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Authors: Beloglazova N., Goryacheva O., Speranskaya E., Aubert T., Shmelin P. , Kurbangaleev V., Goryacheva I.Y., De Saeger S.

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Silica-coated liposomes loaded with quantum dots as labels for multiplex fluorescent immunoassay

Beloglazova N.V.^a, Goryacheva O.A.^b, Speranskaya E.S.^a, Aubert T.^c, Shmelin P. S.^d, Kurbangaleev V.R.^d, Goryacheva I.Yu.^b, De Saeger S.^a

^a *Ghent University, Faculty of Pharmaceutical Sciences, Laboratory of Food Analysis, Harelbekestraat 72, 9000 Ghent, Belgium*

^b *Saratov State University, Chemistry Faculty, Department of General and Inorganic Chemistry, Astrakhanskaya 83, 410012 Saratov, Russia*

^c *Ghent University, Department of Inorganic and Physical Chemistry, Krijgslaan 281 S3, 9000 Ghent, Belgium*

^d *OJSC CSRIT Technomash, "Neuronet", 121108 Moscow, Russia*

Corresponding author. Tel.: +32 9 2648127; fax: +32 9 2648199

E-mail address: beloglazovanv@gmail.com (N.V. Beloglazova)

Abstract

This manuscript describes synthesis and followed application of silica-coated liposomes loaded with quantum dots as a perspective label for immunoassay. The hollow spherical structure of liposomes makes them an attractive package material for encapsulation of multiple water-insoluble quantum dots and amplifying the analytical signal. Silica coverage ensures the stability of the loaded liposomes against fusion and internal leakage during storage, transporting, application and also provides groups for bioconjugation. For the first time these nanostructures were employed for the

sensitive multiplex immunochemical determination of two analytes. As a model system mycotoxins zearalenone and aflatoxin B1 were detected in cereals. For simplification of multiassay results' evaluation the silanized liposomes loaded with QDs of different colors were used. The IC_{50} values for the simultaneous determination of zearalenone and aflatoxin B1 were 16.2 and 18 $\mu\text{g kg}^{-1}$ for zearalenone and 2.2 and 2.6 $\mu\text{g kg}^{-1}$ for aflatoxin B1 in wheat and maize, respectively. As confirmatory method, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used.

Keywords: Liposomes; quantum dots; silica-coated liposomes; multiplex immunoassay; mycotoxin; cereals.

1. Introduction

Since 1965, when Bangham et al. have discovered and described liposomes structures [1], new perspectives for drug delivery [2], bioimaging [3-5], cosmetics and food applications [6], chemical analysis [7] were opened. Liposomes (lipid vesicles) are colloidal spherical composites of lipid bilayers self-assembled in aqueous media. The large surface area and big internal volume allow them to carry plenty of molecules or nanoparticles. Therefore, loaded liposomes are promising labels for chemical analysis able to improve its sensitivity due to their signal-amplification properties.

Loaded liposomes were successfully applied in different immunassays, such as the microtiter-plate sorbent assay [8-11], flow-injection analysis [12-14], lateral flow on-site tests [15], chemiluminescent [16] and electrochemical [17] biosensors and also microarray [18]. Quantum dots (QDs) have been incorporated into liposomes and mostly used for labeling in biochemical and biomedical aims [19-22]. Recently the liposomes loaded with QDs (LQDs)

were successfully applied by our group as label for the high sensitive immunoassay [23, 24]. Nevertheless, liposomes could be sensitive towards external influences in experimental conditions and during the storage [25, 26]. Semipermeability of liposomes membrane, so essential for medical and pharmaceutical aims, could do a bad turn in chemical analysis. The possible osmosis of QDs through the phospholipid bilayer would results in luminescence decrease, which is undesirable for immunolabel. Leakage of the phospholipid membrane can be halted through the coverage of liposomes with a polymer net [27, 28] or silica cover [29, 30]. Silica coverage prevents the aggregation of liposomes and the leakage of their content, increasing their stability and facilitating their desiccation and therefore their storage. Synthesis of liposome-silica derivatives is a widely used method to stabilize the lipid bilayer [31-34].

