A Fluorescent Aptasensor based on DNA-scaffolded Silver Nanoclusters Coupling with Zn(II)-ion Signal-enhancement for Simultaneous Detection of OTA and AFB₁

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Abstract

The simultaneous detection of various mycotoxins is indispensable for food safety since the agricultural products are often contaminated by multiple mycotoxins. Herein, we report a signal-enhancement fluorescent aptasensor based on DNA-scaffolded silver nanoculsters (DNA/AgNCs) for simultaneous detection of Ochratoxin A (OTA) and Aflatoxin B₁ (AFB₁). The OTA aptamer (Ap1) and the AFB₁ aptamer (Ap2) immobilized on magnetic beads surface hybridize with signal probe1 (Sp1) and signal probe2 (Sp2), respectively. In the presence of these two mycotoxins, the single-strand Sps are released after Aps binding with mycotoxins due to the higher stability of mycotoxin-Ap. Through the magnetic separation, the Sps in the supernatant liquid are acted as the corresponding scaffolds to synthesize AgNCs with different photoluminescence emission bands. Intriguingly, we find that the fluorescence intensity is obviously increased after adding Zn(II)-ion into the system, leading to a linear range from 0.001 ng/mL to 0.05 ng/mL both for OTA and AFB₁ with the detection limit of 0.2 pg/mL for OTA and 0.3 pg/mL for AFB₁. The proposed fluorescent aptasensor shows excellent performance towards the analytical application in real samples, providing a new model for the simultaneous detection for other mycotoxins by simple change of the

aptamer.

Keywords: DNA-scaffolded silver nanoclusters, Mycotoxins, simultaneous detection, Zn (II)-ion signal-enhancement.

Introduction

With the improvement of life quality, food safety has attracted the public attention. Mycotoxins, a group of chemical substances produced by some fungal species, have been regarded as one of causes of illness or even death [1]. As two types of highly toxic and widespread mycotoxins, OTA and AFB₁ exist in various agricultural products including corns, wheat, cereals and peanuts [2,3]. Especially, they have been confirmed to be highly carcinogenic, teratogenic and nephrotoxic [4]. More importantly, many studies have indicated that two or more mycotoxins may coexist in a single sample, which can cause increased toxicity by synergistic effects [5,6]. Due to the fact that OTA and AFB₁ often coexist in many kinds of agricultural products [7], developing a convenient, selective and sensitive method for simultaneous detection of coexisting mycotoxins may be significant for improving the practicability of the assay and food safety control.

Currently, the major analytical methods for multiple mycotoxins detection including OTA and AFB₁ are LC-MS/MS, HPLC-MS/MS [8-10]. However, these methods have some disadvantages including operation complexity, high costly and strenuousness. Enzyme-linked immuno-sorbent assays (ELISA) have been introduced for rapid screening of mycotoxin in real samples

[11-13]. However, this approach requires antibodies specific to corresponding mycotoxins, and the low stability of antibodies often influences the accuracy of detection results. Thus, it is urgently to develop a simple, fast and convenient method to analyze mycotoxins. According to the previous studies, aptamers, a group of single-stranded oligonucleotides, possess high affinity and specificity against the targets [14,15]. Thus, aptamer-based biosensors (aptasensors) have been extensively applied for detection of various mycotoxins [16-18]. Nevertheless, these methods only detected single mycotoxin at a time, which limited their extensive application for simultaneous detection in real samples. Wu et al. developed an aptasensor, which was based on multiplexed fluorescence resonance energy transfer (FRET) between upconversion nanoparticles (UCNPs) and graphene oxide (GO) for simultaneous detection of two mycotoxins [19]. In this method, the 980 nm laser was acted as an excitation source to increase the low conversion efficiency of UCNPs. However, water has high photon absorbance at around 980 nm, which prevents this method to be applied in real samples [20]. In addition, Sun et al. used an aptamer-photonic crystal encoded suspension array to simultaneously detect OTA and fumonisin B₁ (FB₁) [21]. But this method required fluorescence-labeled technology, which was

time-consuming and complex. Consequently, it is urgently to develop an aptasensor for simultaneous and rapid detection of multiple mycotoxins to improve testing efficiency and reflect the contaminated matrix in reality.

