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Fast and sensitive detection of ochratoxin A in red wine by nanoparticle-enhanced SPR

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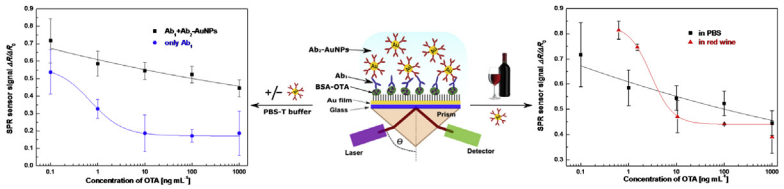
HIGHLIGHTS

- Biosensor for OTA detection using inhibition assay in SPR.
- Detailed description of AuNPs size influence on signal amplification as well as kinetics analysis.
- Real time detection of a small target antigen utilizing AuNPs.
- Simple pre-treatment of the wine sample with the binding agent poly(vinylpyrrolidone) (PVP).
- Sensitive detection of OTA detection in red wine with LOD = 18 pg mL^{-1} and the analysis time of 55'.

GRAPHICAL ABSTRACT



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ABSTRACT

Herein, we present a fast and sensitive biosensor for detection of Ochratoxin A (OTA) in a red wine that utilizes gold nanoparticle-enhanced surface plasmon resonance (SPR). By combining an indirect competitive inhibition immunoassay and signal enhancement by secondary antibodies conjugated with gold nanoparticles (AuNPs), highly sensitive detection of low molecular weight compounds (such as OTA) was achieved. The reported biosensor allowed for OTA detection at concentrations as low as 0.75 ng mL^{-1} and its limit of detection was improved by more than one order of magnitude to 0.068 ng mL^{-1} by applying AuNPs as a signal enhancer. The study investigates the interplay of size of AuNPs and affinity of recognition elements affecting the efficiency of the signal amplification strategy based on AuNP. Furthermore, we observed that the presence of polyphenolic compounds in wine samples strongly interferes with the affinity binding on the surface. To overcome this limitation, a simple pre-treatment of the wine sample with the binding agent poly(vinylpyrrolidone) (PVP) was successfully applied.

1. Introduction

Ochratoxin A (OTA), a highly toxic fungal secondary metabolite of *Aspergillus ochraceus* and *Penicillium verrucosum* is one of the most widely spread mycotoxin that contaminates a large variety of

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agricultural commodities [1]. It exhibits multiple toxicities in animals and mankind, including nephrotoxic, hepatotoxic, immunotoxic, teratogenic and carcinogenic effects, which represent serious health risks to livestock and the general population [2]. It has been linked to the Balkan Endemic Nephropathy (BEN), a kidney disease occurring in some regions in south-eastern Europe (Croatia, Bosnia, Serbia, Croatia, Bulgaria and Romania) and development of tumors in the urinary tract in humans [3,4]. As a result, the International Agency for Research on Cancer (IARC) has classified OTA as a potential carcinogen (group 2B) for humans [5].

The worldwide occurrence of OTA pollution has been already precisely reported. It contaminates a various foodstuffs and beverages including cereal grains, oil seeds, dried fruits, coffee, cocoa beans, grape juice, beer and wine [6–8]. It has been established that wine is the second major source of OTA daily intake in EU, following cereals. After the first report on the occurrence of OTA in wine [9] several studies were performed to assess the pertinence of this toxin [10–12]. Ottender and Majerus noticed the fact that higher level of OTA occurs in a red wine then in a white and rose which may be due to differences in winemaking process [13].