To the best of our knowledge this article is the first one to describe the application of silica-coated liposomes loaded with QDs (SLQDs) as a novel sensitive label for the multiplex immunoassay. The multiplex procedure was based on simultaneous determination of two analytes (mycotoxins zearalenone (ZEN) and aflatoxin B1 (AfB1)) in the single well of microtiter plate by co-immobilization of two specific antibodies there. This technique was the first time described by us using QDs-labeled conjugates [35]. Replacement of the label for the SLQDs resulted in the significant increase of assay sensitivity.

2. Experimental section

2.1 Reagents and materials

Lipoid S75 was purchased from Lipoid GmbH, (Ludwigshafen, Germany). 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), (3-aminopropyl)triethoxysilane (APTES), aflatoxin B1 (AfB1), zearalenone (ZEN), dithiothreitol (DTT), O-(carboxymethyl)hydroxylamine hemihydrochloride (CMO), sodium fluoride, albumin from chicken egg white (OVA), casein sodium salt from bovine milk, skim

milk powder, were purchased from Sigma-Aldrich (Bornem, Belgium). Inject cBSA Immuno Modulator and protein concentrators (9K, 20 mL) were purchased from Thermo Scientific (Rockford, USA). Nuclepore track-etched membranes were purchased from Whatman (Belgium). Microtiter plates (96 flat-bottom wells with high binding capacity; black Maxisorp) were purchased from Nunc A/S (Roskilde, Denmark). All other chemicals and solvents were of analytical grade.

Polyclonal rabbit anti-mouse immunoglobulins (2.1 g L^{-1}) were obtained from Dako Denmark A/S (Glostrup, Denmark). The anti-ZEN monoclonal antibody (1 g L^{-1}) was characterized by a high ZEN (100%) and α -zearalenol (69%) recognition (cross-reactivities for α -zearalanol, zearalanone, β -zearalenol and β -zearalanol were 42%, 22%, <1% and <1%, respectively) [36]. Monoclonal anti-AfB1 antibody (1.3 g L^{-1}) was obtained from Soft Flow Hungary Ltd (Pecs, Hungary) and it was characterized with 79% cross-reaction with aflatoxin M1, 33% with aflatoxin M2, 76% with AfB2, 55% with AfG1, 6% with AfG2 and none at all with AfB2a and AfG2a [37]. CdSe/CdS/ZnS QDs with green and orange emission were prepared and stabilized by octadecylamine [38]. Preparation of AfB1-cBSA was described in [23], whereas the synthesis of ZEN-OVA was presented in [36].

Size distribution of the liposomes was measured by dynamic light scattering method using the Zetasizer Nano ZS (Malvern, England). All measurements were carried out at 25 °C. Bright field transmission electron microscopy (TEM) images were taken using a Cs corrected JEOL 2200 FS microscope operating at 200 kV. An Infinite Tecan Plate Reader (Tecan, Switzerland) was used to measure fluorescence through variation of the emission wavelength, depending on the QDs fluorescence peak position: 540 and 594 nm for green and orange-emitting QDs, respectively.

2.2 Preparation of silica-coated liposomes loaded with QDs (SLQDs)

Preparation of QDs-loaded liposomes was done according to a protocol of thin-film evaporation described by us [23, 24]. The phospholipid material (Lipoid S75, 134 μmol) and the water insoluble

QDs (1.5 nmol) were dissolved in chloroform (2 mL) for their homogeneous mixing. Afterwards chloroform was removed by rotary evaporation at 45 °C and ten mL of carbonate buffer were added to a dried lipid film left on the walls of a flask. The mixture was vigorously stirred in a water bath at 45 °C for 45 min. The lipid suspension was extruded through the polycarbonate membrane (the mean pore size 450, 200, and then 100 nm). The separation of LQDs from non-entrapped QDs and from excess phospholipids was realized by ultracentrifugation (300 000 g, 30 min, 4 °C) in a medium possessing an increasing sucrose density gradient (10-60%). LQDs were concentrated in a 30%-sucrose layer. LQDs were collected and, after removal of the sucrose residue with protein concentrator tubes, the pellet was re-dissolved in carbonate buffer (pH~9.6) and used for further experiments. The obtained LQD solution was stored at 4 °C.

