Research Article

Highly Sensitive FRET-Based Fluorescence Immunoassay for Detecting of Aflatoxin B1 Using Magnetic/Silica Core-Shell as a Signal Intensifier

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Background: Recently, some new nanobiosensors using different nanoparticles or microarray systems for detection of mycotoxins have been designed . However, rapid, sensitive and early detection of aflatoxicosis would be very helpful to distinguish high-risk persons.
Objectives: We report a highly sensitive competitive immunoassay using magnetic/silica core shell as a signal intensifier for the determination of aflatoxin B1 using fluorescence resonance energy transfer (FRET) from Cd/Te quantum dots (anti-aflatoxin B1 antibody immobilized on the surface of Cd/Te quantum dots) to Rhodamine 123 (Rho 123-labeled aflatoxin B1 bound to albumin). The specific immune-reaction between the anti-aflatoxin B1 antibody on the QDs and the labeled-aflatoxin B1 brings the Rho 123 fluorophore (acting as the acceptor) and the QDs (acting as the donor) in close spatial proximity and causes FRET to occur upon photo-excitation of the QDs. Using magnetic/silica core shell to intensify the obtained signal is the novelty of this study.
Materials and Methods: Cd/Te QDs were synthesized by the simultaneous reduction of cadmium chloride and tellurium in the presence of sodium borohydride under nitrogen atmosphere. Magnetic nanoparticles were synthesized using $FeSO_4$ and $FeCl_3$ (1:2 molar ratio) and ammonia as an oxidizing agent under nitrogen atmosphere. The prepared magnetic nanoparticles shelled by silica using tetraethoxysilane in the presence of ammonia. Nanoparticles synthesis and monodispersity confirmed by TEM. Immobilization of Cd/Te QDs to antibodies and labeling of aflatoxin B1-albumin by Rho 123 were performed by EDC/NHS reaction in reaction mixture buffer, pH 6, at room temperature.
Results: By using the magnetic/silica core shell sensitivity of the system changed from 2×10^{-11} in our previous study to 2×10^{-12} in this work. The feasibility of the method established by the detection of aflatoxin B1 in spiked human serum. There is a linear relationship between the decreased fluorescence intensity of Rho 123 with increasing concentration of aflatoxin B1 in spiked samples, over the range of 0.01-0.06 $\mu mol.mL^{-1}$.
Conclusions: This homogeneous competitive detection scheme is simple, rapid and efficient, and does not require multiple separation steps and excessive washing.

Keywords: Aflatoxin B1; FRET; Magnetic/Silica Core shell; Nanobiosensor

1. Background

Aflatoxins, which assume importance due to their destructive effects on human health, poultry and livestock, are a group of potent hepatotoxic, mutagenic, carcinogenic, immunosuppressive metabolites produced mainly by the fungi of *Aspergillus flavus* and *A. parasiticus* (1, 2). There are reasonable economic and safety reasons for establishing highly sensitive, selective, cost-effective and rapid analytical methods for regular screening of aflatoxin B1 in a range of specimens (3, 4). Several methods such as high performance liquid chromatography, thin layer chromatography, enzyme-linked immunosorbent assay have currently used for detection of aflatoxin B1 in different specimens. Recently, some new nanobiosensors using different nanoparticles or microarray systems for detection of mycotoxins have been designed (5-10). However, rapid, sensitive and early detection of aflatoxicosis would be very helpful to distinguish high-risk persons. In recent years, based on such characteristics as easy handling, fast and high sensitivity, QDs in FRET-based nanobiosensor have extensively stud-

ied for the detection and diagnosis of different kinds of molecules and diseases (11-13). Unique optical properties have made QDs one of the most important labeling materials for biological and medical diagnostic applications. QDs are approximately spherical, the shell of which can be capped with various water-soluble functional groups, and are easily dispersed in water (14, 15). These surface functional groups could attach to biomolecules to form QD-nanobioconjugate. An example for this case is the terminal carboxylic groups of thioglycolic acid linked to various biomolecules (16, 17). Moreover, QDs as ideal donors possess many advantages in fluorescence resonance energy transfer (FRET) applications (18, 19). FRET occurs when the electronic excitation energy of a donor fluorophore is transferred to a nearby acceptor molecule.

