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Sensitive and rapid detection of aflatoxin M1 in milk utilizing enhanced SPR and p(HEMA) brushes

Aleksandra Karczmarczyk [a,c,*], Monika Dubiak-Szepietowska [a], Mariia Vorobii [d], Cesar Rodriguez-Emmenegger [d,e], Jakub Dostálek [b], Karl-Heinz Feller [a]

[a] Department of Medical Engineering and Biotechnology, Ernst-Abbe-University of Applied Sciences Jena, Carl-Zeiss Promenade 2, 07745 Jena, Germany
[b] BioSensors Technologies, AIT Austrian Institute of Technologies GmbH, Muthgasse 11, 1190 Wien, Austria
[c] Institute of Analytical Chemistry, University of Regensburg, Josef-Engert Straße, 93053 Regensburg, Germany
[d] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovsky Sq. 2, 16206 Prague, Czech Republic
[e] DWI – Leibniz Institute for Interactive Materials and Institute of Technical and Macromolecular Chemistry, RWTH Aachen University, Forckenbeckstraße 50, 52074 Aachen, Germany

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ABSTRACT

The rapid and sensitive detection of aflatoxin M_1 (AFM_1) in milk by using surface plasmon resonance (SPR) biosensor is reported. This low molecular weight mycotoxin is analyzed using an indirect competitive immunoassay that is amplified by secondary antibodies conjugated with Au nanoparticles. In order to prevent fouling on the sensor surface by the constituents present in analyzed milk samples, an interface with poly(2-hydroxyethyl methacrylate) p(HEMA) brush was employed. The study presents a comparison of performance characteristics of p(HEMA)-based sensor with a regularly used polyethylene glycol-based architecture relying on mixed thiol self-assembled monolayer. Both sensors are characterized in terms of surface mass density of immobilized AFM_1 conjugate as well as affinity bound primary and secondary antibodies. The efficiency of the amplification strategy based on Au nanoparticle is discussed. The biosensor allowed for highly sensitive detection of AFM_1 in milk with a limit of detection (LOD) as low as 18 pg mL^{-1} with the analysis time of 55 min.

1. Introduction

Aflatoxins are a family of extremely toxic and carcinogenic secondary metabolites (mycotoxins) secreted by certain species of *Aspergillus*. In particular, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus* contaminate a large variety of food and feed commodities (Creppy, 2002). Aflatoxin M_1 (AFM_1) is the hydroxylated derivative of aflatoxin B_1 (AFB_1) originating from the activity of cytochrome P450-associated enzyme in liver. It is excreted into the milk of both human and animals that have been fed with AFB_1 polluted diet (Polychronaki et al., 2007; Fallah, 2010). About 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk (Unusan, 2006). This compound elicits a wide spectrum of toxicological and carcinogenic effects causing liver cirrhosis, tumors or liver damage of human as well as animals (Canton et al., 1975; Deshpande, 2002; Zhang et al., 2013). The International Agency for Research on Cancer (IARC) recently reconsidered its carcinogenicity categorization, initially classified as a Group 2B human carcinogen, and changed it to Group 1 (Lyon, 2014). Despite the fact that milk and dairy products, such as cheese and yogurt are an important source of many essential nutrients like proteins, magnesium, calcium or vitamins B12 and A, unfortunately, they are also the most potent providers of AFM_1 among foods. Due to the relative stability during heat treatments (*e.g.* pasteurization) and significant threat to human health, especially to children who are the major consumers of milk, many countries have implemented regulations to control the content of AFM_1 in daily products (Galvano et al., 1996). The European Commission stipulates a maximum permissible level of 50 ng L^{-1} for AFM_1 in milk and dried or processed milk products (Regulation, 2006). In China and United States the regulations mandate AFM_1 levels below 500 ng L^{-1} (US Food and Drug Administration, 1996).

Sampling and analysis of mycotoxins are regulated by the European Commission Directives. The stipulated methods include high performance liquid chromatography with fluorimetric detection (HPLC-FD) coupled with the pre-cleaning by immunoaffinity columns (IF) (Radoi et al., 2008). This procedure

* Corresponding author at: Department of Medical Engineering and Biotechnology, Ernst-Abbe-University of Applied Sciences Jena, Carl-Zeiss Promenade 2, 07745 Jena, Germany.

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relies on extensive sample preparation and the analysis requires couple of hours. Other, currently performed, techniques such as thin-layer chromatography (TLC) (Sassahara et al., 2005; Kamkar, 2006) or enzyme-linked immunosorbent assay (ELISA) (Pe et al., 2009) are also time consuming and require highly trained personnel, expensive equipment deployed in specialized laboratories. In order to simplify the analysis of mycotoxins, research is carried out to provide faster and sensitive techniques suitable for routine assay of milk and other dairy products. Over the last years we witnessed efforts to expedite AFM_1 detection based on immunoassays combined with fluorescence (Sapsford et al., 2006), electrochemistry (Micheli et al., 2005), colorimetry (Garden and Strachan, 2001) or chemiluminescence (Magliulo et al., 2005) readout.

