Analytical Methods

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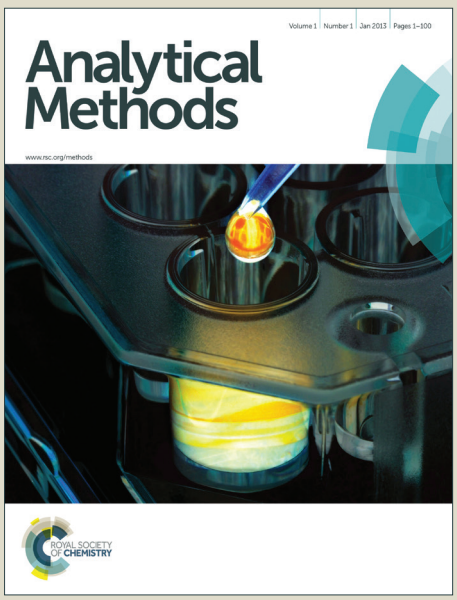

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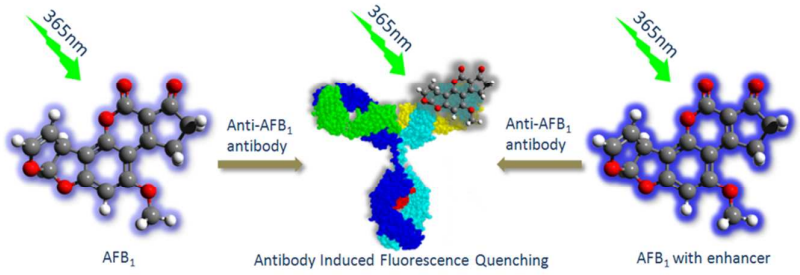
Graphical Abstract

A simple and label-free immunoassay was proposed based on specific antibody-analyte immune binding reaction induced fluorescence quenching of the analyte.



Cite this: DOI: 10.1039/c0xx00000x

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COMMUNICATION

Specific antibody-induced fluorescence quenching for direct and label-free immunoassay

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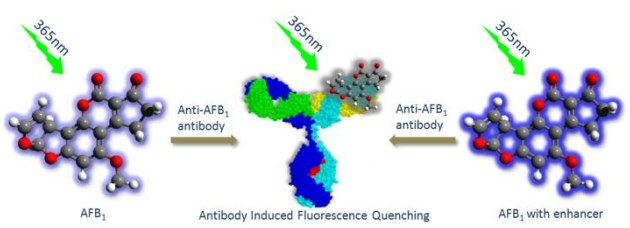
A simple, direct and label-free immunoassay was proposed based on a specific immune binding reaction induced fluorescence quenching of the analyte. Aflatoxin B_1 (AFB_1) was taken as the model analyte, whose intrinsic fluorescence was quenched by the specific anti-AFB_1 monoclonal antibody. This immunoreaction-induced fluorescence quenching was utilized to quantitatively determine AFB_1.

As a rapid, easy and low-cost method, immunoassay is widely used in the analysis of drug metabolites, plant hormones, proteins, microorganisms and other biological interests based on the specificity and selectivity of antibody reagents generated.[1] The immunoassay methods include labelled and label-free technologies, and the former was rapidly developed in recent years along with the technological progress of labelling materials, such as the enzyme, radiation, fluorescent, luminescent and nanoparticle materials.[2] These materials improved the sensitivity of the assay by orders of magnitude, which facilitates its large-scale use. For example, to determine the highly-toxic metabolite aflatoxin B_1 (AFB_1), the competitive and labelled immunoassay method was widely applied for rapid detection.[3] The used marker determines sensitivity of an immunoassay to a large extent but the quality of the used antibody is important as well.