Due to the persistence of OTA in the food chain, high stability and resistance during food processing (*e.g.* cooking, roasting or fermenting), this mycotoxin represents a serious threat for human health. Therefore, the European Commission has established maximum permissible level of OTA in food, feed products, raw materials and beverages (*e.g.* 5 ng mL^{-1} for unprocessed cereals, 3 ng mL^{-1} for products derived from unprocessed cereals, 10 ng mL^{-1} for coffee beans and 2 ng mL^{-1} for all types of wine) (EC No. 123/2005). Such low allowable levels require very sensitive and precise methods of detection. Analysis of OTA is nowadays performed by established analytical techniques including thin-layer chromatography (TLC) [6], gas chromatography (GC) [14] and high-performance liquid chromatography (HPLC) [15] with immunoaffinity columns and fluorescence detection. These technologies provide sufficient detection limit but relay on multiple steps prior to the detection, sophisticated equipment and trained personnel, which cannot meet the requirements of on-site and rapid detection. Therefore, alternative methods such as capillary electrophoresis with diode array detection [16], immunochemical techniques like enzyme-linked immunosorbent assays (ELISA) [17], electrochemical immunosensors [18] or optical techniques either using the optical waveguide light mode spectroscopy or surface plasmon resonance (SPR) has been successfully developed. These methods present good sensitivity and selectivity with the potential for high-throughput screening. In particular, SPR spectroscopy is a powerful, label-free technique enabling monitoring of affinity molecular interactions in a real time and in a noninvasive manner. This opto-electronic phenomenon utilizes refractive index changes to detect mass changes occurring at noble metal surface interfaces [19]. Moreover, the kinetics information on the affinity binding between native biomolecules can be also provided. Nevertheless, SPR biosensors were shown to be suitable for the direct analysis of medium and large molecular weight analytes which induce measurable refractive index changes upon their binding on the surface from samples with the analyte concentration above ng mL^{-1} [20]. Regrettably, mycotoxins are small chemical compounds that possess inadequate mass to cause significant changes in the refractive index. In order to overcome this limitation, sandwich or indirect competitive inhibition assays are developed to detect such molecules. In addition, signal amplification by gold nanoparticles (AuNPs) offers efficient means to increase the SPR response in order to detect binding of minute amounts of target molecules on the surface. It has been already demonstrated that, electronic coupling between the localized surface plasmons of AuNPs and the surface plasmons wave associated with the SPR gold chip can significantly enhance the SPR response [21]. AuNPs exhibit several distinct physical and chemical attributes that make them an excellent scaffold for novel biochemical and chemical sensors. Relatively easy and inexpensive synthesis, stability, unique optoelectronic properties, high surface-to-volume ratio with excellent biocompatibility, safety for humans and small amounts of AuNPs needed in the test allow researchers to develop sensing strategies with higher sensitivity, stability and selectivity [22]. AuNPs have been successfully applied in SPR detection of DNA, proteins and drug molecules. Despite of broad use of AuNPs in SPR biosensors, the role of the size of NPs in the interactions with SPR surface is not fully understood. Studies performed by Uludag and Tothill [23] show higher sensor response with increasing size of AuNPs. On the other hand, Mitchell et al. [24] observed no significant differences in signal amplification for AuNPs with diameter ranging from 25 to 50 nm. The competing effect of enhanced SPR signal, steric hindrance and diffusion mass transfer rate depending on the size of AuNP was investigated by Springer et al. [25].

In this work, we report on the development of fast and sensitive SPR assay for ochratoxin A detection in a red wine. To overcome the matter concerning low molecular weight of the analyte that hampers its detection using SPR, an indirect competitive inhibition assay was performed. Moreover, the signal amplification and sensitivity improvement was achieved by using secondary antibodies conjugated with gold nanoparticles labels. In this study, we also investigate the ability of functionalized AuNPs to enhance the response of an SPR biosensor in a biomolecular detection assay with special attention given to the study of the effect of the size of AuNPs. Furthermore, a detailed analysis of kinetic parameters (association/dissociation constants and association/dissociation rate constants) was made and compared with available literature. To reduce matrix interferences in untreated wine (*e.g.* ethanol, polyphenols) that imposes unspecific sorption and potential inactivation of used antibodies, a very simple pre-treatment of samples with binding agent poly(vinylpyrrolidone) (PVP) was successfully applied.

2. Material 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 (USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Pierce (USA). Ochratoxin A (OTA), the conjugate of OTA with bovine serum albumin (BSA-OTA), poly(-vinylpyrrolidinone) (PVP), PBS buffer tablets and Tween 20 were from Sigma-Aldrich (Austria). The primary rabbit antibody against OTA (Ab_1) was from AntiProt. Goat anti-rabbit secondary antibody (Ab_2) and gold nanoparticles (AuNPs, 10, 20 and 40 nm)-labeled goat anti-rabbit secondary antibody (Ab_2-AuNPs) were from Abcam (UK). 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 of 1.5 and ethanolamine were purchased from Biocore (Germany). The ERM (European Reference Material) BD476 (OTA in red wine) was obtained from the Institute for Reference Materials and Measurements (Belgium). This material was prepared from commercial wine sources intended for human consumption and it was characterized by mass spectrometry and HPLC. The concentration of OTA was determined as 0.52 ± 0.11 ng mL^{-1}.

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2.2. Optical setup

Surface plasmon (SP) resonance measurements were carried out by using the Kretschmann-type of attenuated total reflection configuration, as described previously [26]. Briefly, a monochromatic light beam at a wavelength of $\lambda = 632.8$ nm emitted from a HeNe laser (2 mW) passed through a chopper and a polarizer selecting transversal magnetic (TM) polarization. Then it was made incident at a surface of a sensor chip with gold film that was optically matched using immersion oil to an optical prism. The sensor chip was made of BK7 glass substrate which was coated by sputtering (UNIVEX 450C form Leybold, Germany) with 37 nm thick gold film. To the sensor chip, a transparent flow-cell with a volume of approximately 10 μL was attached in order to flow aqueous samples by using a peristaltic pump at the a flow of 0.5 mL min^{-1}. The assembly of the sensor chip and prim was mounted on a rotation stage in order to control the angle of incidence of the laser beam θ. The resonant coupling to SP is manifested as a narrow dip in the reflectivity spectrum $R(\theta)$. The binding of molecules to the gold layer was observed as a shift of the angular position of 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 (developed at the Max Planck Institute for Polymer Research in Mainz, Germany).

Surface mass density Γ of biomolecules adsorbed to the surface was calculated using Feijter's formula [27]:

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

where the refractive index of the protein sublayer and a buffer is denoted by $n = 1.465$ and $n_b = 1.3337$, respectively. The ratio $\partial n/\partial c = 0.182 mm^3 mg^{-1}$ at a wavelength of 632.8 nm was taken from literature [27]. The thickness d_h was determined by fitting the respective SPR spectrum using following parameters: refractive index of the prism $n_p = 1.845$, complex refractive index for the gold film $n_m = 0.22 + 3.67i$.

For the SPR measurements of kinetics of surface reactions, the angle of incidence was fixed close to the angle $\theta = 55.6°$ where resonance edge with the highest slope $\Delta R/\Delta\theta$ occurs. At this angle, the time dependent reflectivity signal was measured. The reflectivity changes were converted to variations in refractive index by calibrating the sensor with series of standard aqueous samples. These standards were prepared with known refractive index in refractive index units (RIU).

2.3. Sensor chip functionalization and detection format

The gold surface of the sensor chip was modified with a mixed thiol self-assembled monolayer (SAM). First, the gold chip was incubated overnight at room temperature in a molar ratio 1:9 mixture of dithiols: PEG6-COOH and PEG3-OH (dissolved in ethanol at the total concentration of 1 mM). Subsequently, the sensor surface was rinsed with ethanol and dried in a stream of nitrogen. The covalent *in situ* immobilization of BSA-OTA was carried by using amine coupling. By using EDC/NHS (concentrations in deionized water of 37.5 and 10.5 mg mL^{-1}, respectively) carboxylic acid groups at the gold surface were activated. Afterward, BSA-OTA conjugate dissolved in ACT buffer at a concentration of 30 μg mL^{-1} was circulated through the flow cell for 15 min in order to react via their amine groups with the activated carboxyl groups at the sensor surface. Finally, the unreacted active ester moities were deactivated by 10 min incubation in ethanolamine (1 M and pH 8.5).

For the detection of OTA, an indirect competitive immunoassay was applied. Analyzed samples were prepared by spiking the PBS-T buffer or certified red wine standard with purified OTA (at concentrations between 10^{-1} and 10^{3} ng mL^{-1}). To minimize the matrix effect that may interfere with the OTA analysis in wine, samples were spiked with 3% of PVP and subsequently shaken for 5 min at room temperature, filtrated, and the pH of each aliquot was adjusted to 7.4 with NaOH prior to analysis. The standards were then mixed and incubated (for 30 min) with an equal volume of Ab_1 (concentration of 100 ng mL^{-1}), followed by the detection of unreacted Ab_1. Subsequently, the mixture was passed over the chip for 10 min to allow binding the free Ab_1 to the BSA-OTA conjugate. To remove unbounded molecules, the sensor surface was then washed for 2 min with PBS-T buffer. Afterward, the Ab_2-AuNPs antibody was flowed through the sensor for 10 min, followed by 2 min rinsing with PBS-T. After each detection cycle, the substrate surface was regenerated by 5 min incubation in glycine buffer (pH 1.5, 20 mM) followed by rinsing with NaOH (20 mM).

3. Results and discussion

3.1. Sensor chip and assay characterization

Firstly, the surface mass density of *in situ* immobilized BSA-OTA and its ability to bind Ab_1 was evaluated. As shown in Fig. 1, SPR reflectivity curves $R(\theta)$ were recorded upon the subsequent modification of the sensor surface with mixed thiol SAM, BSA-OTA, and after the affinity binding of Ab_1 and Ab_2. Prior to the surface modification by protein molecules, the resonant excitation of SPs occurs at $\theta = 57.04°$ on a gold surface with mixed thiol SAM. This resonance shifts to higher angles by $\Delta\theta = 0.27°$ after the covalent binding of BSA-OTA conjugate to the sensor surface that is associated with increased thickness of an adlayer ($d_h = 2.05$ nm was determined by fitting the reflectivity curve). Based on Eq. (1), the surface mass density of covalently bounded BSA-OTA was estimated to be $\Gamma = 1.48$ ng mm^{-2}. Additional shift of $\Delta\theta = 0.06°$ was observed after the affinity binding of Ab_1 which corresponds to $\Gamma = 0.32$ ng mm^{-2}. These values are much lower than those for the

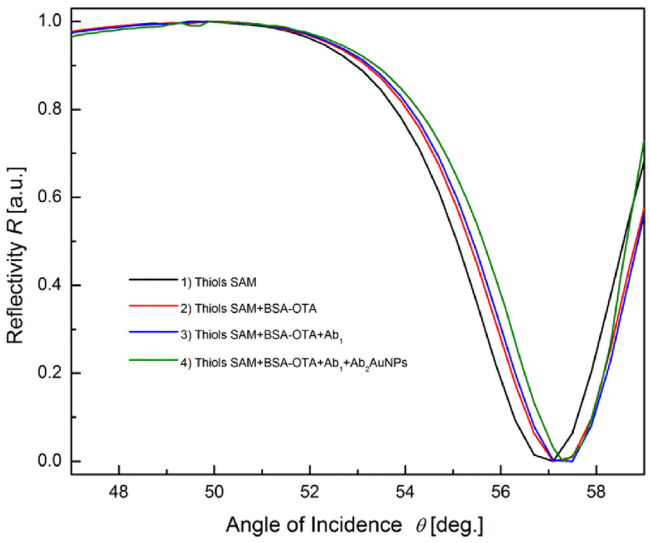

Fig. 1. Angular reflectivity spectra measured for a sensor chip prior to the modification (1, thiol SAM on Au), after the immobilization of OTA-BSA conjugate (2), after affinity binding of Ab_1 (3) and after reaction with Ab_2-AuNPs (4). The spectra were measured for the surface in contact with PBS-T.