In this study, a very high sensitive, simple and rapid FRET-based nanobiosensor designed to detect aflatoxin B1 (Scheme 1). The QDs-labeled anti-aflatoxin B1 antibody and Rho 123-labeled aflatoxin B1-albumin considered as main parts of the nanobiosensor. We also used a novel magnetic/silica core shell that interestingly acts as an intensifier of the related signals. In this study, we decided to make a highly sensitive nanobiosensor to detect aflatoxin B1 in real samples.

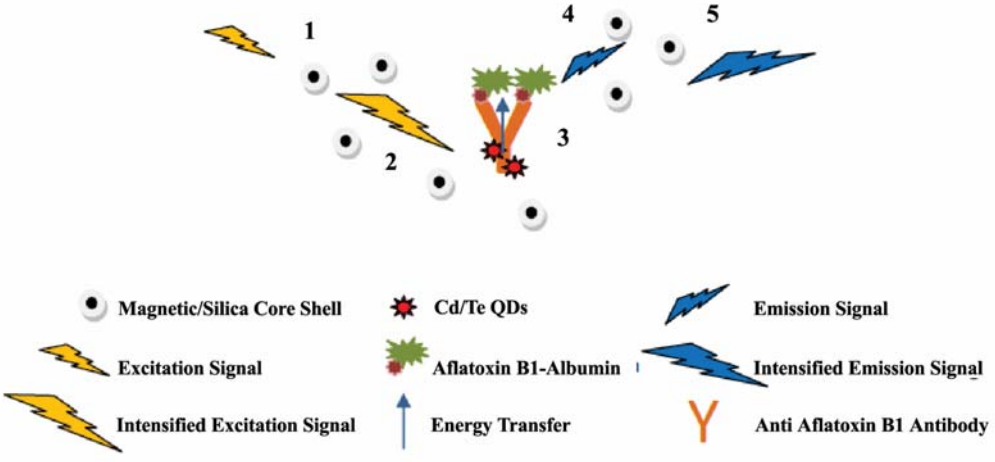
2. Materials and Methods

Aflatoxin B1, aflatoxin B1-albumin conjugate, anti-aflatoxin B1 antibody, cadmium chloride ($CdCl_2$), sodium borohydride ($NaBH_4$), tellurium powder (Te), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) purchased from Sigma chemical company (St. Louis, Mo, http://www.sigmaaldrich.com). Thioglycolic acid (TGA), $FeSO_4$, $FeCl_3$, tetraethoxysilane (TEOS) and all other chemicals obtained from Merck chemical company (Darmstadt, Germany, http://www. merck. de). All the materials were used as supplied without further purification. All the solutions were prepared using double-distilled water.

All fluorescence spectra recorded on a Shimadzu fluorescence spectrometer (Japan, http://www.shimadzu.com). All optical measurements carried out under ambient conditions. A Malvern dynamic light scattering (DLS) apparatus (UK, http://www. Malvern. com) used to investigate the size distribution of the synthesized QDs. All the experiments carried out in triplicate.

2.1. Preparation of TGA-Capped Cd/Te QDs

The Cd/Te QDs was synthesized and characterized according to the procedure reported in our previous work. (12, 13, 18, 19). Briefly, Te powder (0.1 g) was reduced by $NaBH_4$ (0.280 g) in 10 mL double-distilled water under vigorous stirring and continuous N_2 bubbling. After 5 h, color of the solution altered from violet to white. The solution was ultra-filtered to eliminate white precipitate of sodium tetraborate. Then the fresh prepared oxygen-free NaHTe aqueous solution was added into a $CdCl_2$ 2.5 H_2O (0.358 g) in 200 mL nitrogen-saturated double-distilled water at pH 10 in the presence of TGA (200 µL) as a stabilizing and capping agent. The mixture was refluxed under a nitrogen atmosphere while vigorously stirring. Different sizes of QDs could be achieved by prolonging the refluxing time. For removing excess substances, the crude solu-



Scheme 1. Excitation light passes through the magnetic/silica core shell, 2. Excitation light was intensified in contact with the magnetic/silica core shell and excited the Cd/Te QDs, 3. FRET was occurred because of close proximity of the QDs and the Rho 123, 4. Emission of the Rho 123 passes through magnetic/silica core shell, 5. Emission light was intensified

tion was washed three times with absolute ethanol and centrifuged for 15 min at 10,000 ×g. The resulting precipitate was re-dispersed in 250 mL double-distilled water and kept in a dark and cool place. Maximum excitation and emission wavelength of the synthesized QDs were 375 nm and 535 nm, respectively.

