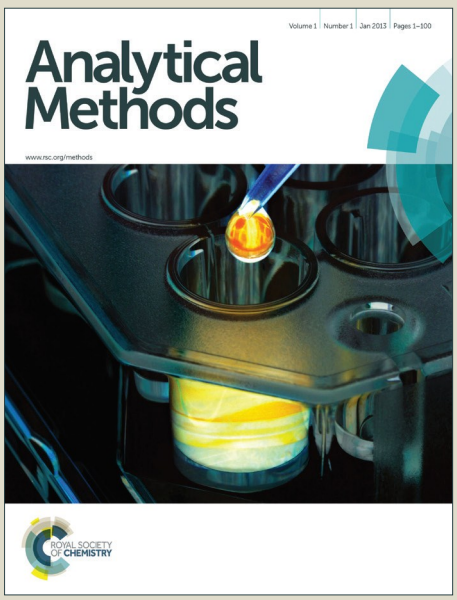
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Analytical Methods

ARTICLE

Magnetic ELISA of aflatoxin B1 - pre-concentration without elution

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A.V. Petrakova, A.E. Urusov, A.V. Zherdev, B.B.Dzantiev

While immunoenzyme assay (ELISA) is widely used for detection of various compounds, its use is significantly limited by the considerable duration (determined by the heterogeneous reaction to form detectable immune complexes) and the restricted detection limit. This study proposes an ELISA variant based on the application of highly dispersed (average diameter - 10 nm) magnetic iron oxide nanoparticles as carriers for the adsorbable antibodies. In conducting the proposed ELISA, the antibodies react with the detectable compound within the sample volume; the formed complexes are preconcentrated by precipitation in a magnetic field and are used for immunoenzyme detection in the wells of a microplate. This approach has been implemented for detection of aflatoxin B1, a low molecular weight compound that needs to be controlled at extremely low concentrations due to its high toxicity. Using magnetic nanoparticles provided a 10-fold lowering of the detection limit and cut the test duration in half, compared to conventional ELISA. Immobilized antibodies exhibited high resistance to methanol when testing aqueous/methanol extracts of contaminated vegetable feed stocks (corn kernels), making it possible to identify aflatoxin B1 at concentrations as low as 2 pg/ml (40 pg/g). The proposed approach is universal and can be used for immunodetection of various compounds.

1. Introduction

Immunoenzyme assay (ELISA) is one of the most widely used analytical approaches in modern medicine, environmental monitoring, quality control, and food safety. Its unquestionable benefits include simplicity, high throughput, reproducibility, and low cost of testing [1]. However, the conventional ELISA protocol prevents the lowering of detectable concentrations of the target compounds and reduction in analysis time. The detection limit in most immunoenzyme test systems is controlled by the equilibrium constant of the antigen-antibody reaction and is limited by the upper limit value of this constant, determined by the immune response induction mechanism [2, 3]. The reason for the extended (several hours) duration of solid phase ELISA is the heterogeneous interaction between immunoreactants in solution and those immobilized on the surface of the carrier. Slow diffusion exchange between liquid layers positioned at different distances from the carrier hinders the reduction of analysis time [1]. In contrast to solid phase ELISA, homogeneous immunoassay systems lack this disadvantage; however, the immune complexes formed therein are not separated from the unreacted molecules, and therefore, highly sensitive detection of the analyte is complicated [1, 4].

In view of the advantages and disadvantages of homogeneous and heterogeneous immunoassay methods, it seems appropriate to combine in a single scheme the rapid formation of immune complexes in solution with their detection effectiveness in heterogeneous systems. The use of highly dispersed carriers provides the ability to increase the surface area for immobilization of reactants and to distribute them uniformly throughout the volume of the reaction medium, thereby speeding up the heterogeneous interactions. Such work is being carried out in several directions.

One of the directions involves the use of charged carriers or polyelectrolytes in immunoassay. Having obtained the conjugate of one of the polyelectrolytes with immunoreactant, the target antigen may be detected in solution just as in conventional homogeneous methods; the formed immune complexes can then be rapidly separated by using a counter-ion or another reagent that would precipitate the polymeric carrier [5-7]. The second option involves the use of a suspension of ultradispersed immunosorbent separated by centrifuging after the specific reaction. For example, the immunosorbent carrier may be polystyrene particles, allowing the immobilization of antibodies by adsorption, as in conventional ELISA [8]. However, centrifuging becomes time-consuming when performing a large number of parallel analyses, and it requires specially adapted equipment. In this respect, the third direction, involving the use of magnetic immunosorbents, appears more promising. Application of a magnetic field after the immunochemical interactions provides a very simple and rapid means of separation of the reactants. This separation is achieved by using holders with permanent magnets of a certain size and strength; it

Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Leninsky prospect 33, 119071 Moscow, Russia. Email address: dzantiev@inbi.ras.ru Tel: 7 495 954 31 42 Fax: +7 495 954 28 04

is easily adapted to different sample volumes and requires no special equipment development [9].