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BSA-OTA due to the very low concentration of Ab_1 and incubation time not long enough to reach saturation. Nevertheless, the application of an additional high mass provided by secondary antibodies Ab_2 labeled with gold nanoparticles (Ab_2-AuNPs, here example with 20 nm AuNPs) caused significant enhancement of the SPR shift $\Delta\theta$ of 0.2°.

The non-specific binding of species in a sample at the sensor surface with tethered ligands [28] can lead to false positive results in screening assays and incorrect determination of analyte concentration. In biomolecular interaction analysis, non-specific binding generally gives results that do not fit to an interaction model and the binding constants are way off the results from the literature. Therefore, the specificity of primary Ab_1 and secondary Ab_2 antibodies used in the detection format was tested. For this purpose, BSA (not conjugated with OTA) was immobilized on a sensor chip, followed by the injection of Ab_1 and Ab_2-AuNPs. The assay showed an excellent specificity as no significant SPR response was recorded for antibodies (data not shown).

Wine is a complex alcoholic beverage and it contains constituents that may have a strong influence on the sensitivity of the detection. Wine matrix is composed of two main fractions, the non-volatile fraction containing ethanol, polyphenols, variety of proteins, and the volatile fraction, that include flavor and aroma compounds [29]. The presence of mentioned polyphenolic compounds may cause inactivation of antibodies and unspecific sorption that blocks the sensor surface. To overcome this matter, a simple pre-treatment of the wine sample with the binding agent poly(vinylpyrrolidone) (PVP) was applied.

In order to evaluate the unspecific sorption of constituents in wine to the surface and its reducing by PVP, SPR observation of surface mass density change was carried out. First, a stable baseline was established upon the flow of PBS-T. Then, 3% PVP was injected for 10 min followed by rinsing with PBS-T. The sensorgram in Fig. 2 shows no measurable change in the SPR response which returned back to the baseline. Afterward, the surface was regenerated and wine sample spiked with 3% PVP was injected. After the subsequent rinsing with PBS-T, a small increase in the SPR response of 4×10^{-5} RIU was observed which can be attributed by a weak non-specific interactions coming from the wine. After the regeneration step, the SPR response returns to the original baseline indicating fully reversible assay cycle. Finally, un-treated wine was injected and after the rinsing with PBS-T a huge SPR sensor signal change of 1.2×10^{-3} RIU was measured. The regeneration allowed to removing most of the deposit but substantial fraction of the adsorbed constituents corresponding to 1.7×10^{-4} RIU fouled the surface irreversibly. These data reveal the excellent ability of PVP to bind polyphenols through hydrogen bonding making it easier to eliminate them from the solution [30].

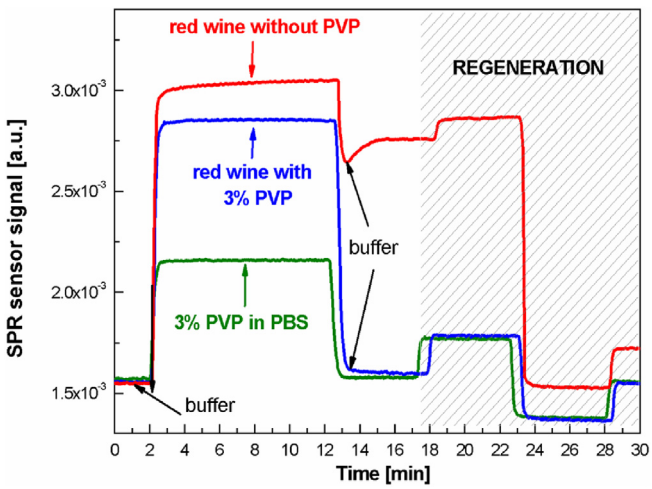

Fig. 2. Sensorgrams showing SPR signal recorded upon injections before and after injection of 3% PVP in PBS-T (green line), wine spiked with 3% PVP (blue line) and pure wine sample (red line) followed by regeneration step. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The SPR was used for the measurement of affinity binding constants of interaction between Ab_1 and the OTA moieties. The equilibrium association and dissociation constants K_A and K_D, respectively, were calculated by the Langmuir binding theory [31] (Fig. 3). K_D is related to the rate of complex formation (described by association rate constant k_{on}) and the rate of breakdown (described by dissociation rate constant k_{off}) such that $K_D = \frac{k_{off}}{k_{on}}$. Association constant ($K_A$) can be then calculated as K_D^{-1}.