It is exciting that DNA-scaffolded AgNCs as a nanometer material have a class of benefits, such as great photostability, nontoxicity, good biocompatibility and uniform subnanometer size [22]. Hence, DNA-scaffolded AgNCs have been increasingly used to develop sensing method for detection of various targets [23,24]. Chen et al. devised an approach based on DNA-scaffolded AgNCs to detect OTA. But this method only detected one mycotoxin so that reduced efficiency of detection, and failed to discriminate coexistence of multi-mycotoxins [22]. According to reports, DNA-scaffolded AgNCs have an interesting characteristic that through rationally change the sequence and length of DNA signal probes, the photoluminescence emission spectra of DNA/AgNCs being able to fine-tuned [25]. Taking advantage of this property, many strategies based on DNA-scaffolded AgNCs have been developed for multiple detection of microRNAs or DNA [26-28]. These assays clearly confirmed that the simultaneous detection for multiple mycotoxins in real sample using DNA/AgNCs was technically possible. Furthermore, Subhadip Ghosh et al. reported

the fluorescence intensity of human serum albumin (HSA)-template AgNCs was increased in the presence of Zn(II)-ion [29]. Similarly, we found the analogous phenomenon that the fluorescence intensity of DNA/AgNCs would be obviously enhanced by Zn(II)-ion.

Stimulated by all above studies, a rapid, simple and signal-on fluorescent aptasensor based on DNA/AgNCs and Zn(II)-ion signal-enhancement was developed for simultaneous detection of OTA and AFB₁. To the best of our knowledge, this method was the first time to use DNA/AgNCs for simultaneous detection of mycotoxins. The fluorescence intensity of this aptasensor exhibited good linear relationship with OTA and AFB₁ in the concentrations both ranging from 0.001 ng/mL to 0.05 ng/mL, and the detection limits were down to 0.2 pg/mL for OTA and 0.3 pg/mL for AFB₁. The experimental results indicated that the proposed aptasensor was great efficient in simultaneous detection of two mycotoxins, providing a promising approach for guaranteeing public health and food safety in the future.

2. Experimental Section

2.1. Reagents and apparatus

All oligonucleotides were synthesized by Sangong Biotech (Shanghai, China) Co.,Ltd., and their base sequences were: OTA aptamer (Ap1): 5'-GAT CGG GTG TGG GTG GCG TAA AGG

GAG CAT CGG ACA-biotin-3' [17]; AFB₁ aptamer (Ap2): 5'-AGG GTG GTT GGG CAC GTG TTG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CC-biotin-3' [18]; Sp1 (hybridization with Ap1): 5'-AAT TCC CCC CCC ACC C-3' and Sp2 (hybridization with Ap2): 5'-CCC ACC CAC CCT CCC A-3' were chosen as templates to synthesize DNA/AgNCs with different emission bands. In order to synthesize the AgNCs with different emission bands and obtain relative stable DNA duplex, we designed several mismatch sites in the sequence of Sp1 and Sp2 according to the Ap1 and Ap2, respectively. The melting temperature (T_m) value of Ap1-Sp1 and Ap2-Sp2 is 48.7 °C and 56.6 °C, respectively (Figure S1. a and b), which indicates that the DNA duplex are stable in the detection condition. The concentrations of all DNA sequences were quantified by OD₂₆₀ based on their individual absorption coefficients. OTA, AFB₁, ochratoxin B (OTB), aflatoxin B₂ (AFB₂), vomitoxin (DON) and zearalenone (ZEA) were purchased from Sigma-Aldrich (Shanghai, China). Streptavidin-modified magnetic beads (MBs 10 mg/mL) were purchased from Dynal Biotech ASA (Oslo, Norway). Enzyme-linked Immunosorbent assay (ELISA) Kits of OTA and AFB₁ were bought from Youlong Biotech Co., Ltd. (Shanghai, China). All other chemicals were analytical grade and all aqueous solutions were prepared with MilliQ water (18 MΩ).

Fluorescence spectra were measured with a Cary Eclipse fluorescence spectrometer (Agilent Technologies).

2.2. Preparation of two aptamers self-assembly and hybridization on magnetic beads

The Aps self-assembly and hybridization were prepared according to our previous report with some slight modifications [22]. Aps self-assembly and hybridization included following procedures. (1) The 50 μ L streptavidin coated MBs were transferred into a 2.0 mL centrifuge vial. Then the MBs were rinsed three times with immobilization buffer (pH 7.5, 10 mM Tris, 1.0 M NaNO₃, 1.0 mM EDTA and 0.01 % Tween 20) under the magnetic field. (2) The rinsed MBs were resuspended in the same immobilization buffer containing 5 μ M OTA aptamer and 5 μ M AFB₁ aptamer that both were biotinylated. The above system was incubated at 37 °C for 30 min with gentle mixing, and two types of aptamers were immobilized onto the MBs surface. (3) Aps-modified MBs were resuspended in the hybridization buffer (pH 7.5, 10 mM Tris, 1.0 M NaNO₃, 1.0 mM EDTA and 0.01 % Tween 20) containing 10 μ M Sp1 and 10 μ M Sp2 to hybridize for 2 h at 40 °C after washed three times with hybridization buffer. (4) Under the magnetic field, the Aps-Sps modified MBs were washed three times with trisodium citrate buffer (10 mM, pH 7.0) to discriminate against unwanted

constituents, including excess of signal probes, and then resuspended in 100 μ L trisodium citrate buffer.