Magnetic nanoparticles (MNP) based on iron oxides were used for ELISA of a variety of targets - hormones, mycotoxins, allergens, proteins, viruses, and bacteria [10-15]. However, most of these developments limit their use to the separation of immunoreactants but fail to utilize another important advantage of these carriers. Magnetic separation allows preconcentrating the analyte from a large volume of the original sample into a small volume in the final stages of analysis [16]. Such a preconcentration, as an ELISA step, is described in several studies [17-19], but it was accompanied by a significant complication of the analysis, a large number of steps and a total duration of up to 2-3 hours. As a rule, the antigen bound to the magnetic immunosorbent was then eluted from it for subsequent detection. Record improvements in the detection limit, as compared with conventional ELISA, are largely associated with the introduction of additional steps in the analysis, rather than preconcentrating the detectable substance. Significant improvements in the detection limit are also achieved by changing the detection method, namely using the MNP as a marker, not as a sorbent [20]. It should also be noted that the performed studies do not answer the question of optimal size of magnetic carriers for ELISA. Typically, researchers work with rather large magnetite particles, with an average diameter of many tens or hundreds of nanometers [21, 22]. Smaller particles are potentially preferred due to increased total surface area of the suspension; however, at a diameter less than 30 nm [23] they become superparamagnetic and their fit for performing all ELISA steps needs further confirmation.

The present study describes the use of magnetic immunosorbents obtained by adsorption immobilization of antibodies on small (average diameter 10 nm) superparamagnetic nanoparticles of iron oxide in ELISA. Aflatoxin B1 (AFB1) – a highly toxic, low molecular weight metabolite of mold fungi, which poses a significant threat to human and animal health [24-26] in case of food and feedstock contamination – was selected as the test compound. AFB1 is commonly detected by chromatographic methods [27-29] and various immunoassay methods dominated by microplate ELISA [30-32].

The given complex of superparamagnetic nanoparticles and antibodies against AFB1 was earlier used for ELISA format which was carried out in volume of microplate wells and by this way applied only separating possibilities of the nanocarriers [33]. The ELISA protocol proposed in the present study integrates separating and concentrating possibilities and by this way allows to reach lower limits of detection for semi-homogeneous and elution-free immunoassay.

2. Experimental

2.1. Materials and Reagents

The work involved the use of iron chloride (II), iron chloride (III), Triton X-100, 3,3',5,5'-tetramethylbenzidine (TMB), methanol (all from Sigma-Aldrich, USA, www.Sigmaaldrich.com), aflatoxin B1 (Hromresurs, Russia, www.hromresurs.ru), bovine serum albumin (BSA) (MP Biomedicals, USA, www.mpbio.com). Other reagents (solvents, buffer solution components, etc.) were of analytical grade or higher. The monoclonal antibodies against aflatoxin B1 and aflatoxin B1 conjugate with peroxidase were from IL Test-Pushchino Ltd., Pushchino, Moscow region.

Buffers used for immunoassay:

- 50 mM phosphate buffer, pH 7.4, containing 100 mM NaCl (PBS):
- PBS containing 0.05% Triton X-100 (PBST);
- PBS containing 0.05% Triton X-100 and 0.1% BSA (PBST-BSA).

During the ELISA procedure, the immunoreactants were incubated in optically clear 96-well polystyrene microplates made by Corning (USA, www.corning.com) and MEDPOLIMER (Russia, www.medp.spb.ru). Microplate washer Fluido 2 (Biochrom Anthos, Great Britain, www.biochrom.co.uk) was used for microplate washing; the optical density of the enzymatic reaction products was measured with a Zenyth 3100 (Anthos Labtec Instruments, Austria, www.anthos-labtec.com) microplate photometer. A permanent neodymium magnet measuring 30 x 30 mm (Russia, www.mirmagnitov.ru) and a MagnetoPURE 96 Chemicell (Germany, www.chemicell.com) magnet for microplates were used for magnetic separation. A JEM CX-100 (JEOL, Japan, www.ndsu.edu) transmission electron microscope and Zetasizer Nano (Malvern Instruments Ltd, UK, www.malvern.com) nanoparticle analyzer were used to characterize the nanoparticle dimensions.

2.2. MNP Synthesis [33]

30% ammonium hydroxide was added dropwise to a concentration of 2.4% to an aqueous solution containing 1.4 mg/ml FeCl2 and 3.6 mg/ml FeCl3. The mixture was incubated for 15 minutes at room temperature with vigorous stirring Intelli-mixer RM-2 (Elmi Ltd., Latvia, www.elmi-tech.com). The chosen rotation mode was F1 in accordance with 70 rpm. The particles were precipitated by applying a magnetic field and after removing the supernatant, were resuspended in the original volume of PBS. The washing was repeated 5 times. The resulting suspension was stored at + 4 °C. (The final suspension concentration was controlled by weighing the compound washed and precipitated five times from distilled water and then fully dried.)

2.3. Immobilization of antibodies on the MNP [33]

A solution of anti-AFB1 antibodies (2.8 mg/ml) was added to 500 µl of a 3 mg/ml solution of MNP in PBS to a final immunoglobulin content of 70 µg/ml. The solution was incubated for 30 minutes with vigorous stirring by the same way as described at Section 2.2. The particles were precipitated by applying a magnetic field and supernatant

was separated with followed washing of the particles three times with PBS. The resulting suspension was stored at + 4 °C.