The limit of detection (LOD) was found below 300 ng mL^{-1} and the measured binding affinity of 2.7×10^{-6} M (806 ng mL^{-1}) was determined for the interaction of Ab_1 to surface immobilized BSA-OTA. This values indicates weaker affinity in comparison to other works reported in literature. Houwlingen et al. [32] obtained affinity values for the binding of ochratoxin A to several antibody fragments ranging between K_D = 12 ng mL^{-1} up to 476 ng mL^{-1}. Bodarenko et al. [33] obtained affinity values between K_D = 14 to < 500 ng mL^{-1} when detecting OTA using a fluorescence polarization immunoassay with various tracers. The deviation from the results obtained in this work can be ascribed to the different antibodies used, and/or the inhibition of affinity by the used wine matrix. In the work of Heusser et al. [34] a monoclonal antibody was used for the detection of ochratoxin A using ELISA. This experiment is most comparable to our results, since authors have obtained an IC50 value of 5.7×10^{-6} M and a LOD of 320 nM, which is slightly higher than the demonstrated LOD in this work, but very comparable since the measured LOD in this work still has a good signal strength and could be even lower if the corresponding concentrations were tested.

3.2. AuNPs enhancement

SPR analysis of small molecules (MW < 1 kDa) or analytes at extremely low concentration is hindered by weak refractive index changes occurring upon their capture on the sensor surface [35]. To overcome this major impediment of SPR biosensor technology, gold nanoparticles have been demonstrated as efficient signal enhancers [36].

In order to maximize the enhancement of SPR biosensor response for the OTA immounoassay, Ab_2-AuNPs conjugates with diameters of 10, 20 and 40 nm were tested. As shown in Fig. 4A and B, the binding of non-labeled Ab_2 dissolved at concentration of 2 μg mL^{-1} to affinity captured Ab_1 induced a change in SPR signal of δR = 0.01 and resonance shift to higher angles θ. This value is approximately two times higher than that recorded for the primary antibodies Ab_1. When the Ab_2 was conjugated with AuNPs and diluted at one order of magnitude lower concentration (0.2 μg mL^{-1}), the sensor response increased to δR = 0.018 for 10 nm diameter and to δR = 0.026 for 20 nm diameter which corresponds to the enhancement factors of 3.2 and 5, respectively. Even when the AuNP-Ab_2 conjugate with the diameter of 40 nm was diluted at the two orders of magnitude lower concentration (0.02 μg mL^{-1}), recorded signal δR = 0.061 was 10-fold amplified compared to that for non-labeled Ab_2. Obtained results are in a good agreement with previously reported studies on the dependence of the optical enhancement on the size of AuNPs [25,37]. The magnitude of the reflectivity shift depends on the particle size and the bigger particles lead to larger shifts. When an object with a high

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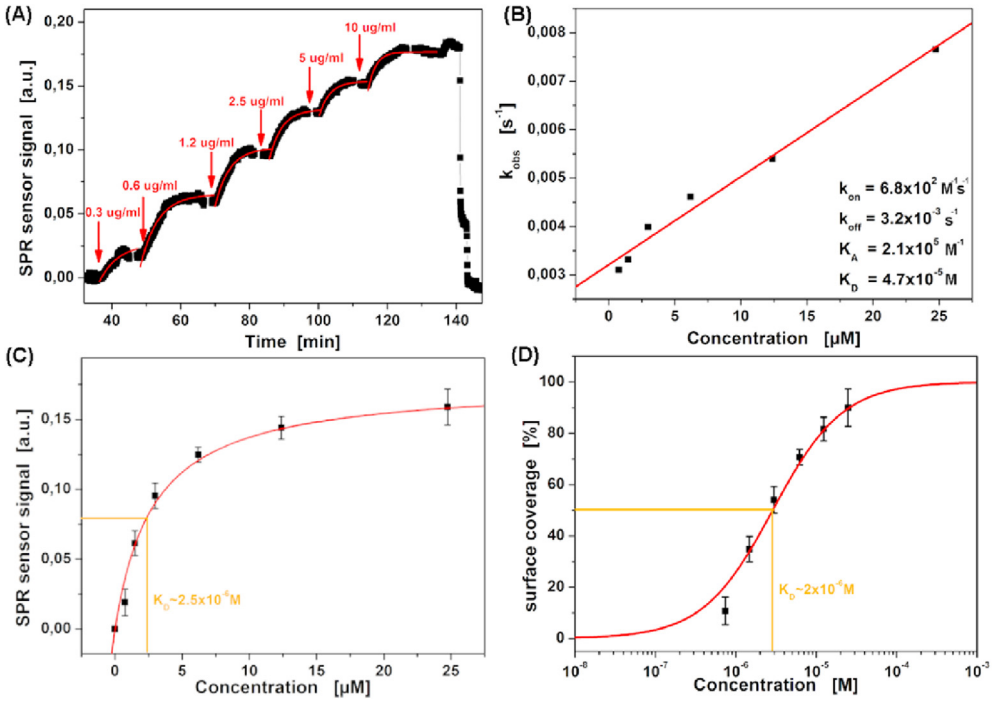

