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Research Article

Magnetic nanoparticles based fluorescence immunoassay for food contaminants

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Abstract

Nanotechnology provides exciting new possibilities for advanced development of new analytical tools and instruments for bioanalytical applications. Magnetic nanoparticles (MNPs) have attracted much research interest in the past decade because they have good biocompatibility and can be readily separated from reaction mixtures with the aid of an external magnetic field. The heterogenic fluorescent immunoassays for determination of different analytes (antibiotics, pesticides, progesterone, aflatoxins, enterotoxins) using MNPs were developed. MNPs were prepared by thermal co-precipitation of Fe^{2+} and Fe^{3+} in aqueous solution. MNPs were functionalized with (3-aminopropyl) triethoxysilane. Two different analyses were made for proving the successful modification of MNPs – FTIR and TEM. The corresponding antibodies were immobilized on modified MNPs. Then corresponding antigen - fluorescent dye were synthesized. The next step was purification of resulting conjugates by gel filtration chromatography and proving by UV-Vis and Fluorescence analyses. The 9 different separate competitive MNPs based immunoassays were developed – for penicillin, sulphonamide, tetracycline, progesterone, aflatoxin M1 and B1, enterotoxin A, paraoxon and dichlorvos. Then multiplex fluorescent immunoassay device based on magnetic nanoparticles for determination of penicillin, sulphonamide and tetracycline in milk sample was developed. Device ensured fast, selective and cheap antibiotic analysis. Very high analytical characteristics for all three antibiotics were received - very low LODs and wide linear range. Practical applications: The developed immunofluorescent methods are used for determination of low toxin concentrations in foods. Magnetic nanoparticles used as carrier for antibody immobilization accelerated the mass-exchange processes and reduced the analysis time. Combining immunofluorescence analysis with an automated device makes the method very practical for ensured food safety.

Keywords: MNP`s, immunoassays, penicillin, pesticides, antibiotics, progesterone, aflatoxins, enterotoxins, food.

Abbreviations:

BSA – bovine serum albumin
FIA – fluorescence immunoassay assay
FITC – Fluorescein isothiocyanate
FTIR - Fourier-transform infrared spectroscopy
GA - glutaraldehyde
GC-MS - Gas chromatography–masspectrometry
HPLC - High Performance Liquid Chromatography
LACTOSCAN IFA - LACTOSCAN Immunofluorescent Analyser
LOD - limit of detection

mAb – monoclonal antibody
MNPs – magnetic nanoparticles
PB – phosphate buffer
PEN - penicillin
RT – room temperature
SDM - Sulphonamide
TC - Tetracycline
TEM - Transmission electron microscopy
UV-Vis - Ultraviolet–visible spectroscopy

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Introduction

Due to the increasing need for improving the quality and food safety and environmental protection the development of advanced and fast method with high sensitivity and selectivity for quantification of a few toxic compounds – drugs, pesticides, hormones, mycotoxins is imperative. For determination of extremely low concentrations of these compounds in analytical laboratories High Performance Liquid Chromatography (HPLC), Gas chromatography–MAs spectrometry (GC-MS) etc. are used (Muratovic et al., 2015). These methods are very appropriate for monitoring of toxic compounds, but are also expensive, time consuming, require well trained staff and are not adapted to conduct analysis on-site and in real time. Methods for food control should be simple, fast and inexpensive. Furthermore, the analysis of contaminants in food should have high screening capacity worldwide. As an alternative to the laborious and expensive instrumental methods for the quantitative determination of toxins immunochemical assays for the quantitative screening have been developed. These methods have many advantages (Kirby and Saad 1996). They are rapid, yield improved precision, and are relatively easy to automate, thus requiring less hands-on involvement. Measurement of a biologic analyte typically involves two major steps: reaction and detection. Immunologic reaction between antigen and antibody are very selective and using the fluorescent detection ensured very sensitive assay. The possibility of simultaneously determining several components in the same sample is a significant advantage to conduct rapid screening (Wang et al. 2013). Heterogeneous competitive FIA methods are widely used for the analysis of low molecular weight compounds in food. An essential element of this process is the choice of matrix for immobilization of antibody or antigen. There are a number of scientific publications for heterogeneous immunoassay using different matrixes (Roque et al. 2009). The magnetic nanoparticles (MNPs) are manifested as an ideal medium for the immobilization of selective reagents because of the possibility to achieve effective separation by applying a magnetic field. Due to their versatility and multiple possibilities of functionalization,

