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# Antibody- and aptamer-based competitive fluorescence polarization/anisotropy assays for ochratoxin A with tetramethylrhodamine-labeled ochratoxin A†

Yapiao Li[ab] and Qiang Zhao *[abc]

Ochratoxin A (OTA) is one of the mycotoxins that often contaminate a variety of food stuffs, and it is a potential carcinogen for humans. Taking advantage of selective affinity binding and simple, rapid, and sensitive fluorescence polarization (FP)/fluorescence anisotropy (FA) analysis, here, we report two competitive FP/FA assays for OTA using tetramethylrhodamine (TMR)-labeled OTA as a fluorescence tracer and either antibody or aptamer as an affinity ligand to recognize OTA. In the absence of OTA, the TMR-labeled OTA binds with a large-sized affinity ligand, showing a high FA value due to the slow rotation of the affinity complex. When OTA is present, OTA competes with the TMR-labeled OTA tracer in binding limited amount of affinity ligand, causing more free TMR-labeled OTA and a significant FA decrease. We found that the antibody showed a stronger affinity towards TMR-labeled OTA compared to the aptamer. The antibody-based FA assay showed higher signal changes than the aptamer based FA assay due to the larger size of antibody over aptamer. The antibody-based competitive FA assay enabled the detection of 2.4 nM OTA, while the aptamer-based FA assay also achieved a detection limit of 2.4 nM OTA at 10 °C with the help of streptavidin conjugation to increase the molecular size and to improve aptamer affinity. These two competitive FA assays were selective, showing capability for analysis in diluted red wine.

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## 1. Introduction

Ochratoxin A (OTA), a mycotoxin produced by *Aspergillus* and *Penicillium* fungi, is a common contaminant of a variety of food and agricultural commodities, *e.g.*, peanuts, corn, grains, grapes, and dried fruits.[1,2] OTA is a potential carcinogen (group 2B) for humans.[1,2] Sensitive and rapid detection of OTA is demanded in environmental analysis, food safety and quality control. High performance liquid chromatography (HPLC) and mass spectrometry (MS) combined with special sample preparation are often used for sensitive quantification of OTA,[3,4] but they usually have some limitations such as time-consuming and laborious processes, expensive instruments, and requirement of professional personnel.

Fluorescence polarization/anisotropy (FP/FA) analysis is a homogenous fluorescence method measuring the rotation change of fluorescent molecule under polarized excitation light, and it is useful for the study of molecular binding and assay developments.[5,6] The FP/FA method provides a rapid, simple, sensitive, and robust approach for target detections.[5–7] FP and FA are two interchangeable terms, and they are frequently used in the assays. We prefer using the FA term unless otherwise stated. The immune-fluorescence polarization (FP) assay (also called fluorescence polarization immunoassay (FPIA)) is an often used FP assay for the detection of small molecules by taking the advantage of an antibody as an affinity ligand and a fluorescently labeled small molecule probe.[7–9] In the immune-FP assay, the fluorescent probe rotates rapidly and has a low FP value in an unbound state. The FP value of the probe increases when the fluorescent probe binds with the antibody due to the increase in the molecular volume and slow rotation. The small molecule target competes with the fluorescent probe in binding antibody, leading to a decrease in FP. A few immune-FP/FA assays have been applied to OTA detection with the antibody and the fluorescein (FAM)-labeled OTA probe, achieving the detection of OTA at nM levels.[8–15] One reported immune-FP assay for the OTA analysis in a red wine sample allowed to detect 1.7 nM OTA.[11]

Aptamer is a nucleic acid affinity ligand, showing some advantages over the antibody, such as easy preparation and

[a]*State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: qiangzhao@rcees.ac.cn*

[b]*University of Chinese Academy of Sciences, Beijing 100049, China*

[c]*School of Environment, Hangzhou Institute for Advanced Study, UCAS, Hangzhou 310000, China*

