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Use of anchor protein modules in fluorescence polarisation aptamer assay for ochratoxin A determination

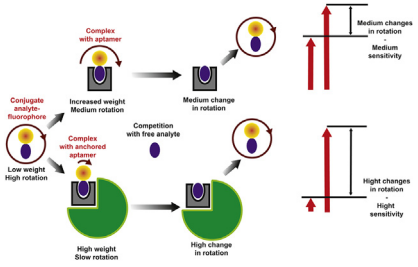
Alexey V. Samokhvalov [a], Irina V. Safenkova [a], Sergei A. Eremin [b], Anatoly V. Zherdev [a], Boris B. Dzantiev [a, *]

[a] A.N. Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Moscow 119071, Russia
[b] Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119991, Russia

HIGHLIGHTS

- Aptamer is anchored with proteins.
- Anchored aptamer is used for fluorescence polarisation assay.
- The use of anchors increases difference in polarisation of fluorescence.
- Limit of detection for ochratoxin A is decreased 40 times by the anchor use.

GRAPHICAL ABSTRACT



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ABSTRACT

A new strategy for sensitive fluorescence polarisation (FP) analysis is proposed which uses aptamer as the receptor and anchor protein modules as the enhancers by including the aptamers in complexes with protein modules. This approach is based on increasing the size differences of bound and unbound fluorophores. The strategy was applied in an ochratoxin A (OTA) assay with the competitive binding of fluorophore-labelled and free OTA with aptamer-based receptors. We showed that the binding of labelled OTA with aptamer included in complexes with anchors led to higher a FP than binding with free aptamer. This allowed the aptamer concentration to be reduced, thus lowering the limit of detection by a factor of 40, down to 3.6 nM. The assay time was 15 min. To evaluate the applicability of the FP assay with aptamer–anchor complex to real samples, we conducted OTA measurements in spiked white wine. The OTA limit of detection in wine was 2.8 nM (1.1 μg/kg), and the recoveries ranged from 83% to 113%. The study shows that the proposed anchor strategy is efficient for increasing the sensitivity of FP-based aptamer assays.

1. Introduction

Aptamers are small single-stranded nucleotide sequences which can bind different compounds with high affinity and selectively [1,2]. Any published aptamer sequence can then be reproduced by chemical synthesis and be strictly modified by different functional groups. The affinities of aptamers and antibodies are comparable [3]; however aptamers, unlike antibodies, are capable of

Abbreviations: AF4, asymmetric field field-flow fractionation; FP, fluorescence polarisation; FPIA, fluorescence polarisation immunoassay; HMW, high-molecular weight; LMW, low-molecular weight; OTA, ochratoxin A; WB, working buffer.

* Corresponding author.
E-mail address: dzantiev@inbi.ras.ru (B.B. Dzantiev).

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renaturation following denaturing effects [4]. Thus, aptamers have significant potential as recognition tools for analytical systems [5,6].

This paper is focused on the application of aptamers in fluorescent polarisation (FP) assays. FP analytical systems are based on the depolarisation of linearly polarised light by fluorophore molecules [7,8]. The main cause of depolarisation is rotational diffusion; in its absence, the emitted light stores the polarisation of the exciting light (see Fig. 1, A).

Low-molecular weight (LMW) fluorophores are subjected to significant rotational diffusion. Under experiments with the parallel linearly polarised excitation of fluorophores, if the lifetime of the excited state is much longer than the time needed to disorient molecules due to rotation, then the fluorophores change their orientation before emission. As a result of this change, the emission intensity recorded by the polariser which is parallel to the direction of the polarised excitation ($I_{\parallel}$) decreases, and the intensity of the emission recorded by the perpendicularly oriented polariser ($I_{\perp}$) increases (see Fig. 1, B). However, if the LMW fluorophores form a complex with a high-molecular weight (HMW) compound, their rotational speed decreases, $I_{\parallel}$ increases, and $I_{\perp}$ decreases (see Fig. 1, C) [9]. A change in FP allows the processes of association and dissociation between fluorophores and HMW compounds to be detected.

An FP immunoassay (FPIA), which is popular for detecting LMW compounds, is based on this principle [10–12]. This method uses a fluorophore-labelled LMW antigen conjugate having low FP values in an unbound state and high values when it is included in the complex with antibodies. The presence of a native antigen in the sample leads to its competition with the labelled antigen for binding sites of antibodies. This competition reduces FP at increased antigen concentrations up to the FP value which corresponds to the state of the labelled antigen [11]. The FPIA, a simple one-stage homogenous assay, allows the rapid detection of target analytes with high sensitivity.