For silanization of QDs-loaded liposomes the 2.5-molar excess of APTES was directly added to the LQDs and the mixture was stirred during 48 hours in the dark. For SLQDs drying sodium fluoride (4% molar excess with respect to the initial concentration silanization agent) was added and the mixture was stirred during 48 h at RT in the dark. Then the sample was dried at 40 °C for 24 h.

2.3 Syntheses of SLQDs-labeled antigens

APTES used for the silanization provides active amino groups on the liposomes' surface. For the synthesis of SLQDs-labeled conjugates a technique based on the modification of analyte-labeled proteins and SLQDs with SPDP and followed their coupling was applied. The analogues techniques were described for the production of ZEN-LQDs [24] and AFB1-LQDs [23]. SPDP (5 µmol) was dissolved in ethanol and dropwise added to the SLQDs solution (2 mL). The reaction mixture was stirred for 2 h at RT. The excess of SPDP was removed by the protein concentrator tube. In parallel, modification of analyte-protein was done. SPDP (6.5 µmol in ethanol) was dropwise added to the ZEN-OVA and AFB1-cBSA solutions (2 µmol) and the reaction mixture was stirred at RT for 30 min. The excess of SPDP was removed by the protein concentrator tube and dithiothreitol (4.2 µmol) was

added to the SPDP-modified protein conjugate. The reaction mixture was stirred during 30 min at RT, and then excess of dithiothreitol was removed by the protein concentrator tube. A portion of the modified SLQDs (1.5 mL) was added dropwise to the obtained thiolated ZEN-OVA and AfB1-cBSA. Reaction was continued under constant stirring overnight at RT. The modified liposomes were separated from excess protein by gel-filtration using Sephadex G-75. The prepared conjugates were kept at 4 °C.

2.4 Fluorescent labeled immunoassay (FLISA)

The 96-well opaque black microtiter plates were coated with rabbit anti-mouse IgG antibody (100 µL/well; 5 µg/mL in 0.05 M sodium carbonate buffer, pH 9.6) for 2 h at 37 °C. Then the plates were washed three times with PBS containing 0.05 % (v/v) Tween 20 (PBST) and blocked for 1 h at 37 °C with PBS containing 2% casein (w/v). Further, the plates were washed two times with PBST. The mixture of anti-ZEN and anti-AfB1 antibodies (50 µL of anti-ZEN antibody in dilutions of 1/2500 and 50 µL of anti-AfB1 antibody in dilution of 1/45000) was added and the plates were incubated for 2 h at 37 °C. The plates were washed three times with PBST. Fifty µL/well of standard mycotoxin solutions (in the range of 0.001 – 1000 ng mL⁻¹ in PBS) or diluted sample extract were added simultaneously with the mixture of ZEN-SLQDs and AfB1-SLQDs (25 µL of each reagent, dilutions of 1/45 and 1/65 for ZEN-SLQDs and AfB1-SLQDs, respectively). After 1 hour-incubation the plates were washed with PBST. The content of each well was re-dissolved in 100 µL of PBS and luminescence was measured using an Infinite Tecan Plate Reader (Tecan, Switzerland).

The standard FLISA sigmoidal calibration curve was plotted on a semilogarithmic scale: absolute or relative luminescence intensity against the logarithm of the analyte concentration. This relation is described by the Rodbard function:

$$y = (A - D) / [1 + (x / C)^b] + D$$

where A is maximum luminescence intensity value, D is minimum luminescence intensity value, C is IC_{50} concentration of analyte, b is slope of the curve in the IC_{50} plot. The limit of detection (LOD) was defined as the concentration that caused the analytical signal to decrease more than three times the signal-to-noise ratio (based on the results of 20 measurements).