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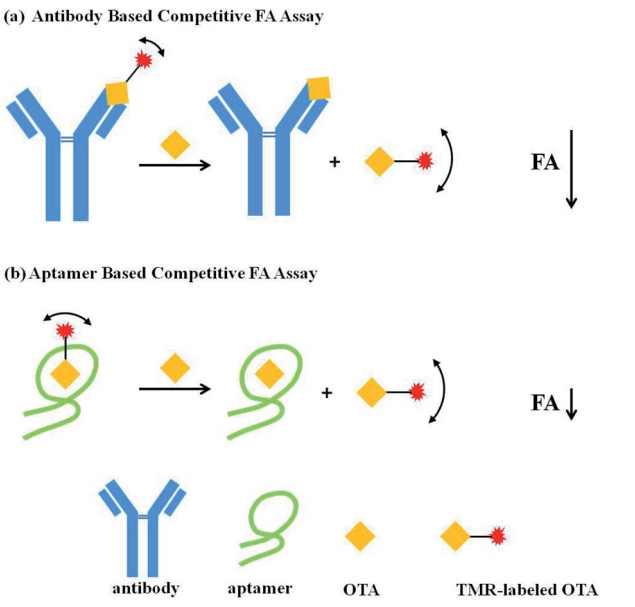
labeling with functional groups, high stability, low cost, and ease of modulating the molecular mass of aptamer.[6,16–20] Recently, aptamer-based FP/FA assays have attracted increasing attention for the detection of small molecules.[7,9,20] Since the selection of DNA aptamer specifically binding OTA was reported,[21] new and diverse formats of FP/FA assays (*e.g.*, direct assay and competitive assay) for OTA detection have been developed.[20,22–25]

Samokhvalov *et al.* reported a competitive FP assay for OTA using the FAM-labeled OTA as a fluorescence tracer and the aptamer-streptavidin-IgG conjugate as a large-sized affinity ligand,[24] and the reported detection limit was about 3.6 nM. Tetramethylrhodamine (TMR) is another one of the commonly used fluorophores in numerous fluorescence assays.[5–7,9] TMR and FAM both have high quantum yield; however, TMR has a relatively shorter fluorescence lifetime than FAM. In neutral solutions, TMR is positively charged, while FAM is negatively charged, and FAM and TMR may have different interactions with protein or DNA.[5–7,9] By now in the competitive FP/FA assays using aptamer or antibody for OTA, the use of TMR-labeled OTA as a fluorescent probe is still rarely reported. It is worth exploring to use TMR-labeled OTA in the competitive FP/FA assays. In addition, the comparison of affinity properties and performances of aptamer and antibody towards OTA in the FA analysis is limited.

In this study, we reported competitive FA assays for OTA using TMR-labeled OTA as a fluorescent probe and the antibody or the DNA aptamer as an affinity ligand. We obtained the TMR-labeled OTA by the reaction between the carboxylic group OTA and the amino group of the TMR fluorophore, which was reported in our previous study.[26] Scheme 1 shows the principle of the antibody-based or the aptamer-based competitive FA assays using the TMR-labeled OTA. The complex of the fluorescent probe and the affinity ligands (antibody (Scheme 1a) and aptamer (Scheme 1b)) has a large molecular volume, so the TMR-labeled OTA probe rotates slowly and shows a high FA signal. With the addition of OTA in the sample solution, OTA competes with the TMR-labeled OTA probes, and more TMR-labeled OTA probes become unbound, which tumble rapidly, so FA signal decreases. We determined the binding affinities of the antibody and the aptamer towards the TMR-labeled OTA *via* the FA analysis. We then developed antibody-based competitive FA assay and aptamer-based competitive FA assays, and compared their performances.




Scheme 1 Schematic of antibody-based competitive FA assays (a) and aptamer-based competitive FA assays (b) for OTA by using the TMR-labeled OTA probe. The binding of the TMR-labeled OTA probe and the affinity ligands (antibody or aptamer) slows down the rotation of the TMR label and causes a high FA value. The presence of OTA in a sample solution leads to a FA decrease because more TMR-labeled OTA probes become unbound due to the OTA competition in binding with the affinity ligand.

## 2. Experimental

### 2.1 Chemical and reagents

The mouse monoclonal antibody specific to OTA was purchased from Abcam. The unlabeled aptamer for OTA (5′-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-3′) and the biotinylated aptamer at the 3′ end were synthesized and purified by Sangon Biotech (Shanghai, China). Ochratoxin A (OTA), ochratoxin B (OTB), fumonisin B1 (FB1), fumonisin B2 (FB2), aflatoxin B1 (AFB1), zearalenone (ZAE) were purchased from Pribolab (Singapore) Co. Ltd. The details of preparation procedures and purification of the TMR-labeled OTA probes were provided in our previous report.[26] We obtained three TMR-labeled OTA probes, denoted as probe 1, probe 2 and probe 3, and the corresponding retention times were 6.3 min, 8 min, and 9.3 min, respectively, during HPLC purification.[26] Ultrapure water was obtained from a Purelab Ultra Elga Labwater system. Other reagents used in the experiment were of analytical grade. Red wine was purchased from the local supermarket (UK).

