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Short communication

Long range surface plasmon-enhanced fluorescence spectroscopy for the detection of aflatoxin M_1 in milk

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ABSTRACT

A novel biosensor for the highly sensitive detection of aflatoxin M_1 (AFM_1) in milk was developed. This biosensor is based on surface plasmon-enhanced fluorescence spectroscopy (SPFS) which was advanced through the excitation of long range surface plasmons (LRSPs). In SPFS, the binding of fluorophore-labeled molecules to the sensor surface is probed with surface plasmons (SPs) and the emitted fluorescence light is detected. This approach takes advantages of the enhanced intensity of electromagnetic field occurring upon the resonant excitation of SPs which directly increases the fluorescence signal. For the detection of AFM_1, LRSP-enhanced fluorescence spectroscopy was combined with an inhibition immunoassay in which a derivative of AFM_1 was immobilized on the sensor surface and antibodies against AFM_1 were used as recognition elements. The developed biosensor allowed for the detection of AFM_1 in milk within 53 min at concentrations as low as 0.6 pg mL^{-1}. The achieved limit of detection was about two orders of magnitude lower than the maximum AFM_1 residue level in milk stipulated by the European Commission legislation.

1. Introduction

Aflatoxins are a class of mycotoxins produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* which grow in a number of agricultural products. Aflatoxin M_1 (AFM_1) is the hydroxylated metabolite of aflatoxin B_1 (AFB_1) and can be found in urine, blood, milk, and internal organs of animals that have ingested AFB_1-contaminated feed (Shreeve et al., 1979). Due to its hepatotoxic and carcinogenic effects (Badea et al., 2004) and the relative stability during pasteurization or other thermal treatments (Govaris et al., 2001), control measurements were established. For instance, the European Commission stipulates the maximum level of 50 pg mL^{-1} for AFM_1 in milk (Commission Regulation, 2001).

Currently, routine analysis of aflatoxin-contaminated samples is mostly performed by high-performance liquid chromatography (HPLC) (Bognanno et al., 2006), thin-layer chromatography (TLC) (Kamkar, 2006), and enzyme-linked immunosorbent assay (ELISA) (Rastogi et al., 2004). However, these techniques require highly trained personnel in specialized laboratories, extensive preparation steps and they are time-consuming. Therefore, research has been carried out to simplify and expedite the detection of aflatoxins over the last years. The majority of novel approaches relies on immunoassays combined with electrochemical (Ammida et al., 2004), scanning densitometry (Ho and Wauchope, 2002), colorimetric (Garden and Strachan, 2001), chemiluminescent (Magliulo et al., 2005), fluorescence (Sapsford et al., 2006) and surface plasmon resonance (Daly et al., 2000) transducers.

Biosensors based on surface plasmon resonance (SPR) are gaining increasing popularity for the detection of chemicals and biological species (Homola, 2008). In these devices, the specific capture of target molecules contained in a liquid sample by biomolecular recognition elements anchored to the metallic sensor surface is probed with surface plasmons (SPs). The binding of target molecules induces an increase in the refractive index on the sensor surface which can be directly measured by spectroscopy of SPs. However, for the detection of extremely low analyte concentrations or small molecules, the induced refractive index changes are too low to be measured directly. In order to improve the sensitivity of the molecular binding detection, SPR biosensors have been combined with fluorophore labeling in a new method referred as to surface plasmon-enhanced fluorescence spectroscopy (SPFS) (Liebermann and Knoll, 2000). In SPFS, fluorophore-labeled molecules captured on the sensor surface are excited with surface plasmons (SPs) and the emitted fluorescence light is measured. This approach takes advantages of the enhanced intensity of the electromagnetic field occurring upon the resonant excitation of SPs which greatly increases the strength of the fluorescence signal.