In contrast with labelled ones, label-free technologies, such as immunodiffusion[4] and FRET[5], require only one reaction step all over the assay procedure. The label-free method achieves a direct and short-time consuming determination. It showed promise in real-time determination and played an important role in analyzing bio-molecules and monitoring the reactions.[6]

AFB_1, with intrinsic fluorescence, was selected as an analyte to search for a simple, label-free and direct immunoassay. As we have known that the fluorescence immunotechnologies for AFB_1 analysis were based on the fluorescence of labeled materials.[7] However, the intrinsic fluorescence signal of AFB_1 was seldom utilized for immunoassay.[8]

Here we aim to perform AFB_1 determination by scanning and evaluating the intrinsic fluorescence signals of AFB_1 before and after immunoreaction with specific anti-AFB_1 antibodies and nonspecific antibodies and proteins. It was unexpectedly found that the intrinsic fluorescence of AFB_1 could be effectively quenched by the specific anti-AFB_1 antibodies. The fluorescence change was shown in Scheme 1, in which the colour intensity around AFB_1 represented its fluorescence intensity.



Scheme 1. Schematic representation of the fluorescence of AFB_1 was quenched by the specific anti-AFB_1 antibody.

To study the selectivity of this antibody-induced fluorescence quenching (AIFQ) phenomenon, the following proteins were utilized to investigate whether they could quench the fluorescence of AFB_1: two anti-AFB_1 monoclonal antibodies (1C11[9] and 3G1[10], whose sensitivities for AFB_1 measured by ELISA are 0.0012 ng/mL and 1.6 ng/mL, respectively), a non-specific monoclonal antibody (1H2, which was specific for ochratoxin A and had no cross-reactivity with AFB_1[11]), a non-specific polyclonal antibody (pAb, rabbit anti-mouse polyclonal antibody) and nonspecific proteins of bovine serum albumin (BSA) and ovalbumin (OVA). For each test, the concentration of AFB_1 was 50 ng/mL and the volume was 1mL. The added amount of each protein was 40 μL in concentration of 1 mg/mL. As shown in Fig. 1, only 1C11 antibody could significantly induce fluorescence quenching and 79.6% of the fluorescence intensity was quenched. Meanwhile, 3G1 can quench it only by 26.2%. 1H2, pAb, BSA and OVA have no quenching effect. These results indicated that only anti-AFB_1 antibodies could quench its intrinsic fluorescence and different anti-AFB_1 antibodies possessed different quenching efficiencies. The non-specific antibodies and proteins could not induce fluorescence quenching.

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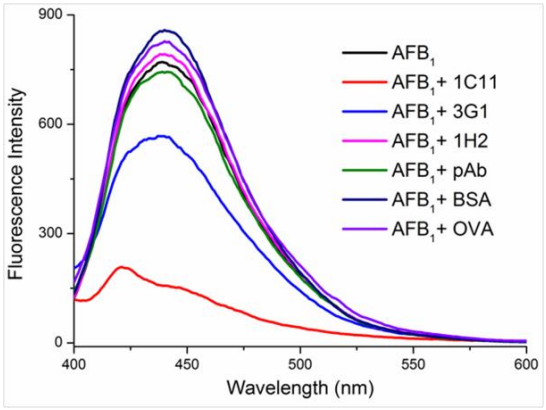

Fig. 1 Fluorescence spectra of AFB_1 in 10% methanol-water before and after the immunoreactions with 1C11, 3G1, 1H2, pAb, BSA and OVA, respectively.

According to our previous report,[12] this immunoreaction-induced fluorescence quenching may mainly be attributed to hydrogen bond and hydrophobic interactions formed by the Ser-H49 and Phe-H103 of the antibody and the benzene ring and its neighboring furan ring of the AFB_1, which changed the conjugated system of AFB_1. However, two different anti-AFB_1 antibodies have different quenching efficiencies. The detailed quenching mechanism and the cause of this difference are under investigation in our recent study.

In this work, this AIFQ phenomenon was proposed to develop a direct and label-free immune technology for quantitative determination of analytes with intrinsic fluorescence. AFB_1 was employed as a model analyte, and the specific antibody 1C11 was selected in the following experiment. Afterwards, AFB_1 analysis in peanut samples was employed to validate this AIFQ immunoassay method.