The ratio of anti-AFB1 antibodies and MNP was chosen based on our previous study [33] and accorded to the monolayer immobilization. The immobilization yield of antibodies (measured by ELISA based on the difference of their content in the initial formulation and in the combined supernatant) was equal to 24%.

2.4. Characterization of the MNP and its conjugate with antibodies

During the transmission electron microscopy the preparations of the MNP and its conjugate with antibodies were applied to 300-mesh grids (Pelco International, USA, www.pelcoint.com) coated with a support film of poly(vinyl formal). The film was formed on a glass plate by its incubation in formvar solution (0.15% in chloroform), removed from the glass and used to cover the grids [34].

The images were obtained with a JEM CX-100 electron microscope operating at 80 kV. The digital microscopic images were analyzed with the Image Tool program (University of Texas Health Science Center, San Antonio, TX, USA).

Particle size determination was performed by dynamic light scattering using a Zetasizer Nano. Before measurement, the preparation was diluted by water to a 1:2000 ratio. Registration was carried out at 25 °C for 10 seconds at scattering angle 12.8°.

Zeta potential measurement was performed using a Zetasizer Nano. Before measurement, the preparation was diluted by water to a 1:100 ratio. Registration was carried out at 25 °C at 2 mm measurement position.

2.5. Microplate ELISA for AFB1

Antibodies against AFB1 were incubated in a microplate for 2 h at 37°C at a concentration of 1 µg/ml in 100 µl of PBS. After four washes with PBST, a solution of AFB1 (50 µl) at concentrations between 3 ng/ml and 4 pg/ml in PBST were added, mixed with 50 µl AFB1-HRP conjugate (100 ng/ml, by HRP) and incubated for 60 min at 37°C. The microplate wells were then washed four times with PBST.

To determine the peroxidase activity, the substrate solution (0.42 mM TMB and 1.8 mM H_2O_2 in a 0.1 M sodium citrate buffer, pH 4.0; 100 µl per well) was injected. After incubation at room temperature for 15 min, the reaction was terminated by the addition of 100 µl of 1 M H_2SO_4. The absorbance of the reaction product was read at 450 nm.

2.6. ELISA for AFB1 with the use of MNP

5 ml of AFB1 solutions at concentrations ranging from 1 ng/ml to 15 fg/ml was mixed with 50 µl of the MNP-antibody conjugate at a concentration of 1 µg/ml (as MNP) in PBST-BSA or in PBST-BSA containing either 5, 10, or 20% methanol, or in corn extract containing 20% methanol and incubated for 30 min at room temperature with vigorous stirring. The MNP was precipitated by applying a magnetic field; the supernatant was removed and 100 µl of PBST-BSA was added (50-fold preconcentration) and the precipitate was resuspended. The resulting concentrated suspension was transferred to a microplate and washed 1 more time by precipitating in a magnetic field, removing the supernatant, and resuspending in PBST-BSA. 100 µl of AFB1-HRP conjugate was then added at a concentration of 600 ng/ml (in preliminary experiments, the concentration was varied in the 200-600 ng/ml range). After 10 minutes of incubation at room temperature with vigorous stirring, the preparation was washed 4 times with PBST-BSA.

To determine the peroxidase activity the substrate solution described above was added to the resulting residue, then it was resuspended. Further manipulations were performed similar to conventional microplate ELISA.

2.7. ELISA data processing

The plot of the absorbance (y) versus the antigen concentration in the sample (x) was drawn with Origin 7.5 software (Origin Lab, Northampton, USA) using the four-parameter function $y = (A-D)/(1+(x/c)^B)+D$. The analytical characteristics of the assay, i.e. limit of detection and working range, were determined based on the resulting function, as described in [35, 36].

2.8. ELISA validation samples

The corn for the extract was bought at a store. Milled grains were mixed with an extraction solution (70% methanol, 30% water) at a ratio of 1:5, and incubated with gentle stirring at room temperature for 1 day (in accordance with Asis et. al. [37], with modifications). After centrifugation, the supernatant was collected and stored at 4°C. The extracts were analyzed by HPLC according to Barbas et. al. [38] and no aflatoxin B1 was detected. Aflatoxin B1 solution was introduced into the obtained extract, immediately prior to performing the ELISA.

3. Results and discussion

3.1. Preparation and characterization of the reagents

When ELISA is carried out in the usual format, the monoclonal antibodies against AFB1 used in this study provided an AFB1 detection limit equal to 20 pg/ml.

Magnetite nanoparticles were obtained by the widely used method based on co-precipitation of Fe^{2+} and Fe^{3+} ions while treated with ammonia[39]. The co-precipitation protocol has been selected on the basis of our previous studies[33]. The dimensional characteristics of the particles in the resulting preparation were determined after synthesis of MNP and their conjugation with antibodies by means of transmission electron microscopy and optical measurements of nanoparticles in bulk using a Zetasizer Nano analyzer. Transmission microscopy shows that the preparations of both MNP and their conjugates consist of small particles that form the aggregates and chains (Fig. 1 (a) and (b)). By selecting images of individual MNP in the micrographs, it can be seen that MNP average diameter was 9.1±3.2 nm (see Fig. 1 (c)) and MNP conjugate average

diameter was 9.8±3.7 nm. The shape of the MNP was nearly spherical (axial ratio was 1.4). The aggregate dimensions varied significantly.