### 2.2 Competitive FA assay

FA measurements were performed by a JASCO fluorescence spectrometer (FP-8300, Japan), which was equipped with a thermostat for the temperature control. An FA signal was determined with excitation at 555 nm and emission at 580 nm. The slits of excitation and emission were both set at 5 nm.

In the antibody-based competitive FA assay, 2 nM TMR-labeled OTA probe 1, 2 nM antibody and various concentrations of OTA were incubated in a 1× PBS buffer (10 mM $Na_2HPO_4$, 1.75 mM $KH_2PO_4$, 2.7 mM KCl, and 137 mM NaCl) (pH 7.5). The detection temperature for the FA analysis was 25 °C unless otherwise stated.

In the aptamer-based competitive FA assay, 20 nM TMR-labeled OTA probe 1, 50 nM aptamer or streptavidin-conjugated aptamer and various concentrations of OTA were mixed with the binding buffer (10 mM Tris–HCl (8.5), 20 mM $CaCl_2$, and 120 mM NaCl). The streptavidin-conjugated aptamer was obtained *via* incubation streptavidin and biotin-labeled aptamer at a ratio of 1 : 1 for 30 min at 4 °C. The detection

temperature for the aptamer-based FA assays was 10 °C unless otherwise stated.

Triplicate analyses were performed in the FA assays, and the error bar in the graphs was the standard deviation from three measurements.

## 3. Results and discussion

### 3.1 Antibody-based competitive FA assay for OTA

We prepared the TMR-labeled OTA by a reaction between the carboxylic group OTA and the amino group of the TMR fluorophore, and the details were reported in our previous study.[26] We obtained three TMR-labeled OTA (probe 1, probe 2 and probe 3), and the retention times of probe 1, probe 2 and probe 3 were 6.3 min, 8 min, and 9.3 min, respectively, during HPLC purification.[26] They were identified as three isomers.[26]

We applied the FA titration analysis to investigate the affinity binding between the TMR-labeled OTA probes and the antibody in the binding buffer (Fig. 1).[6] In the absence of the antibody, the TMR-labeled OTA probes (20 nM) showed a low FA value. With increasing antibody, the FA value gradually increased until it reached a saturated value as the antibody binding caused an increase in the molecular volume and a slowing-down rotation of fluorescent probe. The maximum FA increases caused by the antibody binding were about 0.235, 0.248 and 0.242 for probe 1, probe 2 and probe 3, respectively. Through the FA titrations and nonlinear fittings,[6,25,27] the dissociation constants ($K_d$s) of the antibody were determined to be 2.3 ± 0.4 nM for probe 1, 4 ± 1 nM for probe 2 and 1.6 ± 0.5 nM for probe 3, which were similar to the previously determined $K_d$s of these probes binding to the antibody.[26] The result shows that the antibody has strong binding strength to TMR-labeled OTA and unlabeled OTA.[26] We chose probe 1 as the fluorescent probe in our antibody-based competitive FA assay for OTA detection.

Then, we developed a competitive FA assay for OTA using probe 1 (2 nM) and the antibody (2 nM) by following the principle shown in Scheme 1a. In the absence of OTA, probe 1 bound to the antibody, showing high FA values. Upon OTA addition, the FA value gradually decreased with the increase in

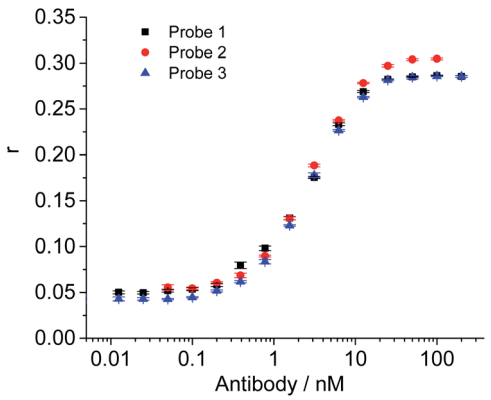



Fig. 1 Fluorescence anisotropy change of the TMR-labeled OTA probes (probe 1, probe 2, and probe 3) (20 nM) upon the increase in the concentrations of the antibody.