2.3. Preparation of the fluorescent aptasensor for OTA and AFB₁ detection

During the AgNCs synthesis, the Aps-Sps modified MBs were dispersed in the trisodium citrate buffer. A certain concentration of OTA and AFB₁ standard solutions were mixed in trisodium citrate buffer containing above MBs. Under the magnetic field, the MBs were isolated. The 50 μ L supernatant liquid was transferred into another centrifuge tube. Subsequently, 20 μ L 1 mM AgNO₃ and 70 μ L trisodium citrate buffer were added into the same centrifuge tube. The mixture was incubated for 10 min at room temperature in dark. Then 50 μ L 200 μ M NaBH₄ and 10 μ L Zn(II)-ion were added into above mixture, and reacted for 5 min at 45 °C. The resulting solution was transferred into spectro fluorometer cuvette. Finally, the fluorescent DNA-scaffolded AgNCs exhibited two fluorescence emission peaks at 630 nm (excitation 580 nm) and/or 770 nm (excitation 720 nm) at room temperature, respectively.

2.4. Pretreatment of cereal sample

Cereal samples purchased from the local supermarket including rice, corn, and wheat were pretreated by the previous procedures with some slight modifications [21]. Briefly, the samples were first

shaken in 10 mL of methanol-water (8:2 v/v) solution for 2 h at 6 g, and then the extracts were centrifuged at 3000 g for 20 min. Subsequently, the solution was further filtered with glass fiber filter paper until the filtrate was clear. Last, the resulting solution was diluted by trisodium citrate buffer.

3. Results and discussion

3.1. Experimental mechanism of the aptasensor

Figure 1 demonstrates the mechanism of this method. OTA aptamer (Ap1, colored green) and AFB₁ aptamer (Ap2, colored purple) both modified with biotin at one end were immobilized on the surface of MBs modified with streptavidin. Then single-stranded Sp1 (colored black) and single-stranded Sp2 (colored red) were added to hybridize with Ap1 and Ap2 to form Aps-Sps duplex structure, respectively. In the presence of OTA and AFB₁, they bound with their corresponding Ap to form G-quadruplex and AFB₁-Ap2 complex. The stability of Ap-Sp duplex was lower than G-quadruplex or complex, which resulted in Sp1 and Sp2 being released. Through magnetic separation, the released Sp1 and Sp2 was left in the supernatant liquid that were acted as the scaffold for the synthesis of DNA-scaffolded AgNCs with producing different fluorescence emission peaks. Subsequently, during the DNA/AgNCs synthesis, adding Zn(II)-ion into the solution, the fluorescence

intensity was significantly increased. The experimental result showed that the Sp1-scaffolded DNA/AgNCs presented the characteristic emission maximum at 630 nm upon the excitation at 580 nm for OTA detection, while Sp2-scaffolded DNA/AgNCs presented the characteristic emission maximum at 770 nm upon the excitation at 720 nm as the signal indicator for AFB₁. As shown in Figure 2 inset, the typical transmission electron microscopy (TEM) image showed that all the DNA-AgNCs were monodispersed and uniform. At the same time, the TEM image of DNA-AgNCs before adding Zn(II)-ion was shown in Figure S2. The shapes of DNA-AgNCs before and after adding Zn(II)-ion were almost not changed. More importantly, the fluorescent emission peaks of two types of DNA/AgNCs did not overlap. In contrast, in the absence of the OTA and AFB₁, Sp1 and Sp2 hybridized with Ap1 and Ap2, respectively, and then were separated by the magnet. Therefore, the supernatant liquid contained no single-stranded DNA Sps, and cannot exhibit fluorescence emission peak. Due to the use of MBs, the operation of the sensor is convenient and efficient. Based on above facts, a simple, selective and sensitive approach for simultaneous detection of OTA and AFB₁ was developed. In addition, aptamer modified MBs were easily recycled and safer to the environment.