Fig. 3. (A) Binding kinetics of the titration of OTA antibody. The arrows mark the injection of the spiked concentrations. The binding kinetics for each injection were fitted with simple exponential fits, obtaining the observed binding parameters k. (B) Plot of the observed binding parameters k for each measured concentration against the respective concentrations. The linear fit gives a direct relation to $k = conc[c]+k_{off}$, therefore association and dissociation rate constants are obtained from the slope and intercept of the linear fit. The calculated binding affinity K_A is shown in the insert. (C) Injected concentration plotted against the sensor response signal. The red line is the best fitted Langmuir isotherm, showing a half saturation concentration of around 2×10^{-6} M, which corresponds to the K_D value. (D) Surface coverage of the biosensor, calculated for the response signals. The estimated K_D from this fitting routine - the IC50 concentration - gives very comparable results to the values obtained from the analysis of the kinetic reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

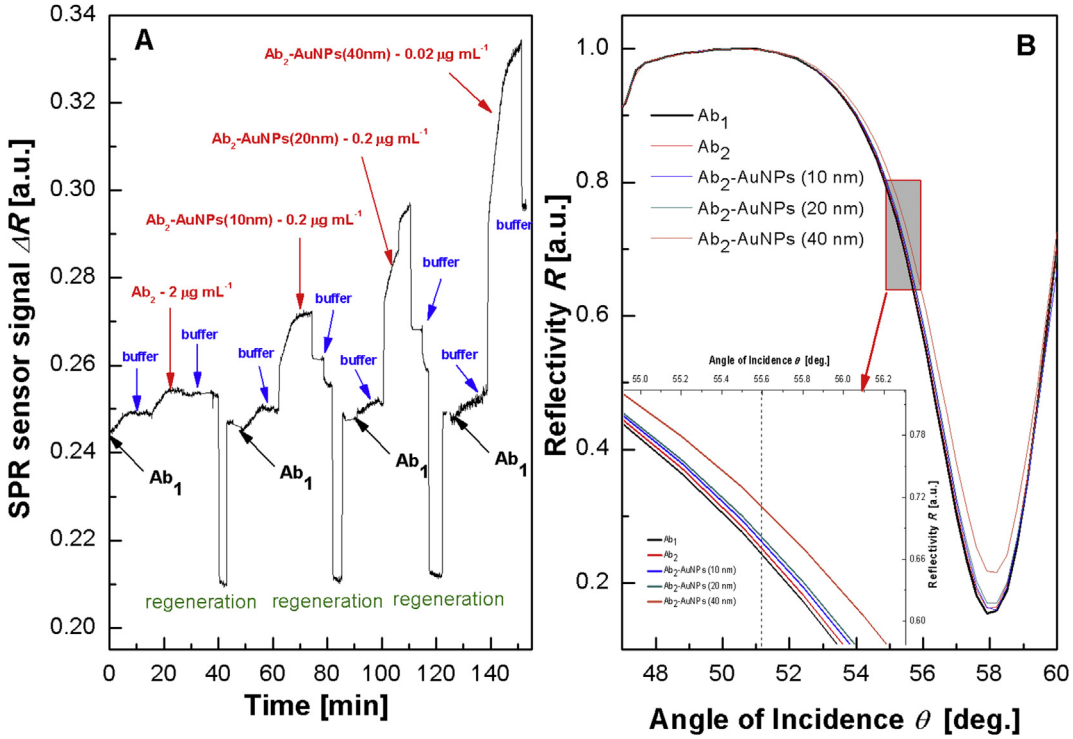

Fig. 4. (A) Sensogram showing affinity binding ofAb_1 and subsequent reaction with non-labeled Ab_2 and Ab_2-AuNPs conjugates (size 10, 20 and 40 nm) (B) Angular reflectivity spectra measured from a sensor chip after affinity binding of Ab_1 (black line) and non-labeled Ab_2 (red line) and Ab_2-AuNPs (10 nm - blue line, 20 nm – green line and 40 nm – orange line). The spectra were measured for the surface brought in contact with PBS-T. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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mass (like AuNPs) is applied when the effect of diffusion-limited mass transfer is weak, higher mass is allowed to bind to the surface which leads to the stronger SPR shift. Nevertheless, SPR response is influenced not only by size of NPs but also by the electromagnetic field coupling between localized surface plasmons of the nanoparticle and propagating plasmon field of the surface [21].

Furthermore the enhancement factor strongly depends on the distance between the NPs and the gold surface. This effect is dominant especially at certain sizes of the NPs. From Leveque and Martin it is known that for distances d < 50 nm most of the energy is concentrated between the particle and the film leading to enhancement factors between 10^2 and 10^3 [38]. Due to the fact that a distribution of distances between NP and gold surface is to be expected the presented results are in good agreement with the theoretical calculations by various authors [21,38]. Work is in progress to verify the distance dependency by measurements of the signal enhancement at various fixed distances between the NPs and the gold surface.