There are only a few works which use the FPIA-based approach with aptamer receptors instead of antibodies; however, they are directed more to affinity and selectivity determination than to use for analytical purposes [13,14]. Most of the studies implement alternative schemes for aptamer-based FP assays. Thus, Cruz-Aguado et al. [15] suggested the use of competition between analytes and short-labelled nucleotide sequences for binding with aptamers. Hafner et al. [16] suggested the use of competition between analytes and labelled aptamers for the protein binding site. In addition, a number of FP assays based on the analyte binding-induced structure switch of aptamers have been developed. Thus, analyte binding leads to segmental mobility change in aptamer-conjugated fluorophores [17–19] or to fluorescence quenching during photo-induced electron transfer [20–22].

The limited number of traditional competitive assays with aptamers is likely due to the fact that aptamers are small in comparison with antibodies. The length of most aptamers does not exceed 80 nucleotides [3], and their binding is not associated with the same changes in rotation speed and FP as antibodies' binding [23]. In this paper, we propose to provide FP-based aptamer assays with increased sensitivity by increasing the difference in sizes of bound and unbound states of labelled analytes.

For this purpose, aptamers can be bound with anchor protein modules. We assume that the optimal protein anchors which allow the obtaining of the highest possible mP for the aptamer–protein complex with fluorescein would be IgG molecules. Nor should a further increase of anchor protein MW lead to a significant change in the FP signal.

The abovementioned is consistent with the theoretical dependence of fluorescein FP on the molecular weight (MW) of its complexes with proteins and DNA quadruplexes (a full description can be found in ESI, Part S1).

As an analyte–aptamer pair, we used a pair of ochratoxin A (OTA)–anti-OTA aptamer. OTA is a secondary fungal metabolite with a molecular weight of 404 Da and has a wide range of toxic effects on animals and humans, including neurotoxic, immunotoxic, teratogenic, reprotoxic and carcinogenic effects [24,25]. OTA is an ubiquitous contaminant of various agricultural products: grains, spices, coffee and cocoa beans, nuts and dried fruits [26]. It is one of the food contaminants with legal limits at the European level [27]. Thus, there is a great demand for simple and rapid methods to detect OTA in food products.

For the aptameric recognition of OTA, different approaches (electrochemical, colorimetric, fluorescent) are used [28–32]. In this paper, we used the FP-based approach for OTA detection by a specific aptamer with the sequence 5′-GAT-CGG-GTG-TGG-GTG-GCG-TAA-AGG-GAG-CAT-CGG-ACA-3′ [33]. Among known anti-OTA aptamers, only this sequence has earlier been applied for FP-based assays [15,22,34–36]. This allows the comparison of the existing assay schemes and the proposed one.

The aim of the study was to develop a new amplification strategy for FP aptamer assays, based on increased differences in the sizes of bound and unbound labelled antigens. The study

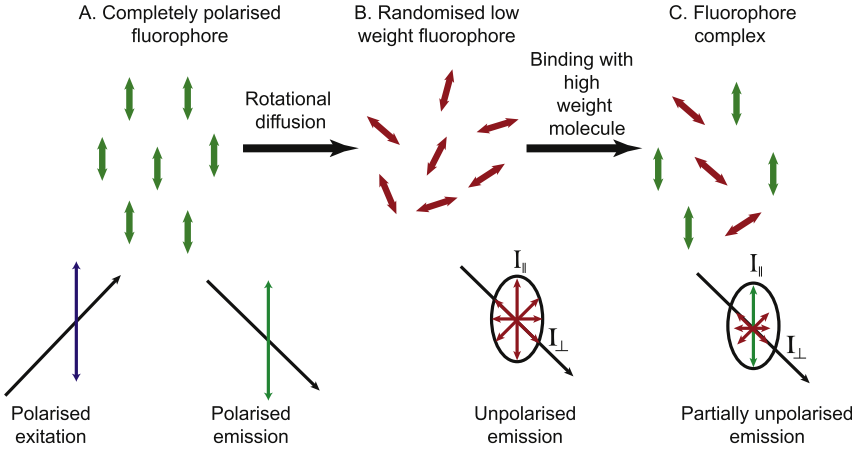

Fig. 1. The influence of rotational diffusion on the polarisation of emitted fluorescence (based on [9] with modifications): A – a fluorophore without rotational depolarisation; B – a LMW fluorophore; C – a complex of LMW fluorophores with a HMW compound. $I_{\parallel}$ and $I_{\perp}$ –intensities of emission fluorescence, registered by polarisers parallel and perpendicular to the direction of the polarised excitation.

objectives included implementing the proposed strategy on an OTA assay and comparing the FP-based aptamer assay characteristics in both new and traditional ways.