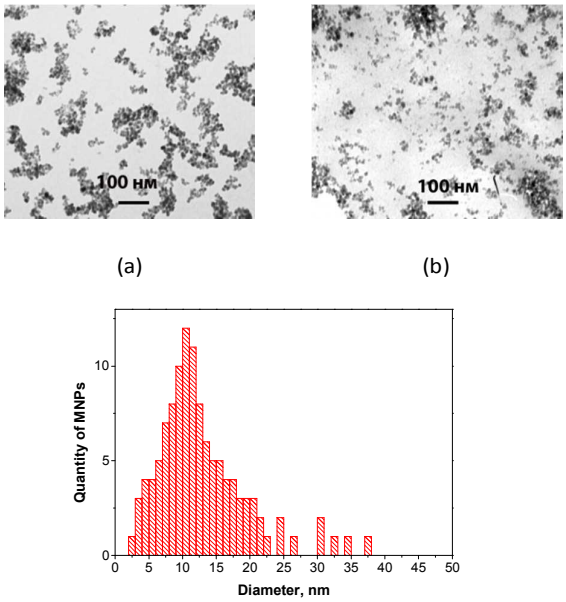


Fig. 1. TEM characterization of MNP and its conjugate. (a) – image of MNP; (b) – image of MNP conjugated with antibody; (c) – distribution of the nanoparticles (n = 116) by their average diameter measured by the TEM technique.

Keeping in mind that the transmission electron microscopy is susceptible to artificial aggregation of detected particle, occurring during their immobilization on the substrate, additional measurement of the true state of nanoparticles in solution was carried out by the dynamic light scattering (DLS) technique using a Zetasizer optical analyzer. It was determined that particle and conjugate diameters recorded by this method varied in the range of 150-500 nm (Fig. 2), which confirms the substantial degree of aggregation. At the same time, performing up to 7 consecutive cycles of magnetic deposition and resuspension did not change the size ranges of the aggregates recorded with the aid of the Zetasizer. It allows to conclude that the aggregation factor does not influence significantly of the assay parameters.

Zeta potential of MNP and their conjugates measured by the Zetasizer equipment was equal to 51.1±6.4 and 42.1±6.2 mV respectively.

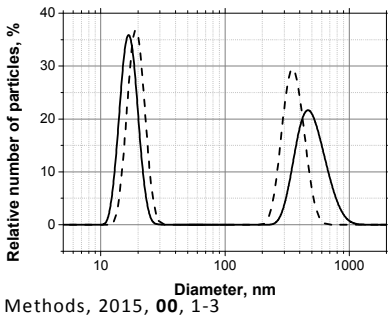


Fig. 2. DLS characterization of MNP and their conjugates. Straight line - distribution of MNP at the average diameter; dash line - distribution of MNP conjugates with antibodies at the average diameter.

Thus, the proposed use of superparamagnetic nanoparticles (instead of the commonly used larger ones) in the analysis, on the one hand, ensured the maximum increase in the total surface area of nanoparticles for the immobilization of antibodies. On the other hand, the aggregation of superparamagnetic particles resulted in the emergence of larger agglomerates, which did not change their structure during several cycles of precipitation and resuspension. Thus, despite the small initial dimensions of the MNP, repeated separation in a magnetic field and washing of their agglomerates becomes possible during the ELISA, just like when working with traditionally used larger nanoparticles of magnetite.

3.2. AFB1 ELISA using MNP

Enzyme immunoassay with magnetic preconcentration was performed according to the sequence of steps shown in Fig. 3. MNP conjugate with anti-AFB1 antibodies was added to a test sample potentially containing AFB1 (Fig. 3, a). After incubation, the resulting MNP-antibodies-antigen complex was separated from the free components by applying a magnetic field (Fig. 3, b); it was preconcentrated 50-fold by resuspension and transferred to a microplate (Fig. 3, c). Next, peroxidase-labeled AFB1 was added to the microplate wells (Fig. 3, d). After incubation, washing and reacting with the substrate (Fig. 3, e), the analyte content was determined on the basis of the measured optical density. The higher the AFB1 content of the sample, the lower the binding of the AFB1-peroxidase conjugate with the immunomagnetic sorbent and, accordingly, the lower the recorded optical density.

