Analytica Chimica Acta 1133 (2020) 20–29

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a DNA-based biosensor for the fast and sensitive detection of ochratoxin A in urine

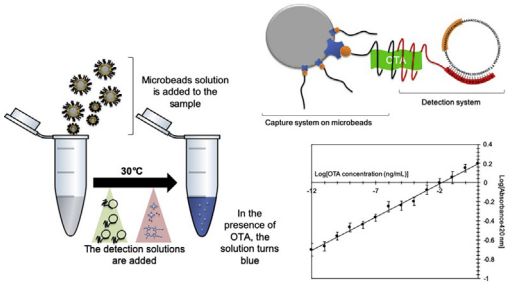
Elisa Santovito [a, *], Donato Greco [a], Vito D'Ascanio [a], Simona Marianna Sanzani [b], Giuseppina Avantaggiato [a]

[a] Istituto di Scienze Delle Produzioni Alimentari (ISPA), Consiglio Nazionale Delle Ricerche (CNR), Via Amendola 122/O, 70126, Bari, Italy
[b] CIHEAM Bari, Via Ceglie 9, 70010, Valenzano, BA, Italy

HIGHLIGHTS

- Development of a novel DNA-based biosensor on paramagnetic microbeads.
- Detection of ochratoxin A by self-catalytic rolling circle DNA amplification.
- Aptamer-based ochratoxin A capture for sample enrichment.
- Visual detection of ochratoxin A in urine samples by a colourimetric reaction.
- High selectivity of the capturing and detection system in mice urine.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 March 2020
Received in revised form
23 July 2020
Accepted 30 July 2020
Available online 12 August 2020

Keywords:
Ochratoxin
Mycotoxins
Rolling circle amplification
Aptamer
Self-catalytic DNA
Urine

ABSTRACT

In this paper, a novel DNA-based biosensor is proposed, which is based on paramagnetic microbeads carrying an ochratoxin A (OTA) capture aptamer. A sandwich-like detection complex is linked to the capture aptamer and is able to trigger, in presence of OTA, an isothermal rolling circle amplification (RCA) reaction. This latter generated autocatalytic units with a peroxidase activity (DNAzyme) that, in presence of a proper substrate, gave a blue-coloured product visible by the naked eye. The capture aptamer, blocked onto magnetic beads, allowed the specific capture of OