide (Kabak, 2009; García-Cañas et al., 2012).

Chromatography techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) coupled with fluorescence detector or mass spectrometry (MS) have been used for mycotoxin analysis. HPLC is recommended as the standard detection method of mycotoxins by the International Organization for Standardization due to its excellent accuracy and high sensitivity.(Kuoa et al., 2002; Ren et al., 2007; Devreese et al., 2012; Ferreira et al., 2012; Njumbe Ediage et al., 2012) However, it requires time-consuming sample pretreatments, bulky and expensive instruments and skillful operations, thus impairing its applications for rapid and especially on-site detection for fast quality screening of food products. Compared to HPLC, enzyme-linked immunosorbent assay (ELISA) relying on the specific affinity between toxin target and its antibody has emerged as a valuable alternative for mycotoxin detection (Kolosova et al., 2005; Liu et al., 2008; Li et al., 2009a, 2009b; Maragos, 2009). It allows quantitatively detecting mycotoxins with good sensitivity and some ELISA kits have been commercially available. However, it also requires clean-lab condition and skillful personnel, and most importantly, it is lack of high-throughput ability for rapid multiplexed detection. To facilitate fast and on-site screening, some lateral flow immunoassay

* Corresponding author. Tel: +8627 86812943.
** Corresponding author at: Southwest University, Institute for Clean Energy & Advanced Materials, 70 Nanyang Drive, Chongqing 400715, China. Tel./fax: +8623 68254969.
E-mail addresses: ecmli@swu.edu.cn, changming12@yahoo.com (C.M. Li), peiwuli@oilcrops.cn (P. Li).
[1] Weihua Hu and Xin Li have contributed equally to this work.

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(LFI) strips and electrochemical immunosensors have been developed with simple sample pretreatment, (Ho and Wauchope, 2002; Pal and Dhar, 2003; Parker et al., 2009; Zhang et al., 2011; Preechakasedkit et al., 2012) but they can only provide qualitative or semi-quantitative detection, and are not a high-throughput approach.

Protein microarray as a renowned high-throughput tool for multiplexed immunoassay by printing various molecular recognition elements on a substrate in an addressable manner has attracted great research interest in recent years.(Zhu and Snyder, 2001; Templin et al., 2002; Hu et al., 2010a, 2010b, 2010c, 2011; Liu et al., 2011) As mycotoxins are small molecules with molecular weights less than 1000, their immunoassay detections mainly rely on a competitive format rather than a sandwich one, which intrinsically cause great difficulties to fabricate a highly sensitive microarray (de Champdore et al., 2007; Li et al., 2009a, 2009b; Shlyapnikov et al., 2012; Li and Hu, 2013). In competitive immunoassay, the target competes with the analog antigen attached on a substrate to bind with the reporting antibody in a certain concentration, and the target is quantified by the amount of antibody bound on the substrate-attached antigen, thus leading to detected signal inversely proportional to the target concentration (Sauceda-Friebe et al., 2011). Up to date due to the detection sensitivity sacrificed from the competitive part, the performance of the competitive immunoassay microarray still demands significant improvements to satisfy its practical applications. An antigen microarray based on commercial agarose-modified substrate has been developed to simultaneously detect multiple mycotoxins, but it only achieves a detection limit of tens pg mL^{-1} to several ng mL^{-1} mycotoxins.(Wang et al., 2012) A displacement-based microarray has been also studied to detect small patulin molecules but only a detection limit of 10 ng mL^{-1} is realized (de Champdore et al., 2007). In order to achieve high sensitivity and broad detection range to high-throughput analyze small target toxins, an antigen microarray should possess high probe loading capacity, low spot-to-spot signal variation and low nonspecific protein absorption for high signal-to-noise ratio. At the same time, monoclonal antibodies with high affinity and excellent specificity are also very critical in multiplexed microarray detection of mycotoxins.

In this work, a non-fouling antigen competitive immunoassay microarray based on a polymer brush is reported to detect multiple mycotoxins by utilizing highly specific monoclonal antibodies of mycotoxins produced in our laboratory. The polymer brush, poly [(ethylene glycol) methacrylate-*co*-glycidyl methacrylate] (POEGMA-*co*-GMA) is synthesized via surface-initiated atom transfer radical polymerization (SI-ATRP) on standard glass slides (75×25 mm^2) according to our previous works (Hu et al., 2010a, 2010b, 2010c; Liu et al., 2011). Abundant poly(ethylene glycol) (PEG) side chains in the brush are able to ultimately suppress the nonspecific protein adsorption in solution while the epoxy groups of glycidyl methacrylate (GMA) residues provide covalent binding sites for spotted proteins in a dry environment (Hu et al., 2010a, 2010b, 2010c; Liu et al., 2011). The polymer brush shows high while uniform protein loading and high resistance to nonspecific protein absorption for highly sensitive immunoassay. As a high-throughput demonstration, AFB1, OTA and ZEN are selected as template targets for simultaneous detections with the designed microarray.