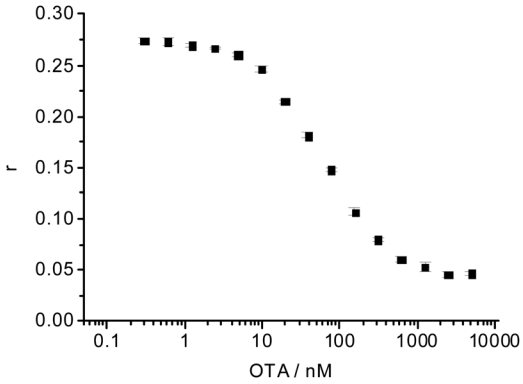



Fig. 2 Detection of OTA with the antibody-based competitive FA assay at 25 °C. Varying concentrations of OTA were incubated with the antibody (2 nM) and probe 1 (2 nM).

OTA because OTA competed with probe 1 in binding to the antibody and more probe 1 became unbound (Fig. 2).

The detection limit was estimated to be about 2.4 nM (0.97 μg $L^{-1}$) OTA based on a distinguished FA signal change was three times standard deviation of the signal of the blank sample. The maximum FA decrease was about 0.228 (the corresponding maximum FP change was about 0.259, Fig. S1, in the ESI†). The dynamic detection range for OTA, which can be detected by the assay with distinguished FA signal changes, was from 2.4 nM to 2500 nM. The detection limit of our antibody-based competitive FA assay was comparable to or better than some of the previously reported immuno-FP assays for OTA (Table S1 in ESI†),[7,11–15] Of note, antibodies from different sources with different affinities and properties can have effects on the assay performance. Our antibody-based competitive FA assay using the TMR-labeled OTA probe showed a larger maximum FA change (or FP change) caused by OTA than the reported immune-FA assay using the FAM-labeled OTA probe.[11–15]

To investigate the assay selectivity towards OTA detection, we tested other mycotoxins such as OTB, AFB1, FB1, FB2 and ZAE in the antibody-based competitive FA assay. As shown in Fig. 3, the tested other mycotoxins (100 nM) did not cause a significant decrease in FA. The presence of OTB, AFB1, FB1, FB2 and ZAE did not interfere with the detection of OTA. These results demonstrate that this immune-FA assay is selective towards OTA detection.

To check the feasibility of this antibody-based competitive FA assay for detecting OTA in a complex sample matrix, we detected OTA spiked in a 100-fold diluted red wine sample (Fig. S2 in ESI†). In the diluted red wine, the FA values of the blank sample were lower than that in the binding buffer. With the increase in OTA, the FA value gradually decreased, and this FA assay achieved a detection limit of 2.4 nM OTA though the signal change was lower than that in the binding buffer. These results indicate that this antibody-based competitive FA assay shows potential for OTA detection in complex sample matrixes.

### 3.2 Aptamer-based competitive FA assay for OTA

Here, we also reported an aptamer-based competitive FA assay for OTA by using a DNA aptamer (5′-GAT CGG GTG TGG GTG

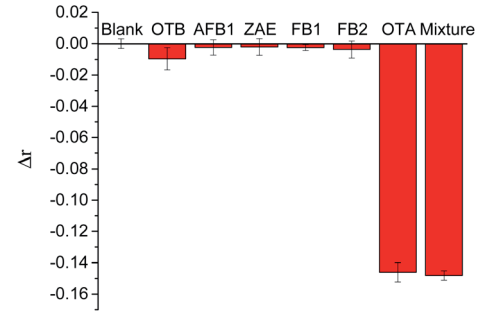



Fig. 3 Selectivity test of the antibody-based competitive FA assay towards OTA. Several different mycotoxins (OTB, AFB1, FB1, FB2, and ZAE) were tested (each of 100 nM) were tested along with OTA (100 nM). The mixture sample contained OTB, AFB1, FB1, FB2, ZAE and OTA (each of 100 nM).