The obtained relationships between the recorded ELISA signal and the AFB1 concentrations were compared different ELISA modes, differing in the concentrations of immunoreactants and in duration of the stages. As a result of optimizing operations, the concentration of the magnetic particles-antibodies conjugate was selected to be

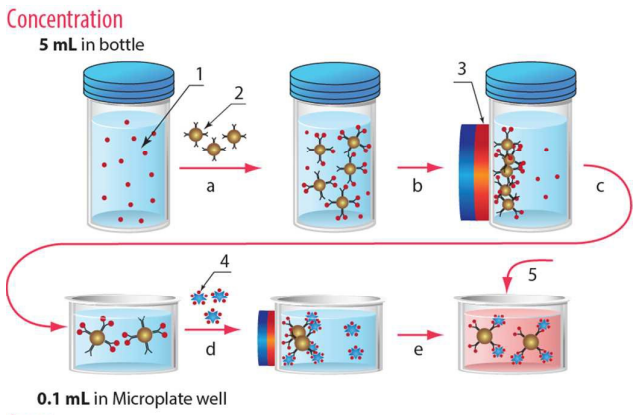


Fig. 3. Proposed scheme of immunoenzyme assay using MNP: a-g – the sequence of steps (1 – antigen-containing sample; 2 – MNP conjugate with specific antibodies; 3 – magnet; 4 –antigen-enzyme for conjugate; 5 – enzyme substrate).

equal to 1 µg/ml (as MNP), and the concentration of AFB1-HRP conjugate– equal to 600 ng/ml (as HRP). Performing the analysis at this ratio of reagents provided sufficient binding analysis at this ratio of reagents provided sufficient binding of the marker for correct quantitative assessment of the content of AFB1, maximum sensitivity and low background noise (the signal due to non-specific sorption of the marker on the MNP surface).

The study of kinetics of the immunochemical reactions in the proposed system is of particular interest. Experimental results that vary the time for interaction between the MNP-antibodies conjugate and the free antigen and for interaction between the magnetic immunosorbent and the AFB1-peroxidase conjugate are shown in Fig. 4 and 5, respectively. As seen in Fig. 4, effective preconcentration of AFB1 from a large sample volume requires 30 minutes of incubation, which, by the way, is less than half of traditional ELISA. A shorter duration of this step only leads to partial binding of the antigen by the immunosorbent in the sample and, accordingly, to a shift in the ELISA calibration towards higher analyte concentrations. However, the increase of interaction time with the analyte to over 30 min does not significantly change the calibration function (see curves 2 and 3 in Fig. 4). Fig. 5 reflects the much more rapid interaction nature of the magnetic immunosorbent with the AFB1-peroxidase conjugate, when carried out in the small volume of microplate wells. The competing curves obtained at 5-, 10- and 60-minute incubation were not significantly different in terms of the operating ranges of detectable AFB1 concentrations. Moreover, a significant increase in incubation time causes unwanted nonspecific conjugate adsorption on the carrier, independent of the presence of antigen (curve 3 in Fig. 5). Accordingly, the optimal time of interaction between the magnetic immunosorbent and the AFB1-peroxidase conjugate was indicated to be 5 minutes.

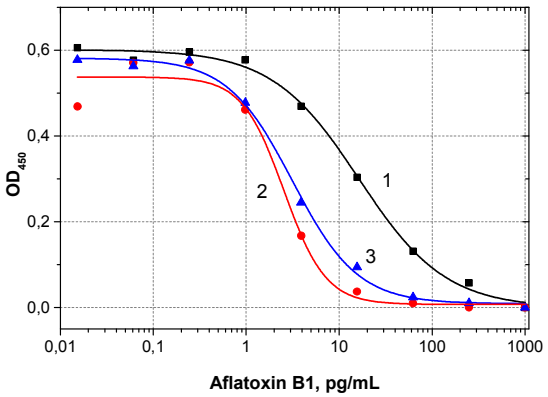


Fig. 4. ELISA of AFB1 using MNP. Recorded signal (optical density) curves as functions of the AFB1 concentration, obtained at different interaction times of the MNP-antibodies conjugate with the free antigen: 1 – 10 min, 2 – 30 min, 3 – 60 min.

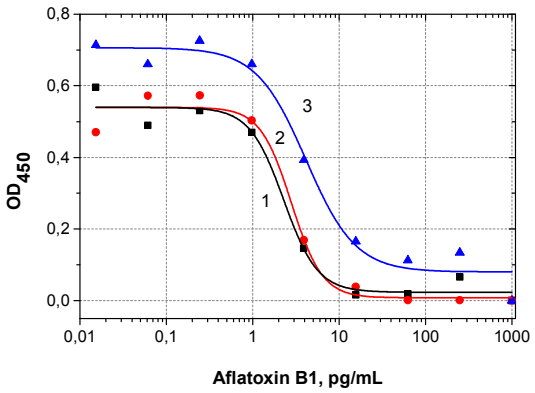


Fig. 5. ELISA of AFB1 using MNP. Recorded signal (optical density) curves as functions of the AFB1 concentration, obtained at different interaction times of the magnetic immunosorbent with AFB1-peroxidase conjugate: 1 – 5 min, 2 – 10 min, 3 – 60 min.

A calibration curve for determining AFB1, shown in Fig. 6, was obtained under the selected ELISA conditions with the use of MNP. Analytical characteristics of this variant of ELISA, as well as those of traditional ELISA are given in Table 1.

It shows that magnetic preconcentration allows reducing the level of detectable concentrations of AFB1 by almost an order of magnitude. The total assay duration in this case is 50 minutes, which is 2.4 times less than in a conventional microplate ELISA. Note that the use of MNP solely as a carrier for antibodies during all steps of the ELISA process in microplate wells, as was done in our previous study [33], did not alter the magnitude of detectable concentrations of AFB1, providing a gain only in the assay duration. Thus, the ELISA format implemented in

this study provides the right combination of activities aimed at the speed and sensitivity of immunoassays.