2.2. Preparation of QDs-anti-aflatoxin B1 Antibody Nanobioconjugate

For preparation of QD-anti-aflatoxin B1 antibody nanobioconjugate, the prepared QDs solution (1 mL) was mixed with solution of EDC (1 mg.2.5 mL^{-1}) and NHS (1 mg.2.5 mL^{-1}) at pH 6.0 and incubated for 1 h at room temperature in a dark place. Subsequently, the ice-cold solution of anti-aflatoxin B1 antibody was added by drop wise addition to the ice-cold carboxyl group activated QDs while gently stirring, and the whole solution was kept for 1 h in this condition in dark. In order to characterize the formation of the QDs-anti-aflatoxin B1 antibody nanobioconjugate, optical density of the sample of anti-aflatoxin B1 antibody and QDs-anti-aflatoxin B1 antibody nanobioconjugate at 280 nm, before and after centrifugation at 10,000 ×g for 20 min at 4ºC, were recorded on a spectrophotometer. The stock solution was kept at 4ºC in dark for further use.

2.3. Labeling of Aflatoxin B1-Albumin Bioconjugate with Rho 123

For preparation of Rho 123-labeled aflatoxin B1-albumin bioconjugate, Rho 123 (1 mg) was dissolved in 1 mL of phosphate buffer solution (50 mM, pH 6) and then chilled. The prepared solution was added to 2 mL of ice-cold phosphate buffer solution (50 mM, pH 6) containing 1 mg of aflatoxin B1-albumin. The homogenized mixture was prepared by repeated and gently pipetting. One mL of solution of EDC (1 mg.2.5 mL^{-1}) and NHS (1 mg.2.5 mL^{-1}) at pH 6.0 was then added to the prepared mixture and incubated for 1 h at 4°C in dark. Consequently, the Rho 123-labeled aflatoxin B1-albumin was dialyzed (cutoff: 10 kDa) against 2×1 l of 50 mM phosphate buffer pH 7.4 to remove any excess substances. In order to characterize the labeling of the aflatoxin B1-albumin with Rho 123, optical density of the dialyzed mixture was measured on a spectrophotometer at 450 nm. The solution of aflatoxin B1-albumin was considered as a blank. Because of photosensitivity of Rho 123, the stock solution kept at 4ºC in dark for further use.

2.4. Synthesis of Magnetic/Silica Core-Shell

Magnetic silica nanoparticles synthesized based on the procedure in our previous work (20). In brief, $FeCl_3$ and $FeSO_4$ dissolved in double distilled water. The solution kept under nitrogen flow. Then, ammonium hydroxide solution added to the solution. The prepared magnetic nanoparticles collected using magnetic bar. Trinatrium citrate used as a stabilizing agent under sonication (Hielscher ultrasonic, Germany. http://www.hielscher.com). The citrate-stabilized magnetic nanoparticles solution sonicated for 20 min. Upon complete dispersion of the magnetic nanoparticles, to keep the solution pH in 9-10 NH_3 added into the mixture and stirred gently. Subsequently, TEOS added while vigorously stirred and kept for 3 h in this condition. Characteristics of the magnetic/silica core-shell were investigated by TEM and SEM.

2.5. Biosensor Evaluation

For evaluation of the ability and sensitivity of the designed nanobiosensor for detecting of aflatoxin B1, a reaction mixture containing Rho 123-labeled aflatoxin B1-albumin bioconjugate (0.06 μM) and QDs-labeled anti-aflatoxin B1 antibody (0.03 μM) in sodium phosphate buffer (50 mM, pH 7.4) and NaCl (100 mM) was prepared. After incubating the reaction for 3 min to ensure the formation of immuno-complex of the Rho 123-labeled aflatoxin B1-albumin bioconjugate and the QDs-labeled anti-aflatoxin B1 antibody, the samples were excited at 375 nm. Emission spectra were recorded in a range of 450 to 620 nm) on a spectrofluorimeter. Excitation and emission bandwidth were selected at 10 nm and 15 nm, respectively. To evaluate the intensifying effect of the magnetic/silica core shell, the prepared magnetic/silica core shell was sequentially added to the above-mentioned media to a final concentration of 10-100 $mg.L^{-1}$ (based on the magnetic concentration). Then the emission of the reaction mixture was recorded as mentioned above. To evaluate the ability of the system to detect aflatoxin B1 in a sample, a stock solution of one $mg.mL^{-1}$ of aflatoxin B1 in sodium phosphate buffer (50 mM, pH 7.4) and NaCl (100 mM) was prepared and then was sequentially added to the reaction mixture. Emission spectra of the samples were recorded after incubating the reaction mixture for 3 min at room temperature in a 1cm pass-length quartz cell, at the same condition as mentioned above.