MNPs are promising components, providing anchoring platform for biomolecules, allowing an improvement in sensitivity and selectivity. Multiplex fluorescent immunoassay based on the magnetic particles is very suitable for detection of antibiotics in milk, because it is possible to simultaneous detect a few antibiotics directly in sample (Cristea et al. 2017). The aim of this study is comparison of some developed MNPs based fluorescent immunoassay for separate and simultaneous determination of different contaminants in food.

Materials and Methods

Materials

Raw materials. For the preparation of MNPs were used $FeCl_3x6H2O$ and $FeSO_4x7H2O$ and for modification of MNPs - APTES from Sigma-Aldrich. Activation of the modified MNPs-NH_2 is carried out with glutaraldehyde (GA) from Merck. Different antibodies were used – monoclonal anti-tetracycline, anti-progesterone, anti-aflatoxin M1, anti-aflatoxin B1 and anti-enterotoxin A from Sigma-Aldrich, Germany, USA and polyclonal anti-penicillin, anti-sulphonamide, anti-dichlorvos, anti-paraoxon prepared in our laboratory (Yaneva et al. 2017; Usleber et al. 1998). For the preparation of fluorescent conjugates were used FITC, ATTO 488, ATTO 630, ATTO 590, and different analytes (Sigma-Aldrich, USA). All reagents were prepared with deionized water from PURELAB Ultra-system (ELGA, UK). All other reagents were with analytical purity.

Improvers. The new MNPs based fluorescent immunoassays were developed for determination of different contaminants of foods. These methods are very rapid, sensitive, and suitable for continuous monitoring. It was achieved very low detection limits for separate analytes. Besides that the simultaneous determination of some analytes in the same sample was developed. The obtained results were very close to the results of separate immunoassays. The possibility of simultaneously determining several components in the same sample

is a significant advantage to conduct rapid screening. The developed immunoassays were automated, by special device LACTOSCAN IFA constructed by company Milkotronic Ltd.

Instrumentation. The ultraviolet–visible spectra of hapten conjugates were recorded by Spectrophotometer 6900 (Jenway, England). Fourier transforms infrared spectra of hapten conjugates were measured by Fourier transform infrared spectrophotometer Tenzor 27-Bruker (Bruker Optik GmbH, Germany). The fluorescence intensity in immunoassay was detected by a Perkin Elmer LS 45 fluorescence spectrophotometer (USA).

Preparation and functionalization of the magnetic nanoparticles. The synthesis and functionalization of the magnetic nanoparticles with (3-aminopropyl) triethoxysilane was performed on a Syrris Globe system (England) by the methods described by Chang et al. 2005 and Ivanova and Godjevargova 2015.

Immobilization of different antibodies on the magnetic nanoparticles. After the activation step, 10 µL of antibody (5 mg.mL^{-1}) was added to MNPs (5mg.mL^{-1}) and incubated for 2 h at 300 rpm and RT. The MNPs-mAb was collected with a magnet, washed three times and the supernatant was used for unbound antibody determination. 1 mL of blocking buffer (0.5% BSA and 0.05% Tween 80 in 10 mM PB pH 7.4) was added to block the unreacted active amine groups for 1 h at RT. The MNPs-Ab were washed four times with the assay buffer and suspended in 1 mL of the same buffer to reach a 5 mg.mL^{-1} stock solution.

MNP based competitive immunofluorescence assay in buffer. Immobilized antibody onto MNPs (0.25 mg) was added to 200 µL of different analyte solutions. The solutions were prepared in 10 mM PB pH 7.4. The suspensions were gently mixed and incubated in a shaker, 15 min, at 37°C. Then, antigen-fluorescent dye (20 µL, with concentration 1--50 µg mL-1) was added.

The competitive immunoreaction was performed in a shaker, 15 min, at 37°C. Then the MNPs were separated by a magnetic separator and the fluorescence intensity of the supernatant was measured (800 V).