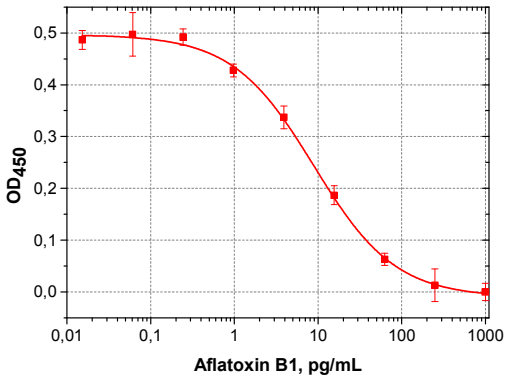


Fig. 6. Calibration curve for AFB1 determination by the developed method.

Table 1. Comparison of analytical parameters of different ELISA versions for aflatoxin B1.

	Limit of detection, ng/mL	Working range, ng/mL	Assay duration, min
MNP-based ELISA with concentration, this study	0.002	0.002-0.2	50
Common format of ELISA, this study	0.015	0.015-0.5	120
MNP-based ELISA without concentration[33]	0.02	0.02-1	20

3.3. Comparing the developed technique with other embodiments of magnetic preconcentration in ELISA

As noted above, in most of the studies on the application of MNP in ELISA the immunomagnetic sorbent is considered only as a means of separating the reactants but not as a means of analyte preconcentration from large samples volumes.

An exception is the work of Kuo et al. [19], who proposed a 1000-fold preconcentration of the analyte by using MNP, which lowered the detection limit 10-fold with a 2 hr. test duration. Preconcentration was also applied in the work of Kim et al. [18], who combined the use of MNP for extraction and preconcentration of the antigen and silicic nanoparticles with adsorbed fluorophore as a means of detection. A 54-fold reduction in enrofloxacin detection limit of was achieved through microscopy-aided recording of analytical results and prolonged incubation of the reagents; the total assay time was 3 hours.

The largest improvement in immunoassay, including analyte preconcentration by using MNP, is described in the work of Cho et al. [17]. However, to achieve their goal, they used a cascade of three conjugates of antibodies with colloidal gold, two types of peroxidase-labeled antispecies antibodies, and analyte preconcentration using MNP. The total duration of the assay was 2.5 hours. Despite the record-lowering of the detection limit – by a factor of 10^5 – the methodology of the assay is very difficult, time-consuming, and is characterized by accumulation of errors at each stage, as well as consumption of significant amounts of various reagents.

Thus, in the similar works by predecessors, the use of MNP provided only a lowering in analyte detection limits, but not a reduction in the duration of the ELISA process. Our proposed embodiment offers a gain both in sensitivity and in the duration of analysis (50 minutes in place of 120).

3.4. Validation of the developed ELISA

A specific feature of AFB1 as an antigen is its extremely low solubility in water. In this regard, organic solvents (mostly methanol) in high – 70% – concentrations are used for its extraction [40]. Such high content of organics in the reaction mixture causes denaturation of proteins (including antibodies and enzymes), hindering the conduct of immunoassays. To prevent this effect, the test sample is diluted with aqueous saline solution before the analysis, resulting in an increase in the minimum concentration of detectable analyte in the sample, proportional to the degree of dilution. Therefore, while transitioning from pure model solutions of AFB1 to actual samples, the influence of the matrices on the assay sensitivity was estimated.

Experiments were performed wherein the reaction between the MNP-antibodies conjugate and the AFB1 containing sample took place in a medium with 20, 10, 5%, and trace amounts of methanol. It was found that the resulting analytical signal curves, as functions of AFB1 concentration, did not differ for the previously characterized water-methanol mixtures, as well as for the buffer solution. The same result was observed by us in the previous study [33], where the possibility was confirmed of performing ELISA using the MNP-antibodies conjugate in a medium with 20% methanol. Thus, immobilization of antibodies on the MNP surface provides their stabilization and greater resistance to denaturing action of organic solvents, which corresponds to the data of other researchers [41, 42]. The observed stability of the magnetic immunosorbent to methanol (AFB1 extraction means from real samples) allows a minimization of the dilution of the test sample and, accordingly, results in a more sensitive analysis.

In view of this fact, the developed ELISA method was validated for AFB1 detection in corn plant extracts. The methanol content in the reaction mixture was 20%. ELISA was characterized by a range of detectable concentrations of AFB1 from 2 to 200 pg/ml, which corresponds to a mycotoxin content in the source grains preparation of 0.04 to 4.2 ng/g of grain. AFB1 recovery ranged from 74 to 120% (see Table 2).

Table 2. Recovery of aflatoxin B1 in the corn extract by applying the developed ELISA method with the use of magnetic nanoparticles.

Introduced concentration of aflatoxin B1, pg/ml	Found concentration of aflatoxin B1, pg/ml	Recovery, %
4.5	5.4	120
8.1	7.1	86
11.4	8.4	74

The proposed assay is an order of magnitude more sensitive (the detection limit was 20 pg/mL) as compared with the previously published one with the same reagents, but without pre-concentration [33].