With a chain of conjugated bonds and heteroatoms, AFB_1 has intrinsic fluorescence and displays a maximum emission wavelength of ca. 440 nm when excited at 365 nm.[13] AFB_1 is highly toxic but of a low concentration in food and feedstuff. European Commission has strictly set the maximum limit of AFB_1 to 5 ng/mL.[14] However, the fluorescence intensity of AFB_1 at a low concentration is not high enough. Thus, the fluorescence quenching phenomenon is not obvious after the combination reaction with the specific anti-AFB_1 antibody 1C11 (Fig. 2). For 20 ng/mL of AFB_1 standard solution, only 26.6% of the fluorescence was quenched. Then, the limit of detection (LOD) of this AIFQ immune method used for AFB_1 detection is of a high level and the method possesses a low sensitivity.

In order to improve the sensitivity of this method, the fluorescence intensity of the initial AFB_1 solution was enhanced. As reported in previous studies, β-cyclodextrin, 2, 6-Di-O-methyl-β-cyclodextrin and other fluorescence enhancers were used to enhance fluorescence signals.[15] In this study, 2, 6-Di-O-methyl-β-cyclodextrin was used to increase the fluorescence strength of AFB_1, making the quenching phenomenon more obvious. The substantial enhancement of the fluorescence emission of aflatoxins with an unsaturated furan ring in the presence of aqueous solutions of 2, 6-Di-O-methyl-β-cyclodextrin is well known. In addition, there are several applications described to use these host-guest inclusion complexes for mycotoxin determination. The changes, i.e., an enhancement of the mycotoxin fluorescence upon inclusion, can be understood being a result of induced significant changes in the physical and chemical properties of AFB_1 as guest molecule. The fluorescence intensity was significantly enhanced with the presence of the enhancer (Scheme 1).

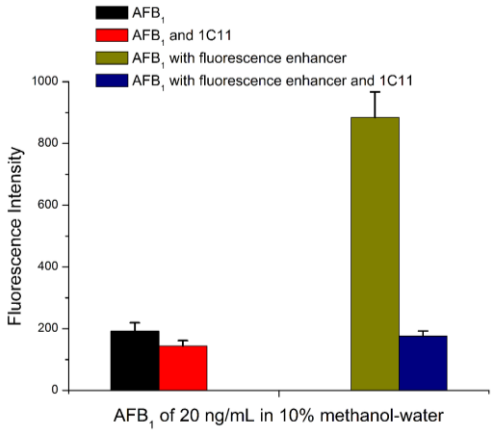

Fig. 2 Fluorescence of AFB_1 at ca. 440 nm when excited at 365 nm, before and after the immunoreaction with 20 μL 1C11 of 1 mg/mL, using and not using a fluorescence enhancer (2, 6-Di-O-methyl-β-cyclodextrin).

The results in Fig. 2 indicated that the fluorescence intensity at 440 nm was enhanced 4.3 times. After the immune reaction with 1C11, 80.1% of the enhanced fluorescence of AFB_1 was quenched. Meanwhile, the maximum fluorescence wavelength for AFB_1 remains unchanged (Supporting Information, Fig. S1).

To employ this method in AFB_1 analysis in real samples, some organic solvents were used in the sample extraction process and these solvents were evaluated to study their effects on fluorescence detection. The extraction of AFB_1 in food and feedstuff is normally performed with 70% (v/v) methanol-water. As high concentration of methanol and other organic solvents may affect the activity of the antibody, the methanol concentration was optimized to minimize the effect on the antibody activity. The evaluation results of 10% and 20% methanol-water indicated that 10% methanol had less negative effect on the antibody than 20% methanol. Therefore, AFB_1 dissolved in 10% (v/v) methanol-water was used for further experiments. The fluorescence of AFB_1 was enhanced by adding 2, 6-Di-O-methyl-β-cyclodextrin, and then the specific anti-AFB_1 antibody 1C11 was added into the mixture to perform immunoreaction. The fluorescence scanning results validated that the fluorescence intensity of AFB_1 decreased sharply after the immunoreaction with 1C11 in the solvent of 10% (v/v) methanol-water.