GCG TAA AGG GAG CAT CGG ACA-3′) as an affinity ligand[21] and the TMR-labeled OTA. We determined binding affinities of the TMR-labeled OTA probes to the aptamer in the FA analysis by titrating TMR-labeled OTA probes (100 nM) with various concentrations of the aptamer in the binding buffer containing 10 mM Tris–HCl (pH 8.5), 20 mM $CaCl_2$ and 120 mM NaCl.[6,25,27] Fig. 4 shows that the FA values of probes increased upon the addition of the aptamer, and the maximum FA increase was about 0.057 for probe 1, 0.056 for probe 2, 0.016 for probe 3 at 25 °C (Fig. 4A), which are much lower than the corresponding FA increase induced by the antibody binding because the molecule volume of the antibody (150 000 g per mole) is 13-fold higher than the aptamer (11 311 g per mole). Through FA titration and nonlinear fitting,[27] the $K_d$s of the aptamer were determined to be 116 ± 35 nM for probe 1, 96 ± 6 nM for probe 2, 297 ± 68 nM for probe 3 at 25 °C, which were higher than the $K_d$ of unlabeled OTA binding to the aptamer ($K_d$ about 50 nM).[23,27,28] It suggests that the conjugation of the TMR had some effects on the binding affinity of OTA towards aptamer. In addition, it demonstrates that the aptamer has a lower affinity than the antibody in binding to the TMR-labeled OTA probes and OTA. At a lower temperature (10 °C), the maximum FA increases caused by aptamers were about 0.072 for probe 1, 0.068 for probe 2, and 0.029 for probe 3, respectively (shown in Fig. 4B), because the low temperature reduces the rotation of the affinity complexes. The $K_d$s of the aptamer at 10 °C were determined to be 39 ± 7 nM for probe 1, 72 ± 12 nM for probe 2, 35 ± 6 nM for probe 3. The results also suggest the binding affinity of the aptamer is enhanced at the lower temperature. Detection at 10 °C was applied for the aptamer-based competitive FA assay because the aptamer has a better affinity towards TMR-labeled OTA at this temperature. We used probe 1 to develop the aptamer-based competitive FA assay for detecting OTA.

In the aptamer-based competitive FA assay, 20 nM probe 1, 50 nM aptamer, and various concentrations of OTA were incubated, and FA signals were measured. In the absence of OTA, the complex of the aptamer and probe 1 showed a high FA value. The presence of OTA induced the competition between OTA and probe 1 in binding to the aptamer. With increasing OTA, more fluorescent probes were displaced from the aptamer, and the FA values gradually decreased (Fig. 5). We achieved the detection of OTA as low as 2.4 nM, with a dynamic detection range from 2.4 nM to 625 nM, and the maximum FA change was 0.041. To further improve the FA change, we used the streptavidin (SA)-labeled aptamer as the affinity ligand to increase the molecular mass with a large-sized protein as previously reported,[20,24]

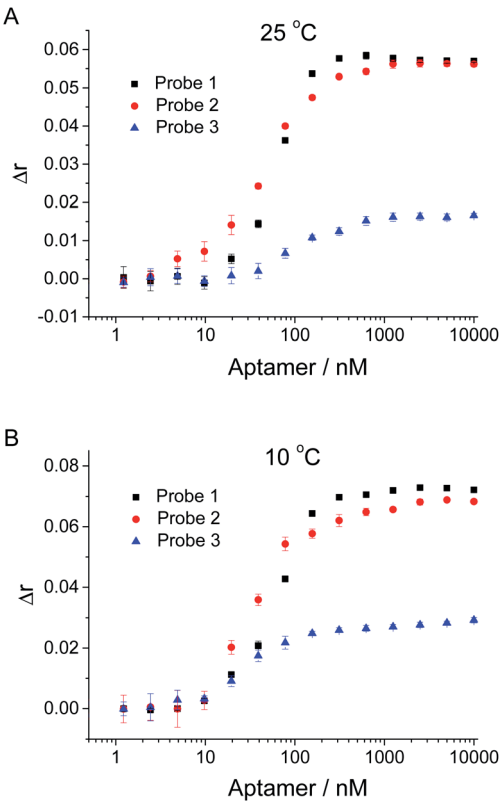



Fig. 4 The net fluorescence anisotropy change of TMR-labeled OTA probes (probe 1, probe 2, and probe 3) upon the addition of various concentrations of the aptamer at 25 °C (A) and 10 °C (B).