Figure 1. should be inserted in here

3.2. Optimization of detection conditions

In this system, several crucial factors that may affect fluorescence intensity were optimized prior to the application of our proposed method, such as incubation time for Aps immobilization, reaction time, temperature of hybridization, and the concentration of AgNO_3 and NaBH_4 . The optimal conditions of maximum fluorescent intensity were as follows: 30 min was used as the optimal reaction time for the aptamers immobilization. The optimal reaction temperature was 37°C , and the time of hybridization was 120 min, respectively. $100\ \mu\text{M}$ AgNO_3 , $50\ \mu\text{M}$ NaBH_4 , and $45\ ^\circ\text{C}$ incubation temperature were chosen to synthesize DNA/AgNCs.

3.3. Fluorescence and stability measurements of Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs

As shown in Figure S3, the synthesized Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs (without and with Zn(II) -ion) produced fluorescence emission at 630 nm and 770 nm upon the excitation 580 nm and 720 nm, respectively. Meanwhile, the aqueous solution of the Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs showed red luminescence under excitation with an ultraviolet analyzer (Figure S3, Inset, a' and b'), and the red luminescence was stronger after adding Zn(II) -ion (Figure S3, Inset, a and b). In order to

acquire the maximum signal enhancement, we studied the relationship between Zn(II) -ion concentration and signal enhancement effect. As shown in Figure S6, the optimal concentration of Zn(II) -ion was 5 mM. In addition, the stability of the fluorescence emission of Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs was studied without and with Zn(II) -ion. The experimental results indicated that there was no obvious decrease in the fluorescence intensities of Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs without and with Zn(II) -ion at room temperature within 2 h (Figure S7 a, b, c and d, respectively). Furthermore, Sp1-scaffolded AgNCs and Sp2-scaffolded AgNCs were interacted with OTA and AFB₁. As shown in Figure S4. the fluorescence intensity was little increase. In a word, the synthesized two kinds of DNA-scaffolded AgNCs had remarkable optical characteristics as well as can be acted as signal indicators for the development of effective AgNCs-based aptasensor for mycotoxins simultaneous detection.

3.4. Fluorescence enhancements of DNA-scaffolded AgNCs by Zn(II) -ion

During the experiment, we paid attention to an interesting phenomenon that the fluorescence intensity was increased significantly after adding Zn(II) -ion into the solution. In order to

illustrate the degree of signal-enhancement by Zn(II) -ion, we compared the change of fluorescence signals in the absence and presence of Zn(II) -ion. As shown in Figure 2, when both OTA and AFB_1 were absent, the fluorescence intensity was almost neglectable after adding Zn(II) -ion into the solution, demonstrating that Zn(II) -ion was no fluorescence (Figure 2, curve a and a'). Subsequently, in the presence of OTA and AFB_1 , upon addition of Zn(II) -ion, the fluorescence intensity of Sp1-scaffolded AgNCs was nearly 10 folds stronger than the case without Zn(II) -ion (Figure 2, curves b and c, respectively), and the fluorescence intensity of Sp2-scaffolded AgNCs was almost 9 folds stronger than the case without Zn(II) -ion (Figure 2, curves b' and c', respectively). In addition, we compared the change of fluorescence intensity in the presence of Zn(II) -ion and other metal ions, such as Cu(II) -ion and Co(II) -ion. The results indicated that Zn(II) -ion provided stronger fluorescence intensity than other metal ions (see Figure S5. in the support information). Based on above facts, we can take advantage of Zn(II) -ion to improve fluorescence intensity, thereby raising the sensitivity of the aptasensor.

The stability of DNA-scaffolded AgNCs is one of the most probable mechanisms explaining the enhancement of fluorescent intensity of DNA/AgNCs by Zn(II) -ion [29]. Single-strand DNA

(ssDNA) contains many heteroatoms, such as nitrogen, oxygen, and phosphor donor atoms. As an acid of borderline hardness, Zn(II)-ion has a strong affinity for above heteroatoms [30] so that Zn(II)-ion is bound to ssDNA to form core shell of Zn-Ag nanoclusters, which makes the DNA/AgNCs become more stable. In order to confirm this opinion, we studied the fluorescence intensities of two kinds of AgNCs without and with Zn(II)-ion within 5 h. The fluorescence intensity of Sp1-scaffolded AgNCs was decrease 16.25 %, but that was only decrease 0.96 % after adding Zn(II)-ion (see Figure S7, a and c). Likely, as shown in Figure S7 b and d, we found the analogous phenomenon for Sp2-scaffolded AgNCs. (the fluorescence intensity was decrease 27.07 % and 3.84 % without and with Zn(II)-ion, respectively). Thus, based on above experiments, the more stable AgNCs under the action of Zn(II)-ion increase the fluorescence intensity. The mechanism is supported by the fact of enhancement in fluorescence intensity. In addition, more mechanisms are being researched in our groups.