To obtain an apparent quenching phenomenon and the highest utilization efficiency of 1C11, the amount of 1C11 was optimized. Serial amounts of 1C11 were added into the mixture of AFB_1 (20 ng/mL, 1 mL) and 2, 6-Di-O-methyl-β-cyclodextrin (0.01 mol/L, 500 μL). After the immunoreaction, the fluorescence of the products was measured. The results in Fig. 3 indicated that 10-200 μg 1C11 showed similar quenching efficiencies. Therefore, 10 μg was used for immunoreaction to save cost. According to the molecular weights of AFB_1 and the monoclonal antibody, the

number of molecules of AFB_1 can be calculated, which equals to that of the mAb. The results indicated that each mAb could only react with one molecule of AFB_1, and after the AFB_1 reacted with the antibody, the fluorescence of AFB_1 was quenched.

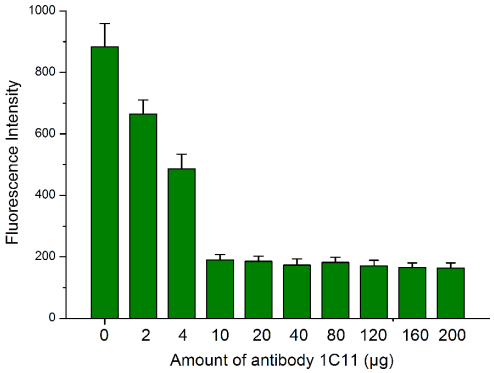

Fig. 3 Optimization of the amount of the anti-AFB_1 antibody 1C11.

To shorten the detection procedure, the quenching efficiencies after different reaction time were studied. The fluorescence of the mixture of AFB_1, the enhancer and the 1C11 antibody was scanned after reaction of 3, 5, 8, 15, 20, 30, 40, 50 and 60 min, respectively. The results in Fig. 4 demonstrated that this antibody could quench most of fluorescence in 3 min. Along with the increase of the reaction time, the quenching efficiency weakly increased. At 15 min, the reaction was almost complete and showed acceptable fluorescence quenching efficiency within the shortest analysis time. Therefore, the reaction time of 15 min was chosen as the optimal time parameter.

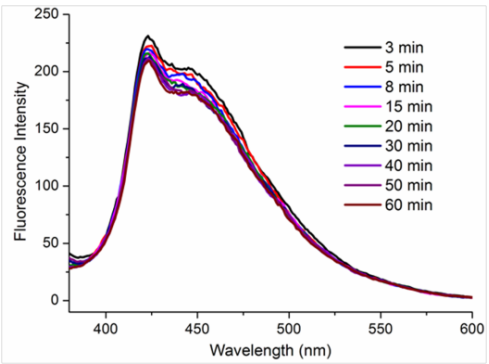

Fig.4 Fluorescence quenching efficiencies in different reaction time for 1C11 antibody.

Based on the optimized parameters, this direct and label-free immune technology was used to quantitatively analyze AFB_1. For AFB_1 standard solutions of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL, the fluorescence intensities before and after the immunoreaction with 1C11 were detected for 10 times separately. The intensity values at 440 nm were shown in Fig. S2 (Supporting Information). Then, a standard quenching curve for AFB_1 standard solution was established. As shown in Fig. 5, the calibration curve showed good linearity (r^2 = 0.9998) for AFB_1 from 1 ng/mL to 20 ng/mL. The LOD for AFB_1 was determined as 0.35 ng/mL, calculated by adding three times the signal-to-noise ratio to the background value.

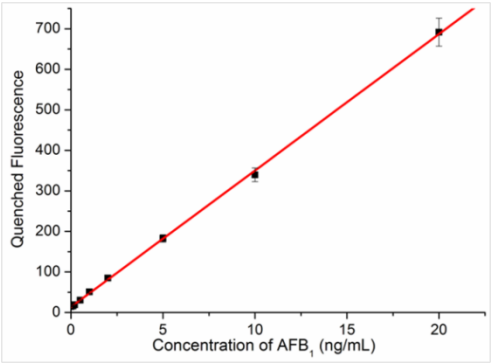

Fig. 5 A standard curve of the quenched fluorescence intensity for different concentrations of the AFB_1 standard, y = 33.67x + 13.68, r^2 = 0.9998, n = 10.

This suggests a sensitive and easy method for AFB_1 analysis. Moreover, this method enables fluorescence quenching with specific antibodies and could overcome the interference from other materials that give similar fluorescence signals. On one hand, this method is significantly simple and time-saving compared with conventional immunoassay methods. On the other hand, this method is environmentally-benign as it does not need highly toxic antigens, which is also friendly to operators and reduces detection costs.