Fluorescent signals of the samples were normalized using the following equation:

$$NS = \frac{B_0 - B}{B_0 - B_x} * 100$$

(1)

where: *NS* – normalized signal (%), B_0 – fluorescent signal of the initial conjugate solution, *B* – fluorescent signal of the spiked sample with analyte, *Bx* – fluorescent signal of the sample without analyte.

Results and Discussion

Fluorescence heterogeneous immunoassay. Two basic types of immunoassays exist. The non-competitive immunoassay uses an excess of labelled specific antibody toward the analyte of interest. The analyte in the unknown sample is bound to the antibody site, and then the labelled antibody is bound to the analyte (Fig.1a). The amount of labelled antibody on the site is then measured. It will be directly proportional to the concentration of the analyte because the labelled antibody will not bind if the analyte is not present in the unknown sample. The competitive immunoassay relies on the competition between the antigen of interest (the analyte) and a constant amount of a similar but labelled antigen for a limited amount of specific antibody (Fig.1b). As in a competitive, heterogeneous immunoassay, analyte in a sample competes with labelled analyte to bind an antibody. Excess unbound labelled analyte, present in the supernatant is measured and its concentration is proportional to the concentration of the analyte.
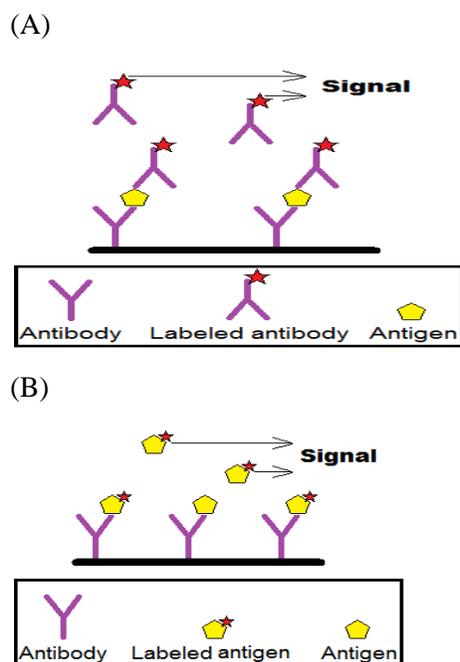
(A)

(B)

Figure 1. Non-competitive (A) and competitive (B) heterogeneous immunoassays

Competitive heterogeneous immunoassays using magnetic nanoparticles. The development of a heterogeneous fluorescent immunoassay using immobilized antibodies on magnetic nanoparticle (MNPs) is a perspective method for conducting multianalysis for determination of drugs, pesticides, hormones, mycotoxins. The use of magnetic particles will result in easier separation of the bound conjugate (immobilized antibody-antigen and the immobilized antibody-antigen-fluorescent marker) by applying a magnetic field. The high specific surface of the magnetic nanoparticles will allow the achievement of high degree of antibodies immobilization, and the implementation of good mass transfer processes, leading to faster and more sensitive assay. When the antigen has a small molecular weight, it has only one epitope to bind the antibody, so the competitive method to the sandwich is preferred to perform the assay. In the competitive method, the antigen-fluorescent dye conjugate of known concentration competes with the analyte in the sample and binds to the free sites

at the antibody surface (Fig.2). Based on the determined residual concentration of the conjugate, the concentration of the analyte in the sample is judged. The relationship between them is proportional.
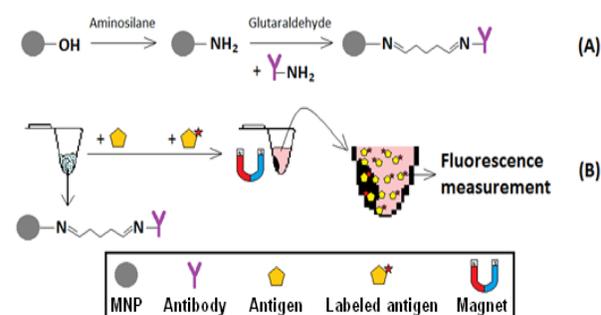

Figure 2. Principe of competitive heterogeneous immunoassays using magnetic nanoparticles: immobilization of antibody (A), immunoanalysis (B)