Figure 2. should be inserted in here

3.5. Sensitivity of the aptasensor

The proposed fluorescent aptasensor can detect OTA and AFB₁ separately and simultaneously. For demonstrating its sensitivity, the following work was implemented. A series of different

concentrations of OTA or AFB₁ in the range from 0.001 ng/mL to 0.05 ng/mL were tested under the optimal condition. In order to detection of each mycotoxin, the fluorescence emission spectra of DNA/AgNCs scaffolded with Sp1 and Sp2 were recorded at two different excitation wavelengths of 580 nm and 720 nm, respectively. As shown in Figure 3A., in the presence of OTA (no AFB₁ presence), a dynamic increase fluorescence emission peaks at 630 nm were observed with the increasing concentrations of OTA ranging from 0.001 ng/mL to 0.05 ng/mL, and the calibration equation obtained from this curve was $F=10234.839C+69.2206$ ($r=0.9930$). However, the fluorescence emission peaks at 770 nm were almost negligible. As for AFB₁, as shown in Figure 3B, for quantitatively sensing AFB₁ (no OTA presence), AFB₁ of different concentrations were investigated. It was clearly indicated that with the increase of AFB₁ concentration, the fluorescence emission peaks at 770 nm increased gradually (calibration equation: $F=7479.708C+36.3623$, $r=0.9962$), while the emission peaks at 630 nm exhibited negligible fluorescence responses.

However, there was a crucial question that whether the method existed fluorescence interference between two types of probes when OTA and AFB₁ were both present. In order to solve this issue, we tested the fluorescence response of a series of OTA concentrations

ranging from 0.001 ng/mL to 0.05 ng/mL with the existence of AFB₁ ranging from 0.001 ng/mL to 0.05 ng/mL. From Figure 3C., it was found that the fluorescence intensity at 630 nm and 770 nm was almost equal with that in the presence of either OTA or AFB₁, and the two fluorescence emission peaks were not overlapped. Moreover, under the optimal condition of simultaneous detection, the calibration equation of OTA and AFB₁ were $F=9947.2C+31.29$ ($r=0.9980$) as well as $F=8595.7C+35.136$ ($r=0.9908$), respectively. Due to above experimental results, the detection limit of AFB₁ was 0.3 pg/mL and OTA was 0.2 pg/mL according to the definition $3\sigma/\text{slope}$ (σ , standard deviation of corresponding the blank sample; slope, the slope of corresponding calibration equation). The precision, expressed as the relative standard deviation (RSD) of OTA detection, was equal to 4.74 % (0.05 ng·mL⁻¹, n=5). The RSD of the AFB₁ detection was equal to 5.86 % (0.05 ng mL⁻¹, n=5), indicating that the developed method exhibited good reproducibility.

These results clearly showed that the aptasensor possessed a remarkable sensitivity compared with other methods (listed in Table S1), and there almost wasn't interference when these two mycotoxins were both present. The whole detection process took 16 min after adding OTA and AFB₁ into the system. Compared with some other methods, our strategy has high efficiency than most

methods (see Table S3 in support information). Although the aptasensor based on Tb³⁺ is more efficient, it cannot achieve the goal that detects two kinds of mycotoxins simultaneously. In addition, the aptasensor based on chemiluminescence resonance energy transfer (CRET) is slightly higher efficiency than this paper. However, as a “signal-off” method, it is susceptible to interference, leading to false positive. Based on above facts, our method is more suitable for simultaneous detection of OTA and AFB₁.

Figure 3. should be inserted in here

3.6. Specificity of the aptasensor

The selectivity was a pivotal issue for the developed aptasensor. Due to interference from other mycotoxins in the real sample, we further compared the change of fluorescent intensity induced by other mycotoxins. As shown in Figure 4A, 0.05 ng/mL OTB, AFB₁, AFB₂, DON, ZEA and a mixture of above mycotoxins (Mix1) were tested under the optimized conditions. The response signals of these mycotoxins were almost neglectable compared with that of OTA. Moreover, when Mix1 was with OTA (Mix2), the sensing signal was almost equal with that of OTA. Likewise, as shown in Figure 4B, we observed that the fluorescence intensity of 0.05 ng/mL AFB₂, OTA, OTB, DON, ZEA and a mixture of above mycotoxins (Mix3) had little change compared with the fluorescence intensity of AFB₁.