2.5 Sample preparation

For the preparation of wheat and maize samples, a portion of ground cereal (10 g) was extracted with 40 mL of methanol/water (80/20, v/v) for 15 min on a horizontal shaker at RT. The supernatant was five times diluted with PBS and submitted to analysis.

2.6 LC-MS/MS procedure

For the LC-MS/MS determination of AFB1 the modified technique described by Monbaliu et al. was employed [39]. A Waters Acquity UPLC system coupled to a Micromass Quattro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA, USA) was used. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. Chromatographic separation was performed, applying a Symmetry C18 column (150 x 2.1 mm i.d. 5 μ m), preceded by a guard column (10 x 2.1 mm) of the same material (Waters, Milford, MA, USA). The limit of quantification (LOQ) was 6.41 μ g kg^{-1} for both matrices. For the LC-MS/MS determination of ZEN the method described by De Boevre et al. was used [40]. A Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface was used. Chromatographic separation was performed, applying a ZORBAX Eclipse XDB C18 column (3.5 mm, 100 mm x 4.6 mm) (Agilent Technologies, Diegem, Belgium). LOQ were 14 and 12 μ g kg^{-1} for ZEN in maize and wheat, respectively.

3. Results and Discussion

3.1 Silanization of QDs-loaded liposomes

Liposomes dispersions are not thermodynamically stable. When vesicles encounter each other in suspension, they can stick together and fuse to form larger particles. Therefore, the liposomes' size could become polydisperse in time. During this fusion process liposomes are prone to leak and lose their content that makes them inappropriate for biolabeling and biomarking. Silanization is a possible way for liposomes' stabilization.

Two techniques for liposomes' silanization were compared. The first technique was based on a direct addition of APTES to the LQDs solution, whereas the second technique called for preliminary hydrolysis of silanization agent. The preparation of silica structures comprises two steps: formation of silanol groups by hydrolysis of ethoxy groups, and subsequently, generation of a siloxane network via polycondensation of these groups. The properties of the silica coating strictly depend on the reaction conditions.

It was proven that a direct addition APTES to the LQDs led to formation of the monodisperse spherical silica-coated particles with an average diameter of about 190 nm (Fig. 1). The molar ratio of APTES and LQDs (2.5/1) and reaction conditions (constant 48-hours stirring in dark) were found as optimal for obtaining of SLQDs. To check the stability of the obtained particles average diameter, measured by DLS, and fluorescence intensity were constantly checking within 6 months when stored at 4 °C. The results of 6-months measurements of average size are presented in Fig. 2. It was proven that the silica-coated liposomes were not prone to aggregation. Silanization of the QDs-loaded liposomes did not result in a significant shift of fluorescence maximum (≤ 2 nm), change in spectrum profile or meaningful decrease of the QY (from 28% to 26%). Therefore, it can be concluded that the obtained particles kept the same size and fluorescence intensity during at least 6 months when stored at 4 °C. Besides, silica-coated liposomes could be dried at 40 °C for 24 h and stored in dry form within at least 8 months with maintaining their properties after dissolution in water.

An addition of the preliminary hydrolyzed APTES result in formation of the so-called “matrioshka architecture”, wherein the loaded liposomes were located inside of the silica capsules. These structures were not suitable to use as labels.

3.2 Development of the SLQDs-based FLISA

The simultaneous determination of two analytes in a single well by QDs-based FLISA was already described in our previous publication [35]. This approach is exclusively possible if conjugates are labeled with particles that luminescent in different part of spectrum. Silica-coated liposomes loaded with green- (λ_{fl} =540 nm) and orange-emitting (λ_{fl} =594 nm) QDs allow modifying of the already existing technique in order to make it even more sensitive. Encapsulation of QDs in phospholipid vesicles and their followed silanization do not lead to significant shift of photoluminescence maximum, light-scattering or remarkable adsorbance (Fig. 1), therefore it is possible to employ them in the multiplex FLISA.