Preparation of modified MNPs. The magnetic nanoparticles (Fe_3O_4) were synthesized by a wet chemical method through the co-precipitation of Fe^{2+} and Fe^{3+} aqueous salt solutions (Chang et al. 2005). The magnetic nanoparticles were modified by aminopropyltriethoxysilane (APTES). The process of surface modification by reaction of silanization is very complex. In this study, an acidic aqueous silanization procedure was used to deposit aminopropylalkoxysilane on the surface of the magnetic core. The silanization reaction occurs in two steps. First, the organosilane was placed into an aqueous solution of an acid that acts as a catalyst. It was hydrolysed, and a condensation reaction occurs to form a silane polymer (Ivanova et al. 2015). In the second step, the polymer associates with the magnetite crystallites (or surface clusters) forming a covalent bond with OH groups. Dehydration as well as adsorption of silane polymers to the metal oxide occurs.

Characterization of modified MNPs. FTIR spectra for APTES coated and uncoated MNPs are shown in Figure 3.
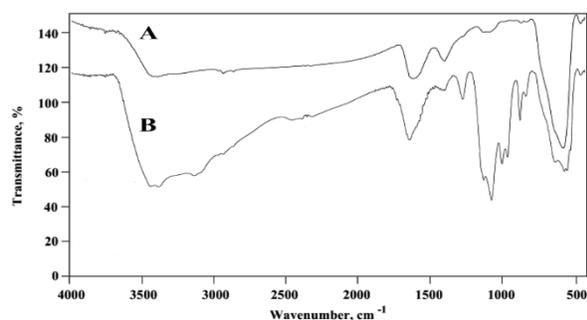
Figure 3. FTIR spectra of uncoated (a) and APTES coated (b) MNPs

Figure 4. TEM measurement of the uncoated (A) and aminosilane coated MNPs (B).

The broad band in the wavelength 585 cm^{-1} – 632 cm^{-1} appears in both spectra. This band has been associated to Fe-O bond. The band at around 3422 cm^{-1} refers to stretching of OH bonds present in the nanoparticles due to their synthesis an aqueous medium and the presence of OH groups on the surface of the material (Costa et al. 2016). Modification of the MNPs with aminosilane is confirmed by absorption bands at 1021 cm^{-1} and 1116 cm^{-1} that are attributed to the ν(Si-O) and Fe-Si, respectively. Amino groups onto modified MNPs appeared on the spectra at 1631 cm-1 and 3422 cm^{-1}, which are due to N-H angular deformation and stretch (Costa et al. 2016). For visual characterization of coated and uncoated MNPs, TEM was used. It is tool for directly imaging nanoparticles to obtain quantitative measures of particle size, and morphology. It is based on imaging the transmission of a focused beam of electrons through a sample, forming an image. TEM analysis highlights a difference in the synthesized products (Fig. 4). TEM images of the both materials demonstrated a sphere-shaped property. The average diameter of uncoated nanoparticles is estimated to be 6.64 nm. The salinized MNP had mean particle size 8.18 nm. Immobilization of antibody on modified MNPs and preparation of conjugates hapten-fluorescence dye.
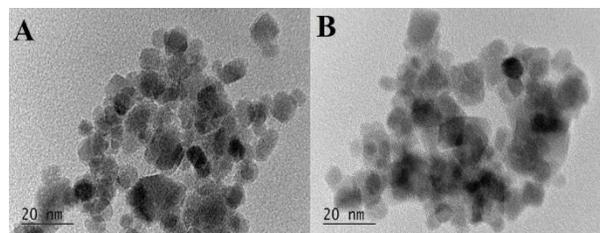
The obtained modified MNPs (MNPs-APTES) were used as a carrier for immobilization of different polyclonal antibodies - anti-penicillin, anti-sulphonamide, anti-dichlorvos, anti-paraoxon and different monoclonal antibody - anti-tetracycline, anti-progesterone, anti-aflatoxin M1, anti-aflatoxin B1 and anti-enterotoxin A. The antibodies against these antigens were covalently coupled by glutaraldehyde through their amino groups to the amino groups of functionalized magnetic nanoparticles. The efficiency of the coupling was evaluated using the Bradford method (Bradford 1976), comparing the IgG concentration in the supernatant before and after the coupling procedure (0.025-0.030 mg Ab per mg MNPs).
Most of the haptens have different functional groups by which to conduct the conjugation with the fluorescent dye: amino, carboxyl, hydroxyl groups. If the hapten does not have a suitable functional group for binding, a modification analogue may be used. The first important condition for conjugate preparation is that the bond between the hapten and the label is a strong chemical bond since the conjugate is stored and used repeatedly for a relatively long period. In addition, they are subjected to separation and purification operations such as gel filtration, liquid chromatography, etc., which require a strong hapten-tagging connection. The second condition is, after binding of the marker, that the hapten retain its specific binding to the antibody. The conjugation between different antigens and fluorescent dye FITC and ATTO was carried by using glutaraldehyde and carbodiimide (Ivanova et al. 2014, 2015; Gabrovska et al. 2013; Yaneva et al. 2017a, b). The next step is to purify