When AFB₁ and Mix3 coexisted (Mix4), the fluorescence intensity was nearly identical with that AFB₁. Based on above experimental results, our fluorescent aptasensor could eliminate the interference from other mycotoxins and present ultra-high selectivity, which indicated that the aptasensor was sufficient for simultaneous detection of OTA and AFB₁.

Figure 4. should be inserted in here

3.7. Simultaneous determination of OTA and AFB₁ in real sample

The recovery rates were performed when the three levels of concentration of OTA and AFB₁ were spiked in wheat samples, rice and corn. As shown in Figure 5, the results indicated that the recovery rate was between $88.5 \pm 6.90 \%$ to $113.5 \pm 5.30 \%$ for OTA and $88.1 \pm 5.70 \%$ to $116.8 \pm 4.90 \%$ for AFB₁, respectively. All recovery rates exceeded the 80 % range. In order to further evaluate the practical applicability and accuracy, the method was used for determining the recoveries by spiking three different concentrations of OTA and AFB₁ into the real wheat sample. The results were shown in Table S2 together with the data determined with ELISA. The results obtained with our biosensor were consistent with those obtained with ELISA, implying that the proposed sensor could therefore be applied to the quantitative determination of OTA and AFB₁ in agricultural commodities samples.

Figure 5. should be inserted in here

4. Conclusion

In conclusion, we constructed a simple, effective and selective fluorescent aptasensor for simultaneous detection of OTA and AFB₁. By rationally changing the DNA sequence, the photoluminescence emission bands of DNA-scaffolded AgNCs can be changed accordingly. During the simultaneous detection, there was no interference between these two types of DNA-scaffolded AgNCs when OTA and AFB₁ were present at the same time. Moreover, adding Zn(II)-ion into this system can increase the fluorescence intensity of DNA-scaffolded AgNCs, and thereby improved the sensitivity of the aptasensor. As discussed above, the proposed method not only detected the two mycotoxins, but also distinguished their coexistence. Therefore, our aptasensor was more reliable and efficient for the application on food safety regarding mycotoxins. Furthermore, it can simultaneously detect more types of mycotoxins by changing corresponding aptamer probe and signal probe. So the proposed aptasensor has a great potential for more extensive application on food safety and public health in the future.

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References

- [1] S. Marin, A.J. Ramos, G. Cano-Sancho, V. Sanchis, Mycotoxins: Occurrence, toxicology, and exposure assessment, *Food Chem. Toxicol.* 60 (2013) 218-237.
- [2] A. Vidal, S. Marin, A.J. Ramos, G. Cano-Sancho, V. Sanchis, Determination of aflatoxins, deoxynivalenol, ochratoxin A and zearalenone in wheat and oat based bran supplements sold in the Spanish market, *Food Chem. Toxicol.* 53 (2013) 133-138.
- [3] D.W. Kolpin, J. Schenzel, M.T. Meyer, P.J. Phillips, L.E. Hubbard, T.M. Scott, T.D. Bucheli, Mycotoxins: Diffuse and point source contributions of natural contaminants of emerging concern to streams, *Sci. Total. Environ.* 470-471 (2014) 669-676.
- [4] F. Wu, J.D. Groopman, J.J. Pestka, Public Health Impacts of Foodborne Mycotoxins, *Annu. Rev. Food Sci. Technol.* 5 (2014)

351-372.

- [5] E.V. Pamel, A. Verbeken, G. Vlaemynck, J.D. Boever, E. Daeseleire, Ultrahigh-Performance Liquid Chromatographic-Tandem Mass Spectrometric Multimycotoxin Method for Quantitating 26 Mycotoxins in Maize Silage, *J. Agric. Food Chem.* 59 (2011) 9747-9755.
- [6] Y.K. Wang, Y.X. Yan, S.Q. Li, H.A. Wang, W.H. Ji, J.H. Sun, Simultaneous Quantitative Determination of Multiple Mycotoxins in Cereal and Feedstuff Samples by a Suspension Array Immunoassay, *J. Agric. Food Chem.* 61 (2013) 10948-10953.
- [7] Z. Ul-Hassan, M.Z. Khan, A. Khan, I. Javed, Immunological status of the progeny of breeder hens kept on ochratoxin A (OTA)-and aflatoxin B1 (AFB1)-contaminated feeds, *J. Immunotoxicol.* 9 (2012) 381-391.
- [8] X.F. Xue, J.N. Selvaraj, L.W. Zhao, H.M. Dong, F.M. Liu, Y. Liu, Y. Li, Simultaneous Determination of Aflatoxins and Ochratoxin A in Bee Pollen by Low-Temperature Fat Precipitation and Immunoaffinity Column Cleanup Coupled with LC-MS/MS, *Food Anal. Methods* 7 (2014) 690-696.
- [9] A. Roland, P. Bros, A. Bouisseau, F. Cavelier, R. Schneider, Analysis of ochratoxin A in grapes, musts and wines by

LC-MS/MS: First comparison of stable isotope dilution assay and disastereomeric dilution assay methods, *Anal. Chim. Acta* 818 (2014) 39-45.