2. Materials and methods

2.1. Reagents

The analytical standard of OTA was obtained from Hromresurs (Russia), 4'-(Aminomethyl)fluorescein hydrochloride was obtained from Thermo Fisher Scientific (USA) and recombinant streptavidin was obtained from IMTEK (Russia). The DNA oligonucleotide 5-GAT-CGG-GTG-TGG-GTG-GCG-TAA-AGG-GAG-CAT-CGG-ACA-biotin-3′ was custom-synthesised, labelled and purified by Syntol (Russia). Monoclonal IgG antibodies specific to the plum pox virus (Research Centre of Biotechnology RAS, Russia) were used. Tris (hydroxymethyl)aminomethane, biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester, dimethyl sulfoxide, fluorescein isothiocyanate, N,N'-dicyclohexylcarbodiimide, dimethylformamide and polyvinylpyrrolidone (PVP-10) were obtained from Sigma-Aldrich (USA). Sodium chloride was obtained from Component Reaktiv (Russia), and potassium chloride, calcium chloride and potassium hydroxide were obtained from Khimmed (Russia). All the chemicals were of analytical or chemical reagent grade. Dry white wine (harvest of 2015, 12.5% alcohol) was bought in a local convenience store.

2.2. Apparatus

A Simplicity Milli-Q® water purification system from Millipore (Germany) was used to obtain ultrapure water for the preparation of buffers and reagent solutions. Black non-binding 96-well microtiter plates from Thermo Scientific NUNC^{tm} (Denmark) were used for FP measurements on a Zenyth 3100 (Anthos Labtec Instruments, Austria) multimode plate reader with the following filter settings: excitation of 485 nm, emission of 535 nm, an estimation time of 4 s, and the G factor set as 0.62.

A Libra spectrophotometer (Biochrom, UK) was used for the determination of aptamer and protein concentrations.

2.3. Measurements of the circular dichroism spectra

The circular dichroism (CD) spectra of the 3 μM biotinylated aptamer (see section 2.1) and its complex with OTA at a molar ratio of 1:1 were obtained using a CD spectrometer Chirascan (Applied Photophysics, UK). All measurements were carried out in a 1 cm path length cuvette in working buffer (WB) (20 mM Tris-HCl; 120 mM NaCl; 5 mM KCl and 20 mM $CaCl_2$, pH = 8.5) at room temperature. Measurements of ΔA [mdeg] were conducted in the range from 220 to 340 nm. CD is measured as the differential absorbance (ΔA) [mdeg] of left (A_{LCP}) and right circularly polarised (A_{RCP}) light, and thus it can be expressed as:

$$\Delta A = A_{LCP} - A_{RCP} \quad (1)$$

The obtained data of differential absorbance values were processed by Pro-Data Chirascan (Applied Photophysics, UK) and OriginPro 9.0 (Origin Lab, USA) software. The molar ellipticity (θ_μ) [deg cm^2 $dmol^{-1}$] of aptamer and its complex with OTA was calculated using the following equation [37]:

$$\theta_\mu = \frac{3298 * \Delta A}{C \times l} \quad (2)$$

where ΔA is the differential absorbance, C is the sample molar concentration and l is the cuvette path length in centimetres.

2.4. Synthesis of the fluorescein-labelled OTA derivative (Flu-OTA)

The carbodiimide protocol [38] was used for the synthesis of fluorescein-labelled OTA; 4 mg of OTA was dissolved in 0.2 mL of dimethylformamide, which also contained 3 mg of N-hydroxysuccinimide and 5 mg of N'-dicyclohexylcarbodiimide, and after stirring, was incubated overnight at room temperature. Then 5 mg of 4'-(aminomethyl)fluorescein was added to the obtained solution; the reaction mixture was placed in a dark place for 2 h.

The mixture was separated by thin-layer chromatography with the chloroform:methanol eluent (4:1 vol ratio) in accordance with reference [39]. The stock Flu-OTA solution was prepared by dissolving the main band (Rf = 0.9) in methanol and was stored at 4 °C. The working solutions of Flu-OTA were prepared using the dilution with 20 mM Tris-HCl buffer at a pH of 8.5. The structure of the synthesised Flu-OTA was confirmed using a liquid chromatography–mass spectrometry in electrospray ionisation mode (see ESI, Part 2).

2.5. Synthesis of the aptamer–protein complexes

Aptamer preparation. 3'-biotinylated aptamer stock solution was diluted in WB to 2 μM and heated to 90 °C for 15 min, followed by cooling at 4 °C for 10 min. Then it was heated to room temperature.

Synthesis of aptamer complex with streptavidin anchor. 500 μL of 4 μM prepared aptamer in WB was mixed with 500 μL of 2, 4 or 8 μM streptavidin in WB. Then the obtained stock solutions were incubated for 1 h at room temperature with gentle stirring.