3. Results

3.1. Characterization of the Prepared Cd/Te QDs

The results show the maximum emission peak of the QDs at 535 nm, while the maximum absorption and emission peaks of Rho 123 are 510 nm and 580

nm, respectively. The full width at half maximum (FWHM) of the QDs emission spectrum was about 35 nm. Emission wavelength of the QDs and absorption wavelength of Rho 123 shows maximum spectral overlap that is critical to obtain optimum FRET phenomenon. In our previous study, data from TEM image and DLS analysis of the prepared TGA-capped Cd/Te QDs showed that, the core shell have a spherical morphology with a particle size of about 3 nm and good monodispersity. The obtained QDs dispersed in the phosphate buffer (pH 7.4) showed high optical stability, without considerable loss of fluorescence intensity during three months (data not shown).

3.2. Characterization of the QDs-Anti-Aflatoxin B1 Antibody Nanobioconjugate

Reduction in optical density (at 280 nm) of the solution of the QDs-anti-aflatoxin B1 antibody nanobioconjugate after high-speed centrifugation confirmed successful covalent coupling between the anti-aflatoxin B1 antibody and the QDs. Immobilization of the anti-aflatoxin B1 antibody on the shell of the prepared QDs did not alter their emission spectra.

3.3. Labeling of Aflatoxin B1-Albumin with Rho 123

To verify the labeling of the aflatoxin B1-albumin with Rho 123, optical density of the dialyzed mixture of aflatoxin B1-albumin-Rho 123 was measured on a spectrophotometer, in Rho 123 maximal absorbance wavelength, at 510 nm. A strong absorption at 510 nm showed the presence of Rho 123 in the sample.

3.4. Preparation of Magnetic/Silica Core Shell

The prepared magnetic nanoparticles and magnetic/silica core shell had a spherical morphology with an average size of 10-15 nm and the magnetic/silica core shell had a spherical morphology with an average particle size of about 70 nm and good monodispersity. The thickness of silica shell was estimated as 50-55 nm. The obtained magnetic/silica core shell dispersed in the phosphate buffer (pH 7.4) showed high stability in optical properties, without considerable agglomeration (data not shown).

3.5. Energy Transfer Between QDs and Rho 123

An intensified FRET system obviously observed in the presence of magnetic/silica core shell when an immunoreaction occurred between the QDs-labeled anti-aflatoxin B1 antibody and Rho 123-labeled aflatoxin B1-albumin. In this study, the final concentration of magnetic/silica core shell was set up at 100 $mg.mL^{-1}$ in the reaction media that gave us maximum signal intensifying. The fluorescence intensity of Rho 123 at 580 nm was enhanced upon sequential addition of a solution containing Rho 123-labeled aflatoxin B1-albumin, from 0.01 $\mu mol.mL^{-1}$ to 0.06 $\mu mol.mL^{-1}$, at a specific period. The observed changes in Rho 123 emission were directly proportional to the final concentration of the Rho 123-labeled aflatoxin B1-albumin until all the antigen binding capacity of the anti-aflatoxin B1 antibody occupied with the Rho 123-labeled aflatoxin B1-albumin. However, a further increase in the concentration of the Rho 123-labeled aflatoxin B1-albumin resulted in no significant enhancement in the emission peak of Rho 123. By adding a specimen containing aflatoxin B1, the Rho 123-labeled aflatoxin B1-albumin could competitively replaced by aflatoxin B1 that leads to reduction in the fluorescence intensity of the system (Figure 1). Figure 2 clearly indicates that there is a good correlation between the decreased fluorescence intensity of Rho 123 at 580 nm with increasing concentration of aflatoxin B1 in specimen, over the range of 0.01 $\mu mol.mL^{-1}$ to 0.06 $\mu mol.mL^{-1}$. The limit of detection (LOD) was estimated as 2×10^{-12} M, based on equation $LOD=3S_D.K^{-1}$, where S_D is the standard deviation of blank measurements (n: 7) and K is the slope of calibration curve.