the resulting conjugates. It is necessary to separate the resulting conjugate from the unreacted components and by-products of the reaction.

MNPs based Competitive Immunoassay for separate determination of different contaminants. The separate fluorescent immunoassays for determination of different contaminants in buffer were developed (Ivanova et al. 2014, 2015; Gabrovska et al. 2013; Yaneva et al. 2017a, b). The comparison of analytical characteristics of the immunoassays is shown in Table 1. The using of monoclonal antibodies ensured determination of low concentration of analytes (pg.ml^{-1}) and with polyclonal antibody – ng.ml^{-1}. The assays have a wide linear range. The same assays were applied in real samples – fresh milk. It was found that the analytical characteristics of separate assay in milk were the same like ones in buffer, except in sheep milk, containing high fat concentration.

Multiplex Competitive Immunoassay of PEN, SDM and TC in Buffer and Milk. Biomarker research has expanded over the years, producing a need to quantitatively measure multiple analytes simultaneously from one sample. Multiplex Immunoassay was based on capture different antibodies to MNPs. The competitive assay format is performed with the three analytes (PEN, SDM and TC) in the same sample. The sample added to the three immobilized antibodies on MNPs, followed by the addition a corresponding conjugates analyte-fluorescent dyes. The fluorescent signal of unbounded fluorescent conjugates in the solution is detected. The fluorescent conjugates are classified based on their emission spectra and the amount of analyte detected is proportional to the intensity of fluorescent signal. Conjugates of the three antibiotics tested (PEN, SDM and TC) with different fluorescent dyes (FITC, ATTO 488, ATTO 590 and ATTO 633) were made. Simultaneous determination of the three antibiotics (PEN, SDM and TC) in a sample is carried out at 37°C, using LACTOSCAN Immunofluorescent Analyser. Table 2 shows the basic analytical characteristics of multi-immunoassay of PEN, SDM and TC in buffer and milk. Very high analytical

characteristics for all three antibiotics were received - very low LODs and wide linear range.

Construction of LACTOSCAN Immunofluorescent Analyser. Multiplex fluorescent immunoassay device based on magnetic nanoparticles for determination of contaminants in food was developed from Milkotronic Ltd. LACTOSCAN IFA consists of three parts: CD based immuno-fluorescent kit, thermostatic block and optoelectronic fluorometer (Fig.5).
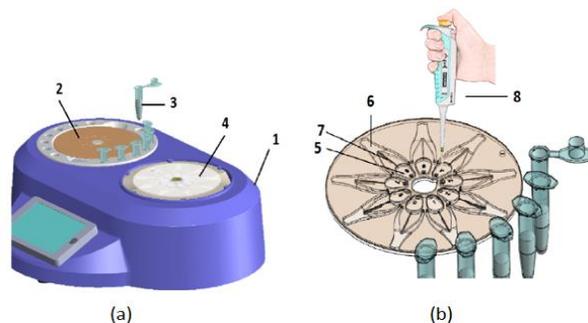

Figure 5. LACTOSCAN Immunofluorescent analyser (a) and CD based microfluidic disk (b). 1- ABS box, - first block , 2 - microfluidic disk in the first block, 3 - microtubes, 4 – second block, 5 - first camera of camera system, 6 - second camera of camera system, 7 - channel, 8 - pipette