Conjugation of SLQDs was performed through the surface amino-groups provided by the silanization agent, APTES. To obtain SLQDs-labeled conjugates a heterobifunctional cross-linker 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP) was used. Conjugation of the silica-covered liposome with ZEN-OVA and AfB1-cBSA comprised the simultaneous activation of analyte-proteins and liposomes with the linker and their followed binding.

The anti-ZEN and anti-AfB1 specific antibodies were co-immobilized into the same well of microtiter plate. The obtained SLQDs tended to non-specific interaction with surface of microtiter plate, therefore PBS-2%-casein was used for its blocking before application a mixture of SLQD conjugates (ZEN-SLQD and AfB1-SLQD, 1/1 v/v, in appropriate dilutions). For preventing undesirable influence of the labeled conjugates on each other and elimination of their non-specific interaction with the plate surface the conjugates were diluted in PBS containing 0.05% (v/v) Tween 20. Besides,

the number of washing steps after the conjugate addition was increased (4 times instead of 3) for complete removal of unbound labeled-conjugates.

Already known that the superior photophysical characteristics of QDs as high fluorescence quantum yield and stability against photobleaching are usually observed in organic solvents [41,42]. Loading QDs into liposomes allows not only making water-insoluble QDs stable in aqueous media, but facilitating their bioconjugation with proteins and dramatically amplifying the analytical signal. Fig. 3 presents the comparison of the calibration curves for determination of ZEN and AFB1 by QDs- [35] and SLQDs-labeled multiplex FLISA. Due to the higher photoluminescence of SLQDs compared with QDs the lower concentration of specific antibodies was used in the same kind of multiplex based on QDs-detection, and it resulted in the significant increase of the assay sensitivity (Table 1): the IC_{50} for ZEN measured by SLQDs-FLISA was four times lower than for QDs-FLISA (0.17 ng L^{-1} vs. 0.71 ng L^{-1}). For AFB1 the IC_{50} were 0.55 ng L^{-1} and 0.09 ng L^{-1} for QDs- and SLQDs-FLISA respectively.

3.3 Validation of the developed multiplex SLQDs-FLISA

Validation of the developed SLQDs-FLISA was done using wheat and maize samples artificially-spiked with the mixture of ZEN and AFB1 (1/1 v/v) with different mycotoxins concentration. For matrix effect correction the calibration curves were set up using the standards prepared in blank wheat and maize extracts (absence of ZEN and AFB1 was confirmed by LC-MS/MS). The change of the assay sensitivity was insignificant for both matrices: IC_{50} values were 0.81 and 0.90 ng L^{-1} for ZEN and 0.11 and 0.13 ng L^{-1} for AFB1 in wheat and maize extracts, respectively (which corresponds to 16.2 and $18 \text{ } \mu\text{g kg}^{-1}$ for zearalenone and 2.2 and $2.6 \text{ } \mu\text{g kg}^{-1}$ for aflatoxin B1 in wheat and maize, respectively). Hereupon for further measurements the calibration curves prepared in PBS were used. The matrix effect was also estimated by recovery experiments using spiked wheat and maize extract. SLQDs-FLISA performed excellent as illustrated by the good correlations between added amounts of mycotoxins and found concentrations in both matrices (Table 2).