It should be noted that the sensitivity of the immunochemical detection of AFB1, equal to 0.042 ng per 1 g of the tested plant sample is a good indicator. According to the COMMISSION REGULATION (EU) No 165/2010 [43], the maximum allowable concentration of AFB1 in food is from 1 to 20 µg per kg, and for baby food it is reduced to 0.1 µg/kg. Consequently, the proposed ELISA method using MNP provides the practically demanded sensitivity of contaminant detection, allowing control of different types of foods and agricultural products.

4. Conclusions

The obtained results confirm that the use of magnetic immunosorbents in ELISA both as carriers of antibodies and as a means of preconcentrating represents an effective approach, offering a significant gain both in assay sensitivity and speed.

Homogenous distribution of MNP with antibodies immobilized on them throughout the reaction medium accelerates the diffusion-dependent processes, allowing the binding of the analyte compound from a greater volume in a shorter time. The magnetic properties of the carrier provide subsequent rapid separation, with virtually no effect on the total duration of the ELISA process. Elimination of analyte elution and the direct use of MNP as carriers for binding and for recording the activity of the enzyme label significantly reduces the complexity of the analysis and further reduces the time needed to carry it out. Also note that the addition of the magnetic separation stage to the ELISA protocol does not require complex and expensive equipment.

In this study, the efficacy of the proposed approach is demonstrated on the example of aflatoxin B1 detection. The use of magnetic nanoparticles in the ELISA process enabled the reduction of the detection limit by an order of magnitude with a more than two-fold reduction in assay duration. The method provides a means for quantitative assessment of contamination of corn samples starting at the level of 0.04 ng per 1 g of grain, meeting the practical monitoring requirements of both conventional and special (baby food) food products.

Acknowledgments

This investigation was financially supported by the Russian Scientific Foundation (Grant No 14-14-01131).

The authors are grateful to S.M. Pridvorova from the A.N. Bach Institute of Biochemistry, Moscow, for performing electron microscopy measurements; V.M. Voznyak and M.V. Voznyak from IL Test-Pushchino Ltd., Pushchino, Moscow region, for giving the monoclonal antibodies against aflatoxin B1 and aflatoxin B1 conjugate with peroxidase.

References

1. D. Wild, ed., *The immunoassay handbook: theory and applications of ligand binding, ELISA and related techniques*, Elsevier Science, Oxford, 2013.
2. T. M. Jackson and R. P. Ekins, *J. Immunol. Methods*, 1986, **87**, 13-20.
3. B. B. Dzantiev and A. V. Zherdev, in *Portable Biosensing of Food Toxicants and Environmental Pollutants*, ed. D. P. V. Nikolelis, Theodoros Erdem, Arzum Nikoleli, Georgia-Paraskevi, Taylor & Francis, London – New York, 2013, pp. 161-196.
4. J. P. Gosling, *Clin. Chem.*, 1990, **36**, 1408-1427.
5. W. Yang, D. Trau, R. Renneberg, N. T. Yu and F. Caruso, *J. Colloid. Interface Sci.*, 2001, **234**, 356-362.
6. B. B. Dzantiev, A. V. Zherdev and E. V. Yazynina, in *Smart polymers for bioseparation and bioprocessing*, eds. I. Galaev and B. Mattiasson, Taylor & Francis, London – New York, 2001, pp. 207-229.
7. E. V. Yazynina, A. V. Zherdev, B. B. Dzantiev, V. A. Izumrudov, S. J. Gee and B. D. Hammock, *Anal. Chem.*, 1999, **71**, 3538-3543.
8. Y. Murakami, T. Endo, S. Yamamura, N. Nagatani, Y. Takamura and E. Tamiya, *Anal. Biochem.*, 2004, **334**, 111-116.
9. I. Safarik, Safariková, M., Forsythe, S.J., *Biomagn. Res. Technol.*, 2004, **2**.
10. Q. Xiao, H. Li, G. Hu, H. Wang, Z. Li and J. M. Lin, *Clin. Biochem.*, 2009, **42**, 1461-1467.
11. G. Pappert, M. Rieger, R. Niessner and M. Seidel, *Microchim. Acta*, 2010, **168**, 1-8.
12. F. Speroni, L. Elviri, M. Careri and A. Mangia, *Anal. Bioanal. Chem.*, 2010, **397**, 3035-3042.
13. M. de Souza Castilho, T. Laube, H. Yamanaka, S. Alegret and M. I. Pividori, *Anal. Chem.*, 2011, **83**, 5570-5577.