Synthesis of aptamer complex with streptavidin–IgG anchor. At first, 10 μL of 12.6 mM solution of biotinamidohexanoyl-6-aminohexanoic acid N-hydroxysuccinimide ester was added to 1 mL of 6.3 μM IgG in 50 mM phosphate buffer (pH = 7.4). The mixture was incubated for 1 h at room temperature with intense stirring [40]. The unreacted excess of low molecular weight reactants was removed by dialysis in 2000-fold volume excess of WB for 8 h at room temperature with intense stirring. Dialysis was repeated two times.

To obtain an aptamer–streptavidin–IgG stock solution at the molar ratio 1:2:1, first, 500 μL of 2 μM biotinylated aptamer in WB and 250 μL of 4 μM biotinylated IgG were mixed. Then 250 μM of 8 μM streptavidin in WB was added. The obtained solution was incubated for 1 h at room temperature with intense stirring.

A stock solution of streptavidin–IgG complexes was also prepared to use as a negative control in the FP assays; 250 μL of 4 μM biotinylated IgG and 250 μM of 8 μM streptavidin were added to 500 μL of WB. All obtained stock solutions were stored at 4 °C.

For the characterisation of the prepared aptamer protein complexes, asymmetric field field-flow fractionation (AF4) was used (see ESI, Part 3).

2.6. FP-based aptamer assays

The competitive FP-based aptamer assays were carried out in black polystyrene microplates; the total volume per well was 200 μL. OTA dilutions in WB were prepared in concentrations ranging from 5 μM to 0.2 nM. The microplate wells were filled with 50 μL of the sample containing the known quantity of OTA and 100 μL of 8 nM Flu-OTA solution, and then 50 μL of the corresponding aptamer receptor (4 μM of aptamers or 2 μM of the aptamer–streptavidin complex or 800 nM of the aptamer–streptavidin–IgG complex) was added. The mixture was incubated for 15 min at 4°C, followed by measurements $I_{\parallel}$ and $I_{\perp}$ and calculations of FP values (P) using the following formula [9]:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (3)$$

The total emission intensity of fluorophores (I_T) was calculated as:

$$I_T = I_{\parallel} + 2I_{\perp} \quad (4)$$

Each analysis was accomplished by several controls: 1) an OTA-free sample was used to determine the maximum degree of polarisation and 2) a solution with an equimolar amount of the corresponding anchor instead of the aptamer receptor was used to determine the minimum degree of polarisation.

The dependence of FP change (y) (difference between the FP of the sample and free Flu-OTA) on the antigen concentration (x) was approximated by a four-parameter sigmoidal function using OriginPro 9 software (Origin Lab, USA):

$$y = A_2 + \frac{A_1 - A_2}{1 + (x/x_0)^p} \quad (5)$$

where A_1 is the FP for an infinitely small analyte concentration (upper asymptote), A_2 is the FP for an infinitely high analyte concentration (lower asymptote), x_0 is the analyte concentration at the inflection point that inhibits the binding of the receptors to the labelled analyte by 50% (IC50) and p is the slope of the curve at IC50.

This function was used to determine the LOD, which corresponded to a 10% inhibition of binding (IC10) [41].

2.7. FP-based aptamer assays in wine samples

An aliquot of white wine was preliminarily prepared according to the protocol suggested by Priesto-Simon et al., with modifications [42]. Namely, the aliquot was discoloured two times by adding 0.02 g mL^{-1} of dry PVP-10. After 5 min of mixing, the aliquot was filtrated through 3 kDa Amicon Ultra-15 centrifugal filter units (Merck Millipore, Ireland) for 30 min under 7000 g. The pH was shifted to 8.5 by 1 M of potassium hydroxide solution. After that, calcium chloride was added to the samples to the final concentration of 20 mM, and centrifugation was performed to remove insoluble calcium salts with organic acids. Then 3 μM of OTA was added to the treated wine, and a range of spiked wine preparations with OTA content from 3 μM to 0.06 nM with serial threefold dilutions was prepared. To carry out the competitive assay, 100 μL of the spiked wine sample was added to 50 μL of Flu-OTA (concentration 16 nM), and then 50 μL of the aptamer–streptavidin–IgG complexes (concentration 800 nM) was added. To study the assay recovery, we prepared nine samples of wine containing different OTA concentrations from 375 to 20 nM and compared the spiked OTA concentration with the concentration found by the obtained calibration curve. Each sample was measured four times. The data were approximated by a four-parameter sigmoidal function.

3. Results and discussion

3.1. Synthesis and characterisation of Flu-OTA

OTA has a free carboxyl group, which can be easily modified. Note that the anti-OTA aptamer used in this study was also selected by binding with OTA conjugated to the carrier by the carboxyl group [33]. Thus, the modification of free carboxyl in OTA molecules should not significantly affect the aptamer's binding.