3.4 Determination of zearalenone and aflatoxin B1 in naturally-contaminated cereals by the developed multiplex SLQDs-FLISA

A set of raw naturally-contaminated cereals samples selected according to the LC–MS/MS was analyzed by SLQDs-FLISA in triplicate applying the above mentioned protocol. This set consisted of 34 maize samples (13 “ZEN positive” samples (ZEN > LOQ of the LC-MS/MS) and 4 “Afb1 positive” samples (Afb1 > LOQ of the LC-MS/MS)) and 19 wheat samples (3 “ZEN positive” samples (ZEN > LOQ of the LC-MS/MS) and 2 “Afb1 positive” samples (Afb1 > LOQ of the LC-MS/MS)). All chosen samples were characterized with different ZEN contents ranging from < LOQ to > 100 µg kg⁻¹ and different Afb1 contents ranging from < LOQ to > 20 µg kg⁻¹. Seventeen negative according to the LC-MS/MS (concentrations < LOQs) samples (6 wheat and 11 maize samples) demonstrated the presence of ZEN (all seventeen samples) and Afb1 (3 wheat and 5 maize samples) in the concentration range ≥LOD FLISA and <LOQ LC–MS/MS. A good agreement was demonstrated with the samples which were found to be contaminated, as determined with LC-MS/MS: an excellent correlation with the data obtained by LC-MS/MS were found ($r^2 = 0.9685$ and $r^2 = 0.9669$ for ZEN and Afb1, respectively, Fig. 4).

4. Conclusions

For the first time, silica-coated liposomes loaded with quantum dots were used as label in a novel sensitive high-throughput and easy-to-operate multiplex immunoassay. To simplify the analysis procedure, both analytes (mycotoxins zearalenone and aflatoxin B1) were determined in the same well of a microtiter plate by co-immobilization of two specific antibodies. Analysis was realized by scanning of the assay outcome at two wavelengths. Silica-coated liposomes, loaded with Cd-based quantum dots, were used to label the target's conjugates. The synthesized label was characterized by an excellent stability during at least 6 months and very high fluorescence intensity. The latter allowed to reach a high sensitivity. Application of silica-coated liposomes loaded with quantum dots as a label

in fluorescent labeled immunoassay (FLISA) resulted in a four-fold increase of the assay sensitivity (decrease in IC_{50} value) for zearalenone and a six-fold increase of the sensitivity for aflatoxin B1 determination compared to the QDs-based FLISA. The IC_{50} values for the simultaneous determination of zearalenone and aflatoxin B1 were 16.2 and 18 for ZEN and 2.2 and 2.6 $\mu\text{g kg}^{-1}$ in wheat and maize extracts respectively. The developed multiplex immunoassay was used to determine mycotoxins in naturally contaminated wheat and maize samples. Liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to confirm these results.

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Fig. 1. TEM image (A.), scheme (B.) and fluorescence spectra (C., the spectra are normalized on peak maximum) of silica-coated liposomes loaded with green emitting QDs (green line) and orange emitted QDs (orange line)