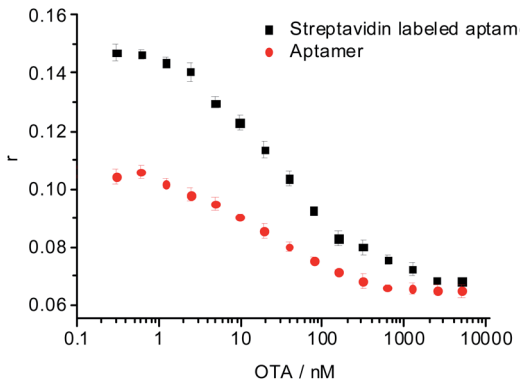



Fig. 5 Detection of OTA by the aptamer-based competitive FA assay. TMR-labeled OTA (probe 1, 20 nM), the aptamer or the streptavidin-labeled aptamer (50 nM) and varying concentrations of OTA were incubated and measured at 10 °C.

which has a larger molecular volume than the aptamer. The SA-conjugated aptamer was prepared by incubating the SA and biotin-labeled aptamer at a ratio of 1 : 1. By using this signal amplification strategy, the maximum FA change was improved to 0.078, and the detection limit of OTA was 2.4 nM with a dynamic detection range from 2.4 nM to 2500 nM. For comparison, the FA assay using aptamer with or without the SA label at 25 °C showed smaller FA signal changes and higher detection limits due to the effects of temperature on the aptamer affinity and FA signal (Fig. S3 in ESI†). At 25 °C, the FA assay using the aptamer gave a detection limit about 10 nM, while the FA assay using the SA-labeled aptamer allowed a detection limit about 5 nM. It shows at 25 °C that the maximum FA signal change from the aptamer-based FA assay is much smaller than that from the antibody-based assay because of the smaller size of the aptamer and the more flexible structure of the aptamer. At 25 °C the signal enhancement from the streptavidin conjugate is not remarkable in the aptamer-based assay, and it may be due to the flexible structure of the aptamer and local rotation of the fluorophore. The aptamer-based FA assay at 10 °C showed a similar OTA detection limit to that of our antibody-based FA assay mentioned above and some of previous FA assays (Table S1 in ESI†).[9,20,22–25] However, the maximum FA change obtained by the aptamer-based FA assay was still lower than that obtained by the antibody-based FA assay (Fig. 2) due to the smaller size of the aptamer (or the SA-labeled aptamer) than the antibody. We found that the aptamer had a weaker affinity to the fluorescently labeled OTA than the antibody and a low detection limit was still observed in the assay using the aptamer at 10 °C. One possible reason is that low temperatures also enhance the affinity of the aptamer towards the unlabeled OTA, and the aptamer still has high affinity towards unlabeled OTA though the aptamer shows a lower affinity towards the TMR-labeled OTA.[23,27,28] In addition, the antibody binds to OTA with a binding ratio of 1 : 2, while the aptamer has a binding ratio of 1 : 1 to the OTA. The difference in binding stoichiometry may also affect the detection limit. The affinity ligand with 1 : 1 binding ratio and high affinity is preferred in the assay to detect low concentration of the target.

This aptamer-based FA assay was selective towards OTA detection (Fig. S4 in ESI†), and was feasible to detect OTA in diluted red wine samples (Fig. S5 in ESI†). When OTA spiked in 100-fold diluted red wine samples, the net FA changes were similar to that obtained in the binding buffer solution. In 100-fold diluted red wine samples, the detection limit of OTA was about 1.2 nM, suggesting that some components in red wine may influence the assay. These results show that this competitive FA assay can be used to detect OTA in the complex sample matrix.

## 4. Conclusions

In summary, we reported two competitive FA assays using the TMR-labeled OTA as a fluorescent probe and employing the antibody or the aptamer as affinity ligands. The antibody showed a higher binding affinity towards OTA than the aptamer. Both of antibody-based FA assay and the aptamer-based FA assay allowed the detection of OTA, and a larger FA change was obtained in the FA assay using the antibody because the antibody has a larger molecular volume than the aptamer. In the aptamer-based FA assay, the FA signal change can be further enhanced by conjugating a large-sized protein (*e.g.*, streptavidin) onto the aptamer, and performing analysis at a low temperature increased the aptamer affinity. Both the antibody-based and the aptamer-based FA assays are selective and promising in applications for the rapid detection of OTA.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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