Based on the above considerations, a fluorophore-labelled OTA was obtained by conjugating the OTA carboxyl group to the amino group of aminomethylfluorescein. The MW (748.18 Da) and structure of the conjugate were confirmed by liquid chromatography–mass spectrometry in electrospray ionisation mode; the results are described in ESI, Part 2.

Then the Flu-OTA concentration was calculated based on the calibration curve for fluorescein (see ESI, Fig. S4). This value was 5.1 μM.

3.2. Characteristics of the aptamers

To bind the aptamer with anchors, we worked with its biotinylated derivative. CD-spectroscopy was performed to evaluate changes in the aptamer's structure caused by this modification. It is known that this aptamer in its native (non-biotinylated) form has an antiparallel G-quadruplex structure [43]. The CD spectrum for biotinylated aptamer has two minima at 233 and 260 nm and two peaks at 243 and 294 nm (Fig. 2). These extremes are typical for single-stranded DNAs, forming a basket-type antiparallel G-quadruplex [44]. Besides this, these peaks were indicated for an unmodified anti-OTA aptamer, as described by Yang C. et al. [43]. Thus, the aptamer's biotinylation at the 3′- end does not affect its quadruplex structure. After the addition of OTA to the biotinylated aptamer, minor changes occurred in the shape of the CD spectrum; however, the antiparallel quadruplex structure with typical extremes was retained (see ESI, Fig. S5).

3.3. Development of an FP-based aptamer assay without anchors

The assay development included successive optimisations of 1) the concentration of Flu-OTA, 2) the concentrations of aptamer and 3) the incubation temperature and time.

The Flu-OTA concentration determines the intensity of the emitted light and affects the formation of a complex of unlabelled analyte with the receptor as a result of competition. Both factors affect the assay sensitivity. To minimise the detection limit, the minimum concentration of the labelled analyte providing reliable signal change after competing with the free analyte should be used [45]. The minimum concentration of Flu-OTA fulfilling the above demand was 4 nM. The fluorescence for the selected Flu-OTA concentration was 10–20 times higher than the background fluorescence of the WB, which was consistent with recommendations represented in other works [46,47].

The next step was the choice of the aptamer concentration. In the competitive assay, a higher concentration of receptors requires

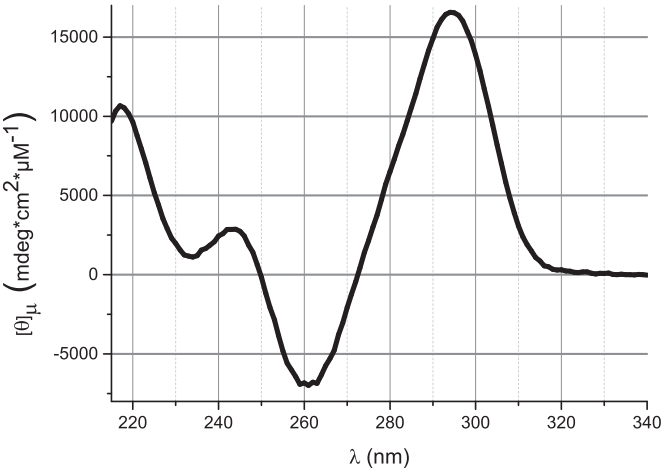

Fig. 2. CD spectrum of a 3′-biotinylated aptamer (3 μM of solution in WB).