2. Experimental

2.1. Chemicals and materials

All chemicals were of analytical reagent grade or higher from Sigma-Aldrich (St. Louis, MO, USA). Deionic (DI) water from a Millipore Milli-Q system was used in all experiments. Mycotoxins including AFB1, OTA, and ZEN and their antigens including AFB1-BSA (bovine serum albumin) conjugate and OTA-BSA conjugate were also obtained from Sigma-Aldrich. ZEN-BSA conjugate came from Aokin (Germany). Cy3-labeled Goat anti-Mouse IgG was purchased from Invitrogen.

2.2. Production of monoclonal antibodies

Monoclonal antibodies including anti-AFB1, anti-ZEN and anti-OTA were produced in one of our authors' laboratories with a hybridorma antibody technology (Zhang et al., 2009; Li et al., 2012). In production, antigens (AFB1-BSA, OTA-BSA and ZEN-BSA) were multiple-site subcutaneously injected into Balb/c mouse separately and four immunizations were carried out to select the hybridomas. In the hybridoma selection process, a two-step ELISA screening procedure was performed to select hybridomas with high sensitivity and good specificity. The hybridoma cells were then intraperitoneally injected into Freund's incomplete adjuvants (FIA) treated Balb/c mice to prepare ascites, which were used to produce monoclonal antibodies.

2.3. Preparation of POEGMA-co-GMA polymer brush modified slides

Standard glass slides from Sigma were ultrasonically cleaned in 0.1 M potassium hydroxide (KOH) solution, DI water, and ethanol for 10 min, respectively. After drying with compressed air, the clean slides were incubated in 5% (v/v) (3-aminopropyl)triethoxysilane (APTES) ethanol solution for 2 h at room temperature, followed by rinsing with ethanol. The APTES modified slides were then subjected to 110 °C thermal curing in a vacuum oven for 2 h, followed by incubation with ice-cold tetrahydrofuran (THF) solution containing initiator α-bromoisobutyryl bromide (BIB, 64 μL per 10 mL) and triethylamine (TEA, 77 μL per 10 mL), for 2 h to attach the initiator, followed by thorough washing with THF. The POEGMA-*co*-GMA polymer brush was then grown with a SI-ATRP technique by immersing the initiator-attached slides in the deoxygenized methonal/H_2O (1:1) growth solution, which contains 20% v/v oligo(ethylene glycol) methacrylate (OEGMA, Mn = 360) and 0.5% v/v glycidyl methacrylate (GMA) with 2.9 mg mL^{-1} CuBr and 6.25 mg mL^{-1} 2,2′-Bipyridine (BiPY). The polymerization was continued for 6 h in an inert atmosphere at room temperature and then the slides were removed from the growth solution. After intensive washing with ethanol and DI water, the slides were dried by gentle nitrogen flow and stored at 4 °C for subsequent usage.

2.4. Printing of antigen microarrays

Three mycotoxin antigens (AFB1-BSA, OTA-BSA and ZEN-BSA) were respectively spotted on a POEGMA-*co*-GMA polymer brush-modified slide to form three 4×4 subarrays by a VersArray Chipwriter™ Compact System (Bio-Rad) equipped with stealth microspotting pins (SMP3). BSA subarray was also printed as a negative control subarray on the slide. The spotted slide was then stored in a dry cabinet at room temperature for overnight incubation, followed by washing with 0.01 M PBS to remove unattached antigens. After drying under a gentle nitrogen flow, the slides were ready for use. A batch of such slides was prepared for evaluation of reproducibility and reliability.

2.5. Optimization of antigen and antibody concentrations

The detection was based on an indirect competitive immunoassay format as schematically shown in Fig. 1. The microarray slide was firstly incubated in a solution containing the corresponding monoclonal antibodies. A fluorescent secondary antibody (Cy3-labeled anti-Mouse IgG) was further used to bind with the bound monoclonal antibodies and fluorescent images of the slide were measured with 543 nm excitation by a Proscanarray