To investigate the matrix effects of the samples on the quantitative analysis, AFB_1 in real food sample extraction was studied by this AIFQ method. The AFB_1-free peanut sample was first validated by HPLC. Then, 20 g blank sample was extracted with 50 mL 70% (v/v) methanol-water by grinding for 2 min. By filtrating with double filter paper, the filtrate was collected in a 50 mL tube, and cleaned twice with the alumina column to alleviate the matrix effect of the peanut. The product was spiked with AFB_1 standard solution and then diluted with water until the final proportion of methanol was 10% and AFB_1 was of the serial concentrations of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng/mL. The fluorescence quenching experiments were performed in the above optimized conditions and the fluorescence of AFB_1 spiking solutions was measured. The evaluation results indicated that the fluorescence quenching was obvious even with the presence of the sample matrix. For fluorescence intensities of serial concentrations of AFB_1, a calibration curve (y = 35.95x + 69.63, r^2 = 0.9959) similar to the curve in Figure 5 was also obtained, which showed good linearity (r^2 = 0.9959) from 1 ng/mL to 20 ng/mL (Supporting Information, Figure S3). The results demonstrated that this method could successfully overcome the matrix effects of real samples and could be employed in accurate determination of AFB_1 in the peanut samples.

To study the application of this AIFQ mothod, the AFB_1 spiked peanut samples were detected with this method. Meanwhile, these samples were detected with HPLC method for validation. The AFB_1-free peanut sample was spiked with AFB_1 at the concentrations of 10, 20 and 50 μg/kg and then was kept at room temperature overnight. These samples' pre-treatment for AIFQ method was the same as the above sample preparation process. The samples' pre-treatment for HPLC detection was according to the reported method.[16] The results obtained by AIFQ and HPLC assay were summarized in Table 1. These results indicated that the recoveries of AIFQ method were consistent with those from

HPLC, showing good application of this AIFQ method in peanut sample analysis. The LOD value of this AIFQ method is not high enough, which may be caused by the fluorescence detector's low resolution. The research on improving the sensitivity will be further studied in the following study.

Table 1. Analysis results of AIFQ and HPLC for AFB_1 spiked peanut samples

Spiked AFB_1 concentration (μg/kg)	AIFQ (μg/kg)	HPLC (μg/kg)
10	ND[a]	9.2
20	21.9	20.7
50	47.3	49.4

[a] ND: not detected

Conclusions

In summary, we proposed a novel label-free immunoassay method based on specific immunoreaction fluorescence quenching, using AFB_1 as an example analyte. This method was applied to perform the detection of AFB_1 in peanut samples and the results indicated that the method could be successfully used to analyze AFB_1 in the complex sample matrix. During the assay, AFB_1 could be specially recognized by its corresponding antibody, which induces a fluorescence quenching phenomenon on AFB_1. The other specific antibodies and proteins, however, cannot react with AFB_1 so as to induce fluorescence quenching. In a direct and label-free way, this fluorescence quenching phenomenon on the target analyte with intrinsic fluorescence signals could be widely employed in determination of AFB_1 and other analytes. This method is easy, fast, low-cost and friendly to operators and the environment. Nevertheless, it is only suitable for the target with intrinsic fluorescence and only specific antibodies could effectively quench the fluorescence. The preparation of specific and sensitive antibodies is essential and very important, which could be obtained by the monoclonal and recombinant antibody technology. The study on the detailed fluorescence quenching mechanism could provide guidance for the production of recombinant antibody. Moreover, the equipment with a higher resolution could be utilized to obtain much higher distinguish ability and better sensitivity.

Acknowledgements

This work was supported by the Project of the National Natural Science Foundation of China (21205133), the National Science & Technology Pillar Plan (2012BAB19B09) and the Special Fund for Agro-scientific Research in the Public Interest (201203094).

Notes and references

† Electronic Supplementary Information (ESI) available: Material, Experiment procedures, fluorescence detection parameters, and the supported results are included in the supporting information.

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