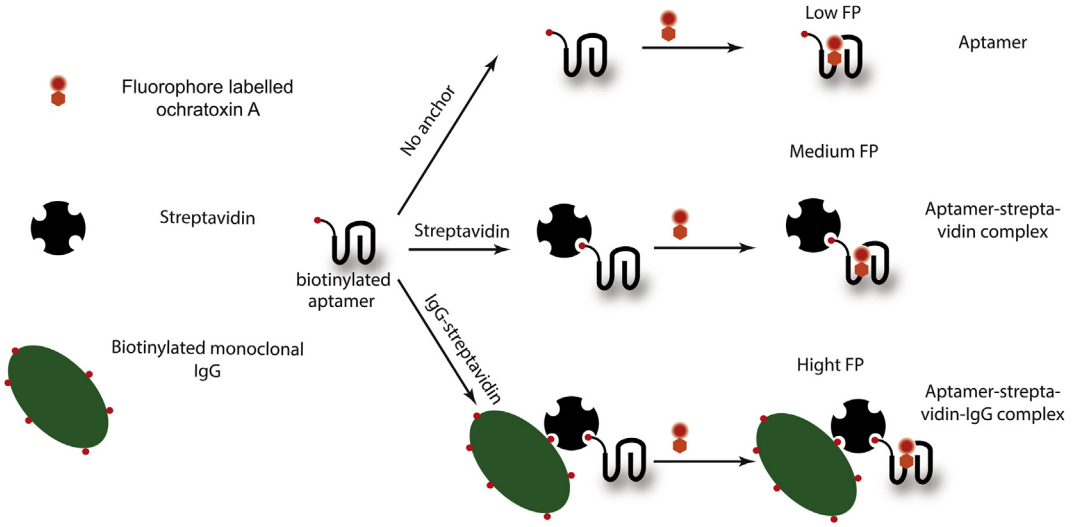

Fig. 3. Scheme of an FP-based aptamer assay without and with protein anchors (streptavidin, streptavidin–IgG).

a higher concentration of analytes in the sample to prevent the binding of labelled receptors, which leads to worse sensitivity [48]. Accordingly, to maximise the sensitivity, the minimum quantity of the receptors providing a valid calibration curve was used. For this purpose, the dependence of FP vs. the aptamer concentration was obtained, and (taking into account the requirement of reliability) a 1 μM aptamer concentration was selected (ESI, Fig. S6).

In the optimisation of the incubation temperature and time, four regimes were tested: 10 min incubation at room temperature and 5 min, 15 min and 60 min incubations at 4 °C. Under these regimes, the FP dependence on free OTA concentrations was obtained (see Fig. S7). The transition from room temperature (curve 1) to 4 °C led to some increases in FP and reductions in LOD. The observed effect can be explained by an inverse relationship between FP and temperature; its quantitative regulations are given in ESI, Part S1.

Taking into account the reliability and LOD criteria, the incubation temperature and time were chosen to be equal to 15 min and 4 °C, respectively (Fig. S7, curve 3), and the competitive assay was carried out under these conditions.

3.4. Development of an FP-based aptamer assay with anchor protein modules

We developed an FP-based aptamer assay with the use of two types of aptamer-containing complexes, based on the streptavidin (53 kDa) and streptavidin–IgG (203 kDa) anchor modules (Fig. 3). The use of these anchors should lead to a significant increase in the FP of their complexes with Flu-OTA see theoretical calculations of MW-FP dependence in ESI, Part S1.

Neither protein interacts with fluorescein or OTA [11,49], and thus they do not influence the implemented FP-based aptamer assays.

The first step was to obtain a complex of biotinylated aptamer (11.7 kDa) with streptavidin. This complex may have various stoichiometries due to the four biotin-binding sites of the streptavidin molecule. Three preparations of aptamer–streptavidin complexes were synthesised using different aptamer:streptavidin molar ratios, namely 1:2, 1:1 (estimated MW of aptamer–protein complex is 65 kDa) and 2:1 (77 kDa). The Flu–OTA FP's dependence on the concentration of these complexes was obtained (see ESI, Part 8). The greatest FP change was observed for the complex with a ratio of 1:2, which was selected for subsequent experiments. Increases in the intermolecular complexes' weight were confirmed by AF4 (see ESI, Figs. S3–1).

The next step was to obtain the ternary complex aptamer–-streptavidin–IgG (~215 kDa). The molar ratio of the reactants was selected based on the optimal value for the previous complex; this was 1:2:1, respectively. The formation of the ternary complex was also confirmed by AF4 (see ESI, Figs. S3–2).

The FP's dependence on the concentration was obtained with the use of the aptamer and its two complexes (Fig. 4).

Based on Fig. 4, the FP change increases in a row: free aptamer → aptamer–streptavidin complex → aptamer–streptavidin–IgG complex. Thus, we have confirmed the amplification effect for FP when including aptamer in the protein complexes. We have also demonstrated that the use of protein anchors with a MW up to 53 kDa is not sufficient to maximise the FP of complexes with Flu–OTA.

The optimal concentrations for aptamer–anchor complexes to perform a competitive assay were selected based on Fig. 4, curves 2 and 3. They were 500 and 200 nM, respectively. Thus, the aptamer concentration required for the assay was lowered two- and fivefold,