Recently, long range surface plasmons (LRSPs) were introduced to SPR-based biosensors (Nenninger et al., 2001; Wark et al., 2005; Dostalek et al., 2007). LRSPs are special surface plasmon modes which propagate along a thin metal film suspended between two

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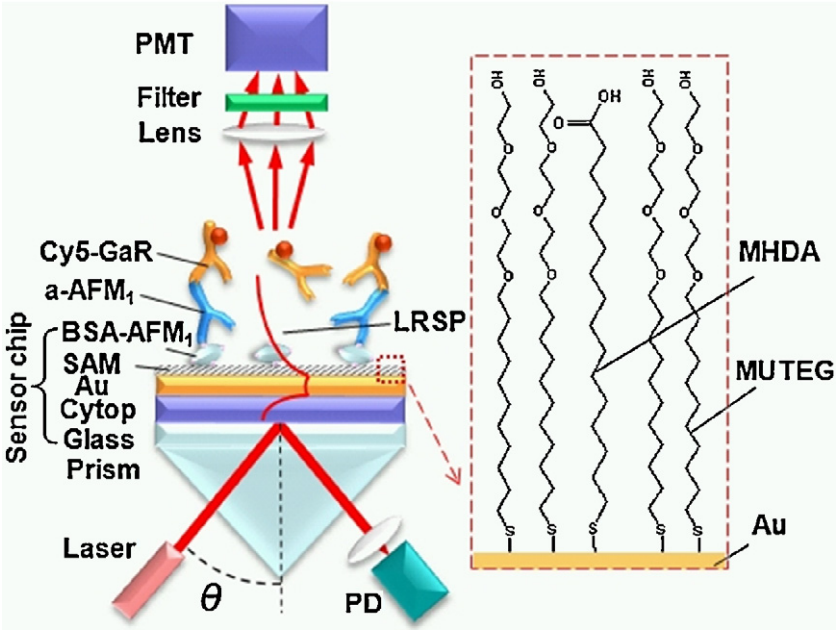

Fig. 1. The schematic drawing of the setup utilizing LRSP-enhanced fluorescence spectroscopy and the interfacial molecular architecture for the detection of AFM_1 by an inhibition immunoassay.

dielectrics with similar refractive indices (Sarid, 1981). Compared to conventional SPs, LRSPs exhibit a greatly reduced damping and thus their excitation is accompanied with a stronger enhancement of the electromagnetic field intensity. This feature enables to further increase the fluorescence signal in SPFS-based detection (Dostalek et al., 2007). In this communication, we report the implementation of LRSP-enhanced fluorescence spectroscopy in an immunoassay-based biosensor for the highly sensitive detection of AFM_1 in milk samples.

2. Materials and methods

2.1. Materials

All reagents were used as received without further purification. 16-Mercaptohexadecanoic acid (MHDA, 90%), (11-mercaptoundecyl)tri(ethylene glycol) (MUTEG, 95%), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU, ≥98.0%), *N*,*N*-dimethylformamide (DMF, 99.8%), aflatoxin M_1 (AFM_1), and the conjugate of AFM_1 with bovine serum albumin (AFM_1–BSA) were purchased from Sigma–Aldrich (Steinheim, Germany). The monoclonal rat antibody against AFM_1 (a-AFM_1, class IgG2b) and Cy5-labeled goat anti-rat antibody (Cy5-GaR, approximately 10.2 dyes per antibody) were obtained from Acris Antibodies GmbH (Herford, Germany). Glycine buffer with a pH of 1.5 and ethanolamine were purchased from Biacore (Freiburg, Germany). Tween 20 was purchased from Serva GmbH (Heidelberg, Germany). The experiments were performed in phosphate-buffered saline at pH 7.4 containing 0.05% Tween 20 (PBS-T). Samples with the composition approximating that of whole milk were prepared by dissolving a milk powder (fat content of 30%) in deionized water at the concentration of 0.1 g mL^{-1}.