3.3. Ochratoxin A detection

Ochratoxin A was detected using indirect competitive format described in section 2.3. For calibration of the OTA biosensor, Ab_2-AuNPs conjugates with the diameter of 40 nm were chosen due to the highest recorded enhancement of the signal from all tested Ab_2-AuNPs (see section 3.3). To evaluate the effect of the SPR sensor signal enhancement by AuNPs, two types of assays were performed. In the first one, only the binding of Ab_1 antibody mixed with analyzed samples was measured. In second assay, the binding of Ab_1 was followed by the reaction with secondary antibodies conjugated with AuNPs. Fig. 5 shows obtained calibration curves normalized with the sensor response measured for the blank sample (not spiked with OTA). The sensor response ΔR was defined as a difference in the SPR sensor signal R before the reaction of the surface with spiked sample and after the rinsing with PBS-T. The sensor response ΔR was measured for series of buffer and red wine samples spiked with OTA at concentrations ranging from 1.5×10^{-1} to 10^3 ng mL^{-1}. The calibration curves were fitted with a sigmoidal function. The limit of the detection (LOD) and the limit of quantification (LOQ) were defined as the concentration of OTA equivalent to three times (for LOD) and ten times (for LOQ) the value of the standard deviation (SD), measured in the absence of OTA (no competition point). The sensitivity of the AuNPs enhanced format was significantly better comparing to the one non-enhanced. For those two assays (performed in PBS-T buffer) LOD was found to be 0.068 ng mL^{-1} and 0.75 ng mL^{-1}, respectively (Fig. 5). The higher signal has clearly reduced the errors in the determination of LOD, and in the case of low antibody concentration this reduction in coefficient of variation (CV, estimated to be 10,2% and 35% for assay with and without AuNPs, respectively) has been so significant that it made the LOD of the enhanced format significantly lower than without enhancement. When such detection format (utilizing AuNPs) was performed in red wine samples, LOD and LOQ of 0.19 ng mL^{-1} and 0.68 ng mL^{-1}, respectively, were calculated. Higher LOD in comparison to the assay established in PBS indicate some non-specific adsorption of wine components to the surface resulting in higher background response (Fig. 5B). Nevertheless, both values are one order of magnitude lower than maximum allowed level of OTA in red wine established by European Commission. Thereby, presented biosensor could be considered as a new, sensitive and fast tool for mycotoxins detection in food and beverages.

Comparing to the conventional methods for OTA detection in foodstuff including TLC [39], HPLC and GC-MS [40] that offer a very good sensitivity at the expense of complicated and time consuming pre-cleaning techniques. Most methods used for determination of a mycotoxin must rely on the correct extraction and clean-up procedures like liquid-liquid extraction (LLE) [41], supercritical fluid extraction (SFE) [42] or solid phase extraction (SPE) [43]. An