14. C.-P. Jia, X.-Q. Zhong, B. Hua, M.-Y. Liu, F.-X. Jing, X.-H. Lou, S.-H. Yao, J.-Q. Xiang, Q.-H. Jin and J.-L. Zhao, *Biosens. Bioelectron.*, 2009, **24**, 2836-2841.
15. J. Baniukevic, I. Hakki Boyaci, A. Goktug Bozkurt, U. Tamer, A. Ramanavicius and A. Ramanaviciene, *Biosen. Bioelectron.*, 2013, **43**, 281-288.
16. M. Hashemi and Z. Taherimaslak, *Anal. Methods*, 2014, **6**, 7663-7673.
17. I. H. Cho and J. Irudayaraj, *Int. J. Food. Microbiol.*, 2013, **164**, 70-75.
18. S. Kim, J. Ko and H. B. Lim, *Anal. Chim. Acta*, 2013, **771**, 37-41.
19. H. T. Kuo, J. Z. Yeh, C. M. Jiang and M. C. Wu, *J. Immunol. Methods*, 2012, **381**, 32-40.
20. A. V. Orlov, M. P. Nikitin, V. A. Bragina, S. L. Znoyko, M. N. Zaikina, T. I. Ksenevich, B. G. Gorshkov and P. I. Nikitin, *J. Magn. Magn. Mater.*, 2015, **380**, 231-235.
21. N. Gan, P. Xiong, J. Wang, T. Li, F. Hu, Y. Cao and L. Zheng, *J. Anal. Methods Chem.*, 2013, **2013**, 1-7.
22. F. Song, Y. Zhou, Y. S. Li, X. M. Meng, X. Y. Meng, J. Q. Liu, S. Y. Lu, H. L. Ren, P. Hu, Z. S. Liu, Y. Y. Zhang and J. H. Zhang, *Food Chem.*, 2014, **158**, 445-448.
23. S. P. Gubin, Koksharov, Yurii A, Khomutov, GB, Yurkov, Gleb Yu, *Russ. Chem. Rev.*, 2005, **74**, 489-520.
24. D. L. Eaton and J. D. Groopman, *The toxicology of aflatoxins: human health, veterinary, and agricultural significance*, Academic Press, San Diego, 2013.
25. A. E. Urusov, A. V. Zherdev and B. B. Dzantiev, *Appl. Biochem. Microbiol.*, 2010, **46**, 276-290.
26. G. J. B. Gnonlonfin, K. Hell, Y. Adjovi, P. Fandohan, D. Koudande, G. Mensah, A. Sanni and L. Brimer, *Crit. Rev. Food Sci. Nutr.*, 2013, **53**, 349-365.
27. P. D. Andrade, J. L. G. da Silva and E. D. Caldas, *J. Chromatogr. A*, 2013, **1304**, 61-68.
28. W. S. Khayoon, B. Saad, T. P. Lee and B. Salleh, *Food Chem.*, 2012, **133**, 489-496.
29. H. Wang, L. Zhao, H. Yang, Q. Guo, H. Shi, H. Pan, L. Zhao and C. Qian, *Anal. Methods*, 2014, **6**, 1545-1549.
30. A. E. Urusov, A. V. Zherdev, A. V. Petrakova, E. G. Sadykhov, O. V. Koroleva and B. B. Dzantiev, *Toxins*, 2015, **7**, 238-254.
31. N. A. Lee, S. Wang, R. D. Allan and I. R. Kennedy, *J. Agric. Food Chem.*, 2004, **52**, 2746-2755.
32. T. He, Y. Wang, P. Li, Q. Zhang, J. Lei, Z. Zhang, X. Ding, H. Zhou and W. Zhang, *Anal. Chem.*, 2014, **86**, 8873-8880.
33. A. E. Urusov, A. V. Petrakova, M. V. Vozniak, A. V. Zherdev and B. B. Dzantiev, *Sensors*, 2014, **14**, 21843-21857.
34. J. Ayache, L. Beaunier, J. Boumendil, G. Ehret and D. Laub, *Sample preparation handbook for transmission electron microscopy: techniques*, Springer Science & Business Media, New York, 2010.
35. G. S. Sittampalam, W. C. Smith, T. W. Miyakawa, D. R. Smith and C. McMorris, *J. Immunol. Methods.*, 1996, **190**, 151-161.
36. J. Ederveen, *A Practical Approach to Biological Method Assay Validation*, Progress, Project Management and engineering, the Niderlands, Hoofddorp, 2010.
37. R. Asis, R. D. D. Paola and M. A. J. Aldao, *Food Agric. Immunol.*, 2002, **14**, 201-208.
38. C. Barbas, U. Monteprincipe, A. Dams and R. E. Majors, Separation of Aflatoxins by HPLC - Agilent Technologies. Available online: http://www.chem.agilent.com/Library/applications/5989-3634EN.pdf).
39. M. Faraji, Yamini, Y., Rezaee, M., *J. Iran. Chem. Soc.*, 2010, **7**, 1-37.
40. R. J. Malone, in *Mycotoxin Prevention and Control in Agriculture*, ed. D. F. K. M. Appell, M.W. Trucksess, American Chemical Society, Washington, 2009, pp. 223-236.
41. J. Hong, P. Gong, D. Xu, L. Dong and S. Yao, *J. Biotechnol.*, 2007, **128**, 597-605.
42. M.-H. Liao and D.-H. Chen, *Biotechnol. Lett.*, 2001, **23**, 1723-1727.
43. COMMISSION REGULATION (EU) No 165/2010, http://faolex.fao.org/docs/pdf/eur92874.pdf).