4. Discussion

In this work, we prepared a highly sensitive FRET-based nonobiosensor for detection of aflatoxin B1 using the anti-aflatoxin B1 antibody, aflatoxin B1-albumin conjugate, Rho 123, Cd/Te QDs core shell and magnetic/silica as an optical intensifier. In this

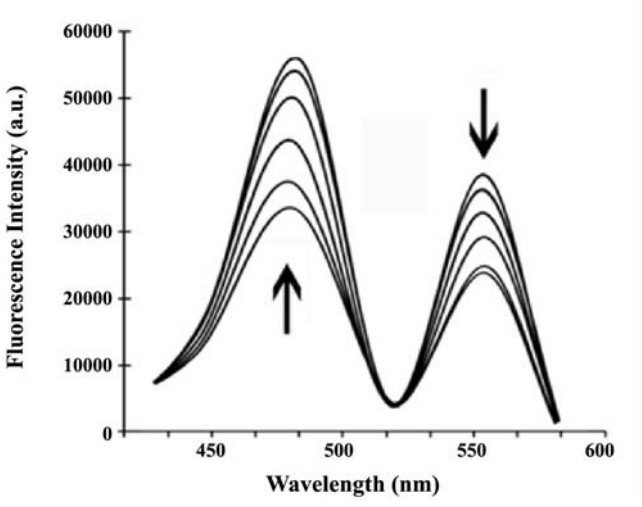


Figure 1. The increase and decrease in the emission of theCd/Te QDs (left) and Rho123 (right), respectively, upon-addition of different concentration of free aflatoxin B1 inthe reaction mixture solution

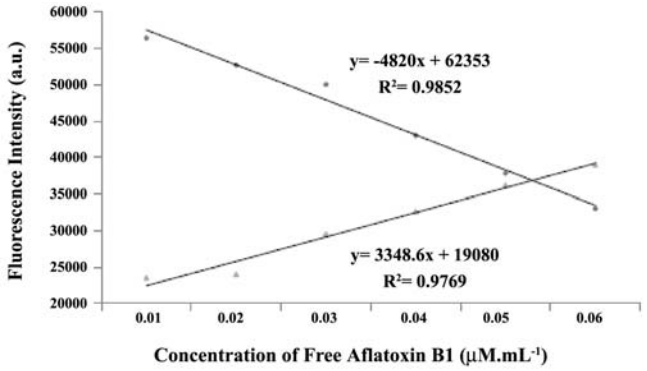


Figure 2. Linear correlation between enhancement (ascending line) and reduction (descending line) in the emission of Cd/Te QDs and Rho123, respectively, upon addition of different concentration of free aflatoxin B1

study, we proposed that magnetic/silica core shell act as nano-mirrors in reaction mixture that could interestingly magnify the optical signal obtained from FRET phenomenon. This may leads to higher sensitivity of the system and could detect even smaller amount of aflatoxin B1 in specimens. In fact, the magnetic/silica core shell used not only magnified the excitation intensity of the QDs, but also intensified the emission of the immobilized Rho123. The magnification of the excitation and the emission of the biosensing system led to an increase in the obtained signal, which in turn enhanced the sensitivity of the system. Based on the results obtained, the designed nanobiosensor showed more sensitivity (10^{-12} mol aflatoxin B1) compared to our previous study (REF). By reviewing other similar studies, we found out that the present nanobiosensor possessed the highest sensitivity, very close to the sensitivity of the impedimetric immunosensor designed by Bacher *et al*. 2012. Water-soluble, highly monodispersed and stable Cd/Te QDs and magnetic/silica core shell were synthesized to fabricate the nanobiosensor. Characterization of the synthesized nanoparticles by spectrophotometer, spectrofluorimeter and TEM confirmed that the particles were suitable for the designed nanobiosensor. As the emission spectra of the synthesized QDs and excitation wavelength of Rho 123 show an appropriate overlap, they were selected as a donor and acceptor, respectively. In fact, we can choose an extended excitation wavelength due to the wide absorption spectrum of QDs. Since Rho 123-labeled aflatoxin B1-albumin showed no significant fluorescence intensity at an emission wavelength of 375 nm, it was selected as the excitation wavelength for the FRET system.