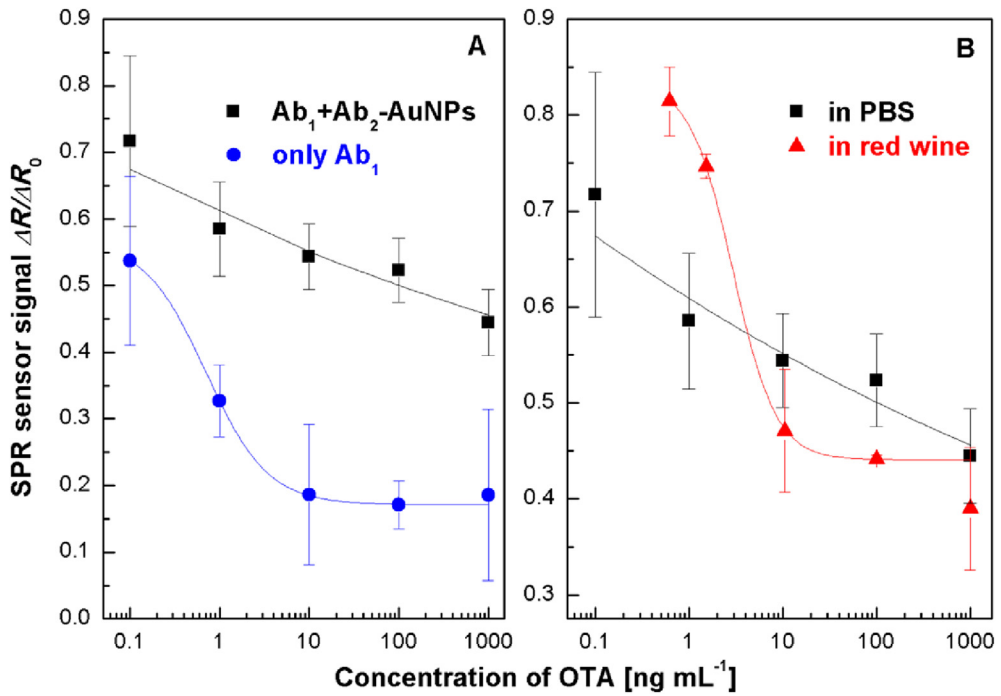

Fig. 5. (A) Normalized calibration curves for the detection of OTA using inhibition immunoassay with Ab_2-AuNPs (40 nm, black squares) and without secondary antibodies measured in buffer solution (blue circles). (B) Comparison of calibration plots performed in PBS-T and red wine (red triangles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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interesting study reported by Maier [44] shows a two-dimensional extraction procedure employed SPE and MIP (molecularly imprinted polymers) for the extraction of OTA. Prior removal of the interfering acidic matrix compounds by C-18 SPE was shown to be successful and direct sample loading onto the MIP resulted in low recoveries. The extracts after the combined SPE protocol enabled OTA quantification by HPLC–FD. However, in this study, a similar result was observed in control experiments in which the MIP was replaced by the corresponding non-imprinted polymer (NIP). In yet another study, detection of OTA using SPME–LC–MS/MS has been reported [45]. High-throughput was achieved by simultaneous preparation of up to 96 samples using multi-fibre SPME (Solid phase micro extraction) device and multi-well plates. A carbon-tape coating was chosen for extracting phase. The SPME technique was reported to be successful as clean-up procedures for OTA extraction from urine with LOD and LOQ of 0.3 and 0.7 ng mL^{-1} in urine, respectively. Pelegri et al. [46] presented a sensitive protocol for OTA detection and quantification using SAX (strong anion exchange) columns in clean up resulting with LOD of 0.02 ng mL^{-1} with HPLC–FD readout. There are several types of very sensitive chromatographic methods (TLC, HPLC, GC) available for mycotoxin analysis however, in all cases sample pre-treatment plays a major part in the analysis. Presented biosensor combine both aspects – detection of toxin of interest at ng mL^{-1} level as well as a very simple and fast treatment utilizing addition of 3% PVP to analyzed sample.

Among other separation techniques, ELISA became very popular recently due to its relatively low cost and easy application [47]. Commercially available ELISA kits offer highly specific as well as simple-to-use tool for mycotoxins detection. The disadvantage of these kits lies in the fact that they are for single use, which can increase costs of bulk screening, require multiple steps that translates to prolonged analysis time, and in the lower sensitivity compared to chromatographic methods [48]. Flajs et al. [49] reported a comparison of ELISA kit for OTA detection in red wine with HPLC and showed that the method of OTA-extraction recommended by the ELISA manufacturer is not appropriate for red wines due to the interference of chromogenes. The introduction additional clean-up with bicarbonate eliminate this interference, and the latter method gives results that correlate well with the results obtained by HPLC. In contrast to HPLC, ELISA could not detect very low OTA concentrations. In other study, Barthelmebs [50] modified typical ELISA by using aptamer instead of antibody. The limit of detection attained (1 ng mL^{-1}), the midpoint value obtained (5 ng mL^{-1}) and the analysis time needed (125 min) for the real sample.

Nowadays, also other alternative methods for OTA detection *eg.* electrochemistry or chemiluminescent has been intensively investigated. Barthelmebs [51] proposed an aptasensor, based on disposable screen-printed electrodes with electrochemical detection using differential pulse voltammetry. The aptasensor obtained using this approach allowed detection limit of 0.11 ng mL^{-1}, and was also validated for real sample analysis. Another very interesting work done by Novo et al. [4] demonstrates an integrated analytical system that conjugates an indirect competitive enzyme-linked immunosorbent assay strategy developed in PDMS microfluidics with integrated microfabricated hydrogenated amorphous silicon photodiodes for chemiluminescence detection. A limit of detection of 0.85 ng mL^{-1} was obtained for OTA detection in a PBS solution using a straight-channel configuration. Comparable limits of detection were obtained for beer extracts but for red wine extracts a higher limit of detection of OTA of 28 ng mL^{-1} was obtained.

Presented in this work sensor offers shorter analysis time (*ca.* 55 min) with sensitivity reaching the most sensitive techniques. Indeed, the LOD of the developed biosensor can be further improved by increasing the binding capacity of the sensor surface and by using higher affinity antibodies. The analysis time can be decreased by the implementation of more sophisticated microfluidic devices with smaller volume of *e.g.* additional means for sample mixing.

4. Conclusion

Fast indirect competitive-based biosensor with application of AuNPs as a signal amplifier for OTA detection in red wine has been successfully developed. The analysis relies on the SPR readout that offers a real-time, label-free aspect of measurements. Estimated LOD of nanogold-enhanced assay of 68 pg mL^{-1} is 10 times more sensitive than non-enhanced one and one order of magnitude lower than the maximum level of OTA in wine specified by the European Commission. The LOD performed in red wine is slightly higher than the LOD of the pure assay indicating a certain amount of unspecific adsorption of red wine components to the surface resulting in higher background signal response. Moreover, to reduce interference of polyphenols which impede the analysis, a simple pre-treatment of the wine samples with the binging agent PVP was applied. It was shown that the addition of 3% PVP to red wine completely reduces non-specific interactions. This is due to the excellent ability of PVP to bind polyphenols through hydrogen bonding and the possibility to eliminate them from the solution with a simple washing step. To overcome a matter of the small size (low molecular weight) of antigen that hampers its detection via SPR, secondary antibodies with metallic nanoparticle (NPs) labels with different sizes of AuNPs have been used as a signal enhancer. The highest signal amplification was obtained for 40 nm AuNPs and for distances $d > 50$ nm between the nanoparticles and the gold surface leading to enhancement factors of more than 100. The precision of the agreement between the theoretical prediction [21,38] and the experimental results with a distribution of the distances is so excellent that the enhancement factor becomes a good measure of the averaged distance.

The low limit of detection, the superior signal response time of less than one hour and low consumption of primary antibodies (reduction of costs) make the developed assay an excellent alternative to conventional methods for the detection of OTA in red wine and other beverages.

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