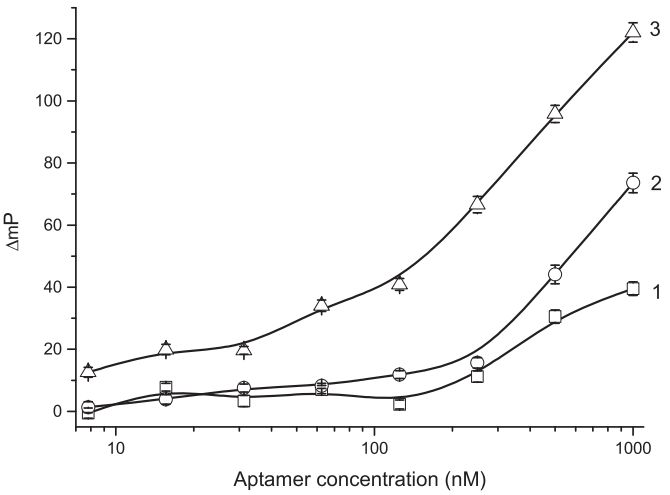

Fig. 4. FP changes dependencies from concentrations of aptamer-based receptors (n = 4): 1 – free aptamer; 2 – aptamer with streptavidin anchor; 3 – aptamer with streptavidin – IgG anchor.

respectively, compared with the non-anchor scheme. We compared the obtained calibration curves for OTA detection (see Fig. 5). The calibration curve for OTA detection for the aptamer is presented in Fig. 5, curve 1. At the selected aptamer concentration of 1 μM, the LOD and IC50 were 130 nM and 1340 nM, respectively.

The calibration curve for OTA detection for the aptamer–streptavidin complex is presented in Fig. 5, curve 2. At the selected concentration of this complex, the LOD was 43 nM, and the IC50 was 533 nM. Due to the inclusion of aptamer in the complex with streptavidin, the LOD of the assay was lowered by three times.

The calibration curve for the aptamer–streptavidin–IgG complex is shown in Fig. 5, curve 3. The LOD was 3.6 nM; the IC50 was 98 nM. Thus, the use of the streptavidin–IgG anchor allows a 40-fold enhancement of the assay sensitivity.

The LOD equivalent by weight was 1.45 μg of OTA per kg of the tested sample, less than the maximum residue level of OTA, namely 2 μg/kg for OTA in wine [27].

3.5. FP-based aptamer assays with anchors of OTA in real samples

Wine is one of the main products affected by OTA contamination [50,51]. White dry wine was used to confirm the efficiency of the proposed FP-based aptamer assay. Pretreatment of the wine included primary discoloration with PVP-10 two times to sorb the polyphenols contained in wine [42,52]; the pH adjustment and the addition of calcium chloride were fully described in part 2.7 of the Material and Methods section. The treated wine samples were used to obtain calibration curves at optimised conditions (4 °C and 15 min of incubation time). The obtained calibration curve for OTA concentration in the wine samples is shown in Fig. 6; the LOD for OTA was 2.8 nM (1.1 μg/kg), and the IC50 was 72 nM. These correspond to the values obtained in the model system. Thus, the chosen pretreatment allows a decrease in the effect of the matrix on the FP assay, and the obtained LOD is lower than the maximum residue level (MRL) of OTA in wine.

To investigate the potential practical applications of the proposed FP assay, OTA-positive white wine samples were prepared by spiking OTA into OTA-free wine samples at different concentrations, and these samples were tested. In Table 1, the recovery data for OTA detection are summarised. The recoveries of the spiked samples ranged from 83% to 113%. Accordingly, the proposed FP assay had appropriate reliability and could be applied to detect OTA in the actual white wine samples.

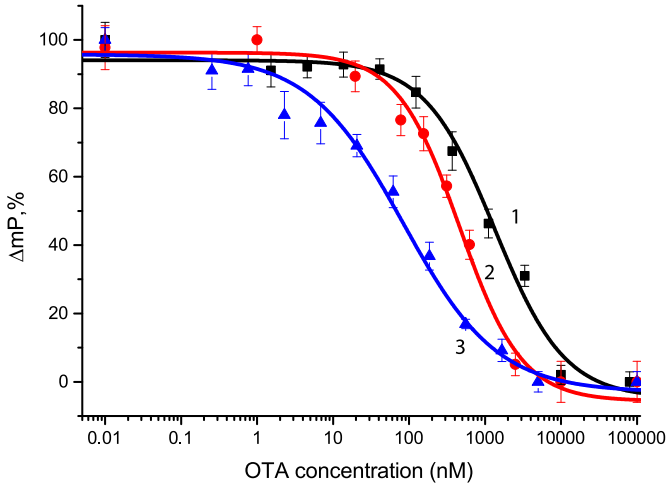

Fig. 5. Calibration curves for FP-based aptamer assays of OTA (n = 4): 1 – free aptamer; 2 – aptamer with streptavidin anchor; 3 – aptamer with streptavidin–IgG anchor.