- [10] L. Wang, Z. Wang, W. Gao, J. Chen, M. Yang, Y. Kuang, L. Huang, S. Chen, Simultaneous determination of aflatoxin B1 and ochratoxin A in licorice roots and fritillary bulbs by solid-phase extraction coupled with high-performance liquid chromatography-tandem mass spectrometry, *Food Chem.* 138 (2013) 1048-1054.
- [11] D. Saha, D. Acharya, D. Roy, D. Shrestha, T.K. Dhar, Simultaneous enzyme immunoassay for the screening of aflatoxin B1 and ochratoxin A in chili samples, *Anal. Chim. Acta* 584 (2007) 343-349.
- [12] Y. Wang, X. Hu, Y. Pei, Y. Sun, F. Wang, C. Song, M. Yin, R. Deng, Z. Li, G. Zhang, Selection of phage-displayed minotopes of ochratoxin A and its detection in cereal by ELISA, *Anal. Methods* 7 (2015) 1849-1854.
- [13] B.H. Liu, Y.T. Hsu, C.C. Lu, F.Y. Yu, Detecting aflatoxin B1 in foods and feeds by using sensitive rapid enzyme-linked immunosorbent assay and gold nanoparticle immunochromatographic strip, *Food Control* 30 (2013) 184-189.
- [14] A.B. Iliuk, L. Hu, W.A. Tao, Aptamer in Bioanalytical

Applications, Anal. Chem. 83 (2011) 4440-4452.

- [15] J. Zhao, X. He, B. Bo, X. Liu, Y. Yin, G. Li, A “signal-on” electrochemical aptasensor for simultaneous detection of two tumor markers, Biosens. Bioelectron. 34 (2012) 249-252.
- [16] Z. Lu, X. Chen, Y. Wang, X. Zheng, C. Li, Aptamer based fluorescence recovery assay for aflatoxin B1 using a quencher system composed of quantum dots and graphene oxide, Microchim Acta 182 (2015) 571-578.
- [17] J. Zhang, X. Zhang, G. Yang, J. Chen, S. Wang, A signal-on fluorescent aptasensor based on Tb³⁺ and structure-switching aptamer for label-free detection of Ochratoxin A in wheat, Biosens. Bioelectron. 41 (2013) 704-709.
- [18] X. Guo, F. Wen, N. Zheng, Q. Luo, H. Wang, H. Wang, S. Li, J. Wang, Development of an ultrasensitive aptasensor for the detection of aflatoxin B1, Biosens. Bioelectron. 56 (2014) 340-344.
- [19] S. Wu, N. Duan, X. Ma, Y. Xia, H. Wang, Z. Wang, Q. Zhang, Multiplexed Fluorescence Resonance Energy Transfer Aptasensor between Upconversion Nanoparticles and Graphene Oxide for the Simultaneous Determination of Mycotoxins, Anal. Chem. 84 (2012) 6263-6270.
- [20] Q. Zhan, J. Qian, H. Liang, G. Somesfalean, D. Wang, S. He, Z.

Zhang, S. Andersson-Engels, Using 915 nm laser excited $\text{Tm}^{3+}/\text{Er}^{3+}/\text{Ho}^{3+}$ -doped NaYbF_4 upconversion nanoparticles for in vitro and deeper in vivo bioimaging without overheating irradiation, *ACS Nano* 5 (2011) 3744-3757.

[21] Y. Sun, J. Xu, W. Li, B. Cao, D. Wang, Y. Yang, Q. Lin, J. Li, T. Zheng, Simultaneous Detection of Ochratoxin A and Fumonisin B1 in Cereal Samples Using an Aptamer-Photonic Crystal Encoded Suspension Array, *Anal. Chem.* 86 (2014) 11797-11802.

[22] J. Chen, X. Zhang, S. Cai, D. Wu, M. Chen, S. Wang, J. Zhang, A fluorescent aptasensor based on DNA-scaffolded silver-nanocluster for ochratoxin A detection, *Biosens. Bioelectron.* 57 (2014) 226-231.