2.2. Sensor implementation

In the experiment, we used a sensor instrument for the excitation of LRSPs and for the detection of fluorescence light as described previously (Dostalek et al., 2007). The instrument utilizes the angular spectroscopy of LRSPs in the total attenuated reflection (ATR) method of the Kretschmann configuration. As seen in Fig. 1, a transverse magnetically (TM) polarized beam from a HeNe laser (λ = 632.8 nm) was coupled to a LASFN9 glass prism. Onto the prism base, a BK7 glass sensor chip with a layer structure supporting LRSPs was optically matched using immersion oil. This layer structure consisted of a low refractive index buffer layer (Cytop, refractive index and thickness of n = 1.340 and d = 800 nm, respectively) and a gold film on the top (thickness of d = 20 nm). To the sensor chip, a transparent flow-cell with the volume of approximately 12 μL was attached. Aqueous samples (with a refractive index close to n = 1.333) were pumped through the flow-cell using a peristaltic pump at the flow rate of 0.5 mL min^{-1}. The volume of analyzed samples which circulated within the fluidic system was V = 800 μL. The fluorescence light emitted from the sensor surface was collected through the flow-cell by a lens, passed through a band-pass filter (transmission wavelength of λ = 670 nm) and was detected by a photomultiplier tube (PMT). The optical excitation of LRSPs was observed through the measurement of angular reflectivity spectra by using a photodiode detector (PD). The whole sensor system and the supporting electronics were controlled by using the customized software Wasplas.

2.3. Sensor chip functionalization

As Fig. 1 shows, the gold surface of the sensor chip was modified with a mixed thiol self-assembled monolayer (SAM) (Ostuni et al., 2001) to which the AFM_1–BSA conjugate was coupled. Firstly, the sensor chip was immersed in a 9:1 mixture of MUTEG and MHDA dissolved in ethanol (net thiol concentration of 1 mM) overnight at room temperature. Subsequently, the sensor chip was successively rinsed with ethanol and water and dried in a stream of nitrogen. The MHDA carboxylic groups were used to anchor AFM_1–BSA conjugates and tri(ethylene glycol) groups of MUTEG chains provided a non-fouling background. The carboxylic terminal groups were activated by immersing the chip in TSTU dissolved in DMF (1 mg mL^{-1}) for 2 h. Afterwards, the chip was rinsed with water and dried in a nitrogen stream. Then, AFM_1–BSA conjugate (1 mg mL^{-1} in PBS) was dropped on the surface, covered with a glass side and incubated overnight at the temperature of 4 °C. Finally, the sensor chip

was rinsed with water, dried in the nitrogen stream and stored at 4 °C. Prior the use, the unreacted active ester groups were blocked by 3-min incubation in ethanolamine (1 mM and pH 8.5).

2.4. Detection format

For the detection of AFM_1, an inhibition immunoassay was used. In this assay, the analyzed samples were incubated with a-AFM_1 antibody (concentration of 100 ng mL^{-1}) for 15 min followed by the detection of the amount of unreacted a-AFM_1 antibody. The mixture a-AFM_1 antibody with a sample was pumped through the sensor instrument for 10 min to let the free a-AFM_1 bind the surface with immobilized BSA–AFM_1 conjugate. Afterwards, the sensor surface was washed with PBS-T buffer for 3 min and the labeled Cy5-GaR antibody (at a concentration of 1 μg mL^{-1}) was pumped through the sensor for 10 min. Finally, the sensor surface was washed for 5 min with PBS-T buffer and the fluorescence signal owing to the binding of Cy5-GaR to the captured a-AFM_1 was measured. The concentrations of a-AFM_1 and Cy5-GaR antibodies were chosen so as to provide the fluorescence signal for a blank sample (not spiked with AFM_1) of about 5×10^4 counts per second (cps) which was about 400 times higher than the standard deviation of the background signal. After each detection cycle, the sensor surface was regenerated by 5-min incubation in glycine buffer (pH 1.5) followed by 20-min incubation in 100 mM sodium hydroxide.

For the calibration of the developed biosensor, a series of samples (in milk and PBS-T buffer) with concentrations of AFM_1 ranging from 10^{-2} to 10^4 pg mL^{-1} were prepared by spiking from a AFM_1 stock solution (AFM_1 was dissolved at a concentration of 10 μg mL^{-1} in a mixture 90:10 (v/v) of PBS and methanol). Milk samples containing AFM_1 were centrifuged at 5000 rpm for 10 min at the temperature of 4 °C, the upper fat layer was removed and the obtained supernatant was used for the further analysis.