To combine the QDs with anti-aflatoxin B1 antibody, the QDs were attached to the anti-aflatoxin B1 antibody via covalent interactions between carboxyl groups of the TGA-capped QDs and amino groups of the antibody. To obtain maximal FRET signal between QDs and Rho 123, different concentration of Rho 123-labeled aflatoxin B1-albumin and QDs-labeled anti-aflatoxin B1 antibody were tested for the nanobiosensor preparation. Optimum immunoreaction between the QDs-labeled anti-aflatoxin B1 antibody and the Rho 123-labeled aflatoxin B1-albumin was achieved at a molar ratio of about 2:1. At this ratio, the immunoreaction of two molecules of aflatoxin B1 with one molecule of antibody is predictable. At lower molar ratios, excess Rho 123-labeled aflatoxin B1-albumin do not participate in the formation of the aflatoxin B1-antibody immuno-complex, which lead to the reduction of FRET signal. In this condition, because of the existence of the free antibody, which in turn binds to aflatoxin B1 in specimen, it underestimates the concentration of aflatoxin B1 in specimen. At higher molar ratios, excess of the Rho 123-labeled aflatoxin B1 lead to loss of accuracy and sensitivity of the nanobiosensor. In this condition, because of existence of the excess Rho 123-labeled aflatoxin B1-albumin in the prepared nanobiosensor, more aflatoxin B1 must be added to overcome competitive replacement in binding sites of the anti-aflatoxin B1 antibody. This leads to loss of the accuracy and the sensitivity of the nanobiosensor especially in the detection of lower concentration of aflatoxin B1 in specimen. As mentioned and summarized in Table 1 and in comparison with the similar investigations, our detection limit (2×10^{-12} M) is very close to other researches. Therefore, based on the results and those with more complementary experiments, it is possible to estimate aflatoxin B1 in patient's specimens or agricultural products by comparing the obtained signals with a standard curve. in one hand, the biomolecules of anti-aflatoxin B1 antibody and aflatoxin B1-albumin that were labeled with QDs and Rho 123, respectively leads to a considerable energy transfer between the QDs and the Rho 123 and on the other hand, the presence the magnetic/silica core shell that in turn intensify the optical signal of the nanobiosensor the designed nanobiosensor showed higher sensitivity in comparison with our previous work. In this situation, even a few amount of aflatoxin B1 could be detected in specimen.

5. Conclusions

Based on the results of this study, magnetic/silica core shell effectively intensified the signal of the sys-

Table 1. Comparison of the limit of detection and linear working range of different detection methods used for aflatoxin B1

Detection Method	Title of the published Paper	LD	LWR	References
Immunochromatographic Assay (Gold-Labeled Antibody)	Preparation of gold-labeled antibody probe and its use in immunochromatography assay for detection of aflatoxin B1	2.5 $ng.mL^{-1}$	ND	Xiulan *et al*.
Lateral flow immunodipstick	Lateral flow immunodipstick for visual detection of aflatoxin B1 in food using immuno-nanoparticles composed of a silver core and a gold shell	0.1 $ng.mL^{-1}$	ND	Liao JY *et al*.
Array Biosensor (Indirect Competitive Immunoassay)	Indirect competitive immunoassay for detection of aflatoxin B1 in corn and nut products using the array biosensor	0.3 $ng.mL^{-1}$	0.6-5.1 $ng.g^{-1}$	Sapsford *et al*.
Immunochromatographic Assay	Development of an immunochromatographic assay for detection of aflatoxin B1 in foods	0.05-0.1 ppb	2-50 ppb	Xiulan *et al*.
Microarray System	An antibody-based microarray assay for the simultaneous detection of aflatoxin B1 and fumonisin B1	3 $ng.mL^{-1}$	ND	Lamberti *et al*.
Impedimetric Immunosensor	A label-free silver wire based impedimetric immunosensor for detection of aflatoxin M1 in milk	1 $pg.mL^{-1}$	6.25-100 $pg.mL^{-1}$	Bacher *et al*.
FRET-Based Immunosensor	Highly Sensitive FRET-Based Fluorescence Immunoassay for Aflatoxin B1 Using Cadmium Telluride Quantum Dots	2×10^{-11} M	$0.1\text{-}0.6\times10^{-9}$ M	Zekavati *et al*.
Intensified FRET-Based Immunosensor using Magnetic/Silica Core Shell	Highly Sensitive FRET-Based Fluorescence Immunoassay for Detecting of Aflatoxin B1 Using Magnetic/Silica Core-Shell as a Signal Intensifier	2×10^{-12} M	$0.01\text{-}0.06\times10^{-9}$ M	Present Study

tem. So it could be used in other optical-based detection system to make the system more sensitive.

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