LACTOSCAN IFA is a new apparatus and used for multiplex determination of antibiotics - penicillin, sulphonamide and tetracycline in milk. Milk sample or buffer was put into an Eppendorf tube that contains a mixture of lyophilized immobilized antibodies onto super magnetic nanoparticles (antibodies against PEN, SDM и TC). At a temperature of 37^{0}C for a period of 3 minutes an immunological reaction proceeds. After incubation the sample is taken out and put in the first camera of CD based microfluidic disk where the lyophilized conjugates antibiotic-fluorescent dye is placed. The fluorescent conjugates binds to the free antibodies. According to the antibiotic concentration in the sample different amount of the conjugate remains unbound. After 3 min incubation the disk is moved into the second block (optomechanic measurement

block – fluorimeter). The magnet, located only under the first cameras from the camera system separates the magnetic particles from the sample. The sample is then centrifuged at 4000 RPM for 3min. The supernatant passes through the hydrophobic channel and enters in the second camera from the camera system. Under the centrifugal forces the sample is separate in three layers (upper layer - fat, middle layer - supernatant,

down layer - somatic cells). After the irradiation of the supernatant with the three LED sources, signals which are proportional to the concentration of antibiotics in the milk are received. The results are automatically presented on the display unit and printed. It was able to record the results and possibly generate reports of results.

Table 1. Basic Analytical Characteristics of separate immunoassay for different analyte

№	Ab	Analytes	Linear interval	Linear equation	R^2	LOD, ng/ml
1.	pAb	Penicillin	2-10 ng/ml	$y = -6.896\ln(x) + 106.96$	0.9960	1,8 ng/ml
2.	pAb	Sulfonamide	10-500 ng/ml	$y = -15.02\ln(x) + 123.23$	0.9870	7.5 ng/ml
3.	mAb	Tetracycline	10-500 ng/ml	$y = -19.72\ln(x) + 128.54$	0.9950	9.8 ng/ml
4.	mAb	progesterone	0.05-10 ng/ml	$y = -15.17\ln(x)+45.614$	0.9990	0.026 ng/ml
5.	mAb	aflatoxin M1	3.0–50 pg/mL	$y = -27.64\ln(x) + 110.88$	0.9884	2.1 pg/mL
6	mAb	aflatoxin B1	1.0-100 pg/mL	$y = -0.21\ln(x) + 1.0006$	0.9946	0.9 pg/mL
7.	mAb	enterotoxin A	0.25-10 ng/ml	$y = -7.3588x + 81.382$	0.8273	0.23 ng/ml
8.	pAb	paraoxon	2–250 ng/ml	$y = -19,42\ln(x) + 113.97$	0.9907	1.9 ng/ml
9.	pAb	dichlorvos	2-250 ng/ml	$y = -18,11\ln(x) + 115.08$	0.9830	1.9 ng/ml

Table 2. Basic Analytical Characteristics of Multi-immunoassay of PEN, SDM and TC in buffer and Milk

Analyte	Sample	Linear range, ng/mL	Linear equation	R^2	LOD, ng/mL
PEN	Buffer	2-40	$y = -25.58\ln(x) + 100.64$	0.990	1.8
	Cow,s milk	2-50	$y = -23.29\ln(x) + 109.43$	0.998	1.8
SDM	Buffer	5-500	$y = -16.22\ln(x) + 107.83$	0.991	4.8
	Cow,s milk	5-500	$y = -15.59\ln(x) + 118.95$	0.996	4.8
TC	Buffer	5-500	$y = -17.97\ln(x) + 116.39$	0.993	4.8
	Cow,s milk	5-500	$y = -16.34\ln(x) + 124.36$	0.995	4.8

Conclusions

The separate MNPs based fluorescent immunoassays for determination of 9 different contaminants were developed. Multiplex fluorescent immunoassay based on magnetic nanoparticles for determination of penicillin, sulphonamide and tetracycline in buffer and in milk samples was developed. Very high analytical characteristics for all investigated contaminants were received - very low LODs and wide linear ranges. The new analytical device was constructed for performing of these assays. Device ensured fast, rapid, selective and cheap analysis for different contaminants in food.

Acknowledgements

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