3. Results and discussion

Fig. 2A shows the angular reflectivity and fluorescence spectra measured before and after the analysis of a PBS-T buffer sample spiked with AFM_1 at a concentration of 1 pg mL^{-1}. It reveals that the excitation of LRSPs is manifested as a resonant dip in the angular reflectivity spectrum and that the fluorescence light reaches its maximum intensity at the virtually identical angle for which LRSPs are resonantly excited. At this angle of incidence, the maximum enhancement of the electromagnetic field intensity occurs and thus the strongest excitation of captured chromophores is seen. In further experiments, the angle of incidence was fixed in the vicinity if the resonance ($\theta = 48.9°$) and the fluorescence signal F was measured as a function of time. Fig. 2B shows the typical time evolution of the fluorescence signals measured upon the analysis of milk samples spiked with AFM_1 at concentrations of 0, 1, 10, 10^2 and 10^3 pg mL^{-1}. Samples incubated with a-AFM_1 were pumped through the sensor ($t = 0$–10 min) followed by the washing step with buffer ($t = 10$–13 min), flow of labeled antibody Cy5-GaR ($t = 13$–23 min) and the washing with buffer ($t = 23$–28 min). The sensor response ΔF was determined as the difference between the fluorescence signal before the sample injection ($t = 0$) and after the final washing step with the buffer ($t = 28$ min). The measurements were performed in cycles and after each analysis the sensor surface was regenerated. We found that after 30 detection cycles measured over 4 days the sensor surface showed good reproducibility with the relative standard deviation (R.S.D.) of 4.6%.

The sensor response was measured for series of buffer and milk samples spiked with AFM_1 at concentrations ranging from 10^{-2} to 10^4 pg mL^{-1}. Fig. 3 shows the obtained calibration curves normalized with the sensor response for the blank samples (not spiked with AFM_1). It shows that the sensor response ΔF gradually decreased when increasing the concentration of AFM_1 in the sample attributed to the blocking of a-AFM_1 binding sites. For each concentration, the sensor response was measured in triplicate and

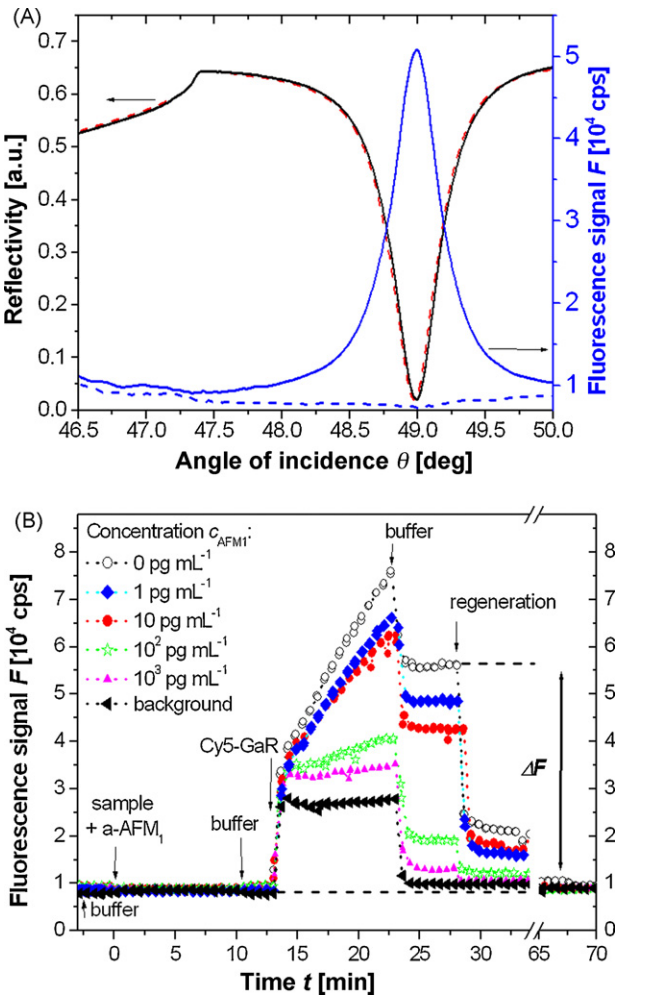

Fig. 2. (A) Angular spectra of reflected intensity (wavelength of $\lambda = 632.8$ nm) and fluorescence intensity (wavelength $\lambda = 670$ nm) measured before (dashed line) and after (solid line) the analysis of a buffer sample spiked with AFM_1 at a concentration of 1 pg mL^{-1} AFM_1. (B) Time evolution of the maximum fluorescence intensity F upon the analysis of a series of milk samples with AFM_1 at concentrations from 0 to 10^3 pg mL^{-1} (concentration indicated in the graph). In addition, the response due to non-specific binding of Cy5-GaR antibody to the surface without a-AFM_1 antibody is shown (noted as background).