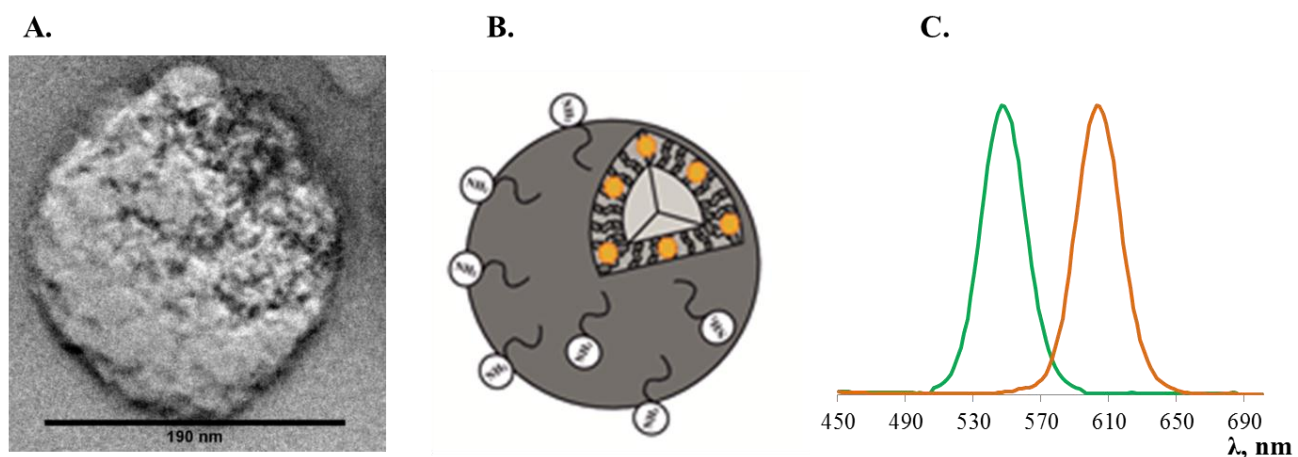


Fig. 2. Dependence of the liposomes' average diameter on time (n=5)

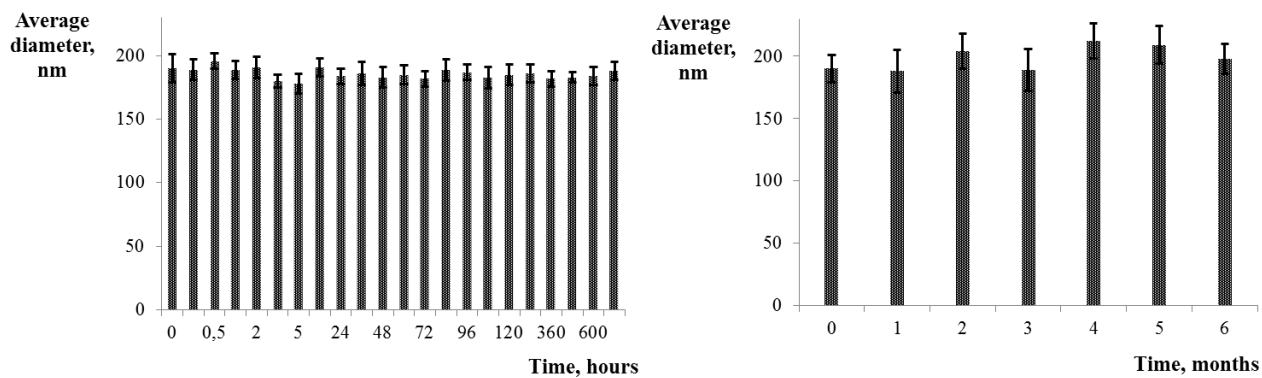


Fig. 3. Calibration curves for zearalenone (A.) and aflatoxin B1 (B.) determination in standard solutions by the QDs- and SLQDs- FLISA (n=5). A/A_0 is the relative luminescence: $\lambda = 540$ nm for green QDs (A.) and 594 nm for orange QDs (B.)