[23] P. Shah, P.W. Thulstrup, S.K. Cho, Y.J. Bhang, J.C. Ahn, S.W. Choi, M.J. Bjerrum, S.W. Yang, In-solution multiplex miRNA detection using DNA-templated silver nanocluster probes, *Analyst* 139 (2014) 2158-2166.

[24] G.Y. Lan, C.C. Huang, H.T. Chang, Silver nanoclusters as fluorescent probes for selective and sensitive detection of copper ions, *Chem. Commun.* 46 (2010) 1257-1259.

[25] J.T. Petty, S.P. Story, J.C. Hsiang, R.M. Dickson, DNA-Templated Molecular Silver Fluorophores, *J. Phys. Chem.*

Lett. 4 (2013) 1148-1155.

- [26] X. Tian, X.J. Kong, Z.M. Zhu, T.T. Chen, X. Chu, A new label-free and turn-on strategy for endonuclease detection using a DNA-silver nanocluster probe, *Talanta* 131 (2015) 116-120.
- [27] M. Zhang, Y.Q. Liu, C.Y. Yu, B.C. Yin, B.C. Ye, Multiplexed detection of microRNAs by tuning DNA-scaffolded silver nanoclusters, *Analyst* 138 (2013) 4812-4817.
- [28] Y. Zhang, C. Zhu, L. Zhang, C. Tan, J. Yang, B. Chen, L. Wang, H. Zhang, DNA-Templated Silver Nanoclusters for Multiplexed Fluorescent DNA Detection, *small* 11 (2015) 1385-1389.
- [29] S. Ghosh, U. Anand, S. Mukherjee, Luminescent Silver Nanoclusters Acting as a Label-Free Photoswitch in Metal Ion Sensing, *Anal. Chem.* 86 (2014) 3188-3194.
- [30] B. Becker, A. Dołęga, A. Konitz, W. Wojnowski, Zinc tri-*tert*-butoxysilanethiolates. Syntheses, properties and crystal and molecular structures of $[\text{Zn}\{\mu\text{-SSi}(\text{OBu}')_3\}(\text{acac})]_2$ and $[\{(\text{Bu}'\text{O})_3\text{SiS}\}(\text{H}_2\text{O})_2\text{Zn}\{\mu\text{-SSi}(\text{OBu}')_3\}\text{Zn}(\text{acac})\{\text{SSi}(\text{OBu}')_3\}]$, *Polyhedron* 20 (2001) 949-956.

Figure Captions

Figure 1. Principle of the aptasensor

Figure 2. The ability of signal-enhancement. Curve a and a' is the case that Zn(II)-ion is involved in the solution, while OTA and AFB₁ being absence. Curve b and b' represent OTA and AFB₁ are presence, while Zn(II)-ion being absent. Curve c and c' represent OTA, AFB₁ and Zn(II)-ion are presence in the system. Inset: a typical TEM image of the DNA-AgNCs.

Figure 3. (A) Fluorescence spectra of the aptasensor with different concentrations of OTA: 0.000, 0.001, 0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 ng/mL. Inset: A calibration curve demonstrating peak fluorescence intensity versus OTA concentration. (B) Fluorescence spectra of the aptasensor with different concentrations of AFB₁: 0.000, 0.001, 0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 ng/mL. Inset: A calibration curve demonstrating peak fluorescence intensity versus AFB₁ concentration. (C) Fluorescence spectra of the aptasensor in the simultaneous presence of different concentrations of OTA and AFB₁: 0.000, 0.001, 0.005, 0.010, 0.020, 0.030, 0.040, and 0.050 ng/mL. Inset: A calibration curve demonstrating peak fluorescence intensity versus OTA concentration and AFB₁ concentration.

Figure 4. (A) The histogram of selectivity. Each data point

represents the average value of three independent experiments with error bars indicated. Selectivity of the aptasensor toward OTA against other mycotoxins at the same concentrations (0.05 ng/mL). Blank, OTB, AFB₁, AFB₂, DON, ZEA, Mix1, Mix2 and OTA. **(B)** The histogram of selectivity. Each data point represents the average value of three independent experiments with error bars indicated. Selectivity of the aptasensor toward AFB₁ against other mycotoxins at the same concentrations (0.05 ng/mL). Blank, AFB₂, OTA, OTB, DON, ZEA, Mix3, Mix4 and AFB₁.

Figure 5. Recovery of OTA and AFB₁ in cereal samples (n=5).

Figure 1.

Figure 2.

Figure 3. (A)

Figure 3. (B)

Figure 3. (C)

Figure 4.

Figure 5.

Biographies

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