A promising alternative to fluorescence assays are label-free biosensors based on surface plasmon resonance (SPR) (Homola, 2008). This technology exploits the measurement of changes in the reflective index occurring upon the affinity binding of molecules in the proximity of a metallic surface. As the response of SPR sensor is proportional to the mass of target molecule, direct detection of small molecules, such as mycotoxins, and/or analytes at very low concentration is challenging. Therefore, alternative assay formats are required for mycotoxin detection by using SPR. In order to enhance the sensor response, it was utilized a competition for binding to the surface between an antigen conjugated with a high molecular weight label and the unlabeled sample antigen. An alternative way is to immobilized the same molecule —antigen— which will be measured to the sensor surface, followed by injection of the primary antibodies and sample containing free antigen mixture. In this latter case, the signal can be further amplified by using the secondary antibodies labeled with metallic nanoparticles (NPs), magnetic nanoparticles (MNPs), fluorophores or quantum dots (QDs) (Wang et al., 2009, 2011; Campione and Capolino, 2011; Hu et al., 2014). Recently, several studies have reported the signal amplification by the implementation of gold nanoparticles (AuNPs). It can be designed to harness several effects including enhanced surface area, refractive index changes by the particles mass, and electromagnetic field coupling between the plasmonic properties of the particles and propagating plasmons (Mitchell et al., 2005; Szunerits et al., 2014).

Another challenging aspect in SPR analysis of complex samples such as milk and dairy products is the non-specific interaction at the surface that is associated with the deposition of non-targeted molecules or entities. SPR biosensors require an interface design for anchoring specific bioreceptors that is at the same time resistant to non-specific adsorption (Rodriguez-Emmenegger et al., 2011). To overcome the problem with fouling, different strategies of surface modification were proposed: grafting of carboxymethyl dextran (O'Shannessy et al., 1992), passivation with albumin (Homola et al., 2002) or a relatively new, however, very promising modification with various types of non-fouling polymer brushes. Such surface architecture has been shown to provide significantly higher fouling suppression (Vaisocherová et al., 2008; Rodriguez-Emmenegger et al., 2011; Kuzmyn et al., 2014; de los Santos Pereira et al., 2015), demonstrating their applications in a real-world biosensing (Vaisocherová et al., 2009; Brault et al., 2010; Rodriguez-Emmenegger et al., 2011; Hu et al., 2014). Considering milk analysis, it has been reported remarkably ultra-low fouling properties of antibody functionalized poly(2-hydroxyethyl methacrylate) p(HEMA) for the direct detection of *Cronobacter* in milk (Rodriguez-Emmenegger et al., 2011).

In this work, we describe an SPR biosensing for rapid, sensitive and specific detection of low molecular weight analyte AFM_1 in complex milk samples. The resistance to non-specific adsorption from milk was assessed by using the p(HEMA) brushes and the assay performance was compared to recent state-of-the-art antifouling polyethylene glycol (PEG) moieties. An indirect competitive immunoassay was developed for the analysis of low molecular analyte and the amplification of the sensor response by using secondary antibodies with metallic nanoparticles labels.

2. Materials and methods

2.1. Reagents

All reagents were used as received without further purification. Dithiol PEG6-COOH and dithiol PEG3-OH were purchased from SensoPath Technologies. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Pierce (USA). *N*,*N*-dimethylformamide (DMF, 99.8%), 4-(dimethylamino) pyridine (DMAP), *N*,*N*'-disuccinimidyl carbonate (DSC), aflatoxin M_1 (AFM_1), the conjugate of AFM_1 with bovine serum albumin ($BSA–AFM_1$), PBS buffer tablets and Tween 20 were from Sigma-Aldrich. The primary rabbit antibody against AFM_1 (Ab_1) was from AntiProt and gold nanoparticles (AuNPs, 20 nm)-labeled goat anti-rabbit secondary antibody ($Ab_2–AuNPs$) from Abcam. The experiments were performed in PBS-Tween buffer (PBS-T) (pH 7.4) prepared by adding Tween 20 (0.05%) in PBS buffer solution. 20 mM acetate buffer (ACT, pH 4) was prepared from sodium acetate trihydrate and acetic acid (both from Sigma-Aldrich) and the pH was adjusted by HCl and NaOH. Glycine buffer with pH 1.5 and ethanolamine were purchased from Biocore (Germany). The ERM (European Reference Material) BD282 (zero level of AFM_1) was obtained from the Institute for Reference Materials and Measurements (Belgium).

2.2. Optical setup

An optical SPR biosensor setup utilizing angular spectroscopy of surface plasmons (SPs) was used. A transverse magnetically (TM) polarized beam with a wavelength of $\lambda = 632.8$ nm emitted from a HeNe laser (2 mW) was coupled to a right angle LASFN9 glass prism. To the prism base, a sensor chip was optically matched by using immersion oil. The sensor chip was prepared on the top of a BK7 glass substrate that was coated by sputtering (UNIVEX 450C form Leybold, Germany) with 41 nm thick gold layer. Then, the gold surface was either modified by a bicomponent SAM of thiols or polymer brushes for subsequent covalent immobilization of $BSA–AFM_1$ conjugates. A transparent flow-cell with a volume of approximately 10 μL was pressed against the surface of the sensor chip in order to flow liquid samples over the sensor surface by using a peristaltic pump at a flow rate of 500 μL min^{-1}. The intensity of the laser beam reflected from the sensor surface was measured using a photodiode detector. The resonant coupling to the SP is manifested as a resonance dip in the angular reflectivity spectrum $R(\theta)$. The binding of molecules to the gold layer was observed as a shift in the reflectivity dip, $\Delta\theta$, and evaluated by fitting with a transfer matrix-based model implemented in the software Winspall (developed at the Max Planck Institute for Polymer Research in Mainz, Germany). The whole sensor system and the supporting electronics were controlled by using the customized software Wasplas.

Since the refractive index is a linear function of concentration over a wide range of concentrations, the absolute amount of biomolecules bound at the surface (Γ) can be calculated using Feijter's formula (De Feijter et al., 1978):

$$\Gamma = \frac{(n_h - n_b)d_h}{\partial n/\partial c} \quad (1)$$

where n_h and n_b are the refractive indices of a protein monolayer ($n_h = 1.5$) and a buffer, respectively. The factor of $\partial n/\partial c = 0.18$ cm^3 g^{-1}