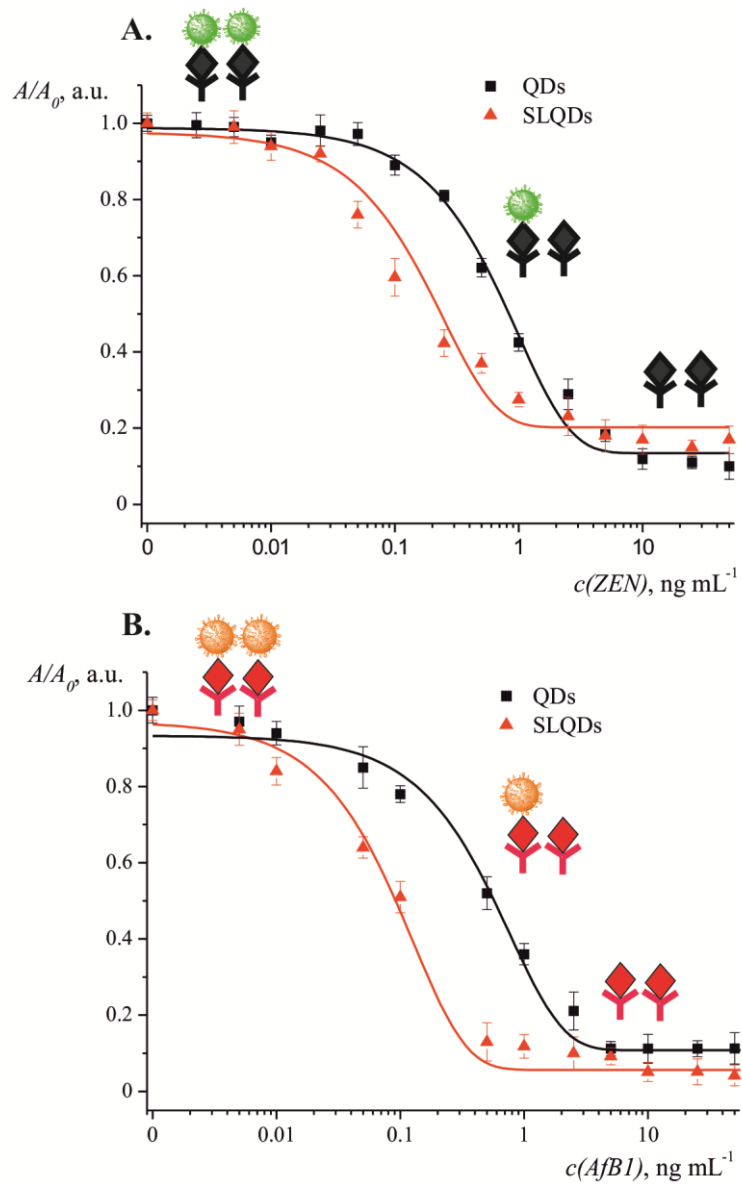


Fig. 4. Linear regression derived using the SLQDs-based multiplex FLISA and the LC-MS/MS data for mycotoxin screening in naturally-contaminated cereal samples, found to be positive by LC-MS/MS (n=5)

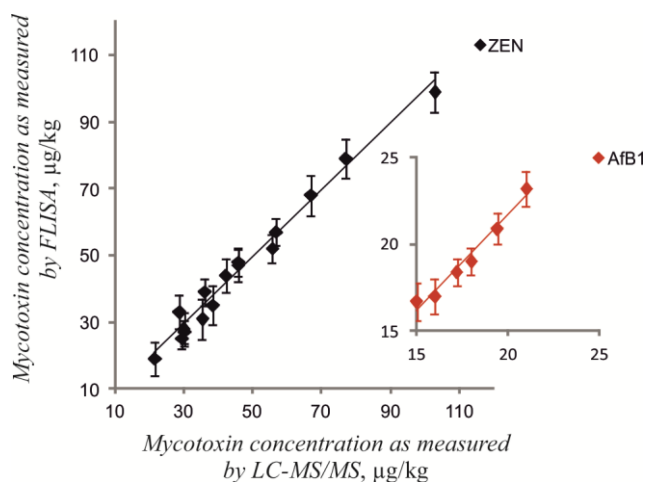


Table 1. Analytical parameters of zearalenone and aflatoxin B1 determination in standard solutions (n=5)

	ZEN	AfB1
LOD (ng L ⁻¹)	0.02	0.01
IC ₅₀ (ng L ⁻¹)	0.17±0.05	0.09±0.01
Linear range (ng L ⁻¹)	0.06-0.85	0.03-0.21

Table 2. Recovery tests for the simultaneous determination of zearalenone and aflatoxin B1 by the SLQDs-FLISA in artificially-spiked wheat and maize samples (n=5)

Added amount, µg kg ⁻¹	Recovery, %			
	Wheat		Maize	
	ZEN	AfB1	ZEN	AfB1
0.5	106±12	93±12	114±13	115±12

1	92±11	118±16	121±10	107±11
2.5	119±14	104±10	90±14	93±11
5	95±8	87±13	103±17	115±14
10	109±10	98±15	87±11	108±10