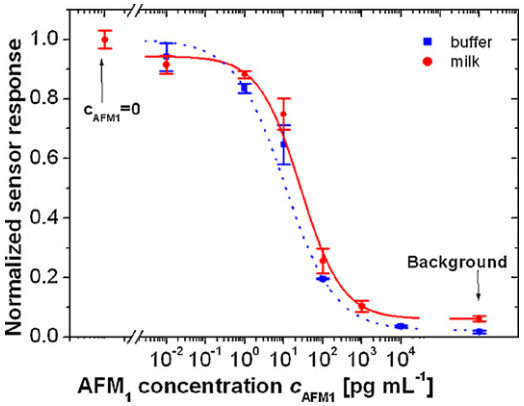

Fig. 3. Normalized calibration curves for the detection of AFM_1 in buffer (squares) and milk (circles) samples.

the standard deviation was determined (shown as the error bar). The standard deviation of the sensor response measured for blank samples was 3%. The background response due to non-specific binding of Cy5-GaR to the surface was 1.8% and 6.2% for buffer and milk samples, respectively. The approximately threefold higher background response observed in milk samples was probably due to the non-specific adsorption of milk compounds to the surface. The measured calibration curves were fitted with a sigmoidal function and the limit of detection (LOD) was determined as the concentration for which the normalized sensor response decreases by three times the standard deviation (9%) with respect to that for blank samples. For the buffer and milk samples, LOD was determined as 0.4 pg mL^{-1} and 0.6 pg mL^{-1}, respectively. The maximum detectable concentration (MDC) was of 1.8×10^3 pg mL^{-1} (for milk samples) and 1×10^4 pg mL^{-1} (for buffer samples), calculated as the concentration for which the measured signal reaches three times the standard deviation above the background signal.

The presented biosensor provides several orders of magnitude higher sensitivity with respect to other reported methods for the detection of aflatoxins including SPR biosensors (LOD = 3 ng mL^{-1}) (Daly et al., 2000), an electrochemical immunoassay (LOD = 25 pg mL^{-1}) (Micheli et al., 2005), HPLC (LOD = 5 pg mL^{-1}) (Magliulo et al., 2005) or ELISA (LOD ~ 10 pg mL^{-1}) (Velasco et al., 2003). Compared to the chemiluminescent immunoassay for detection of aflatoxin M_1 reported by Magliulo et al. (2005) (LOD = 0.25 pg mL^{-1}), the developed biosensor offers a similar limit of detection and shorter detection time of 53 min. This relatively long detection time can be decreased by combining the developed biosensor with microfluidics. By reducing the volume of the fluidic system and analyzed samples, the reaction times as well as the efficiency of molecular binding to the sensor surface can be improved.

4. Conclusion

A biosensor for the highly sensitive detection of aflatoxin M_1 (AFM_1) in milk was developed. This biosensor combined long range surface plasmon-enhanced fluorescence spectroscopy with an inhibition immunoassay and it allowed for the detection of AFM_1 at sub pg mL^{-1} levels within 53 min including the centrifuging of the milk sample, incubation of the sample with the antibody against AFM_1 and the detection of the level of unreacted antibody. The sensor could be regenerated more than 30 times without a change in its performance. The demonstrated limit of detection was about two orders of magnitude lower than the maximum residue level required by the European Commission. Such highly sensitive devices can be, for instance, used for early detection of rising AFM_1 concentration in milk products prior to it reaches levels for which the whole production has to be discarded. Future research will include further validation and optimization of the biosensor. Particularly, the research will be aimed at shortening the detection time and the development of a compact sensor instrument capable of the operation in field.

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