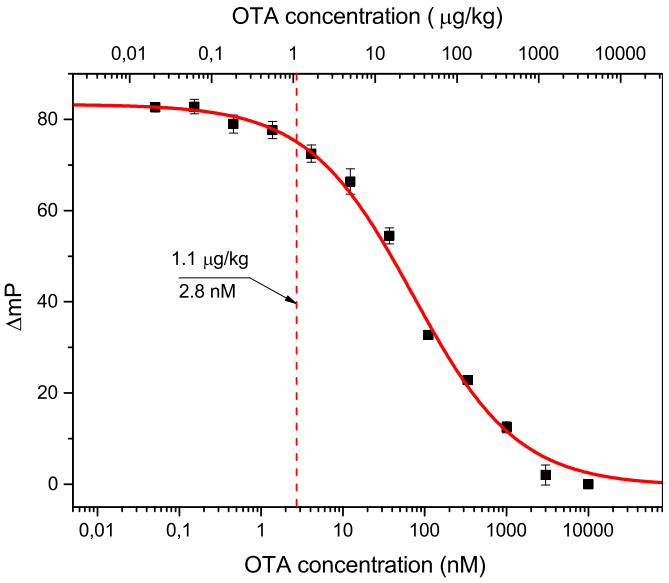

Fig. 6. Calibration curve for FP-based aptamer assays of OTA with aptamer–streptavidin–IgG complex in white wine (n = 4).

3.6. Comparison of the obtained results with characteristics of other FP-based aptamer assays

Five papers on the FP-based aptamer detection of OTA have been published. Their comparison is summarised in Table 2.

As we can see, the LOD value of the proposed FP assay is lower than or matches the earlier proposed methods. Only Huang et al. [54] obtained a lower LOD of 25.2 fM in their model system; however, this FP assay was based on a cascade enzymatic enhancement, which made it more complex and longer than other methods.

With the exception of our article, the suitability of FP-based aptamer assays for OTA detection in natural matrixes has only been demonstrated by Zhao et al. [22].

Thus, our approach allows a combination of high sensitivity and practical applicability, but it requires a preliminary preparation step.

Aptamers have been included in HMW complexes in several alternative schemes for FP-based assays to detect different compounds. Kang et al. [49] proposed a complex scheme with

Table 1
Assay results for detection of different concentrations of OTA in white wine samples (n = 4).

Spiked, nM	Found, nM	Recovery, %
375	328.6	87.6
200	213	106.5
187	160.2	85.7
175	194.9	111.4
100	109.3	109.3
93	77.6	83.4
50	54.3	108.6
44	48.4	110.0
25	28.2	112.7

Table 2
Main parameters of the proposed and existing FP-based aptamer assays for OTA.

	Enhancing tools	Assay time, min	Matrix	LOD, nM	Reference
1	No	5	Buffer	5	[15]
2	Enzymatic cleavage protection	55	Buffer	70	[53]
3	Photo-induced electron transfer	<5	Buffer	3	[22]
			10-fold diluted urine sample	12	
			50-fold diluted red wine	12	
4	Anchor nanoparticles, enzymatic cascade reaction	60	Buffer	0.0000252	[54]
5	Protein anchor	15	Buffer	3.6	This paper
			White wine	2.8	

enzymatic decomposition by DNase, where a fluorophore-labelled aptamer was included in the complex with streptavidin (53 kDa). As a result of binding the streptavidin anchors, the LOD for adenosine was reduced tenfold. Zhu et al. [55] proposed displacing the fluorescently labelled aptamer by adenosine from its complex with single-strand DNA-binding protein (protein anchor, 19 kDa). Cui et al. [23] suggested the direct FP-based assay of adenosine and cocaine based on a chimeric aptamer which consisted of hybridised fluorophore-labelled specific and anti-thrombin parts. This chimeric aptamer was designed so it could bind thrombin (protein anchor, 40 kDa) only in the presence of small analyte molecules.

As we can see, all these assays are based on complex rows of interactions. In addition, working with 19–53 kDa anchors does not ensure a maximal level of FP change (as follows from the theoretical calculations above) or the maximum sensitivity of the assay.

In this work, the application of the anchor with a total weight of ~215 kDa allowed the LOD to be reduced 40-fold. The presented strategy is versatile, simple and one-stage, and it makes it possible to perform assays in natural matrixes. Thus, the strategy can become an effective solution for the FP aptamer-based detection of various LMW analytes.

4. Conclusion

We have demonstrated enhanced assay sensitivity as a result of the increased size of aptamer receptors; a 40-fold difference in the LOD for the traditional and the proposed FP assays of OTA has been achieved. The obtained LOD is not inferior to the LODs for alternative schemes of FP-based OTA detection. At the same time, the proposed assay is one-stage and easy to use; its duration is 15 min. Its applicability to test white wine was confirmed.

The suggested amplification strategy ensures a significant increase in the sensitivity of FP-based aptamer assays and can be applied to detect other LMW molecules. It combines the advantages of aptamer receptors and conventional FP-based assays.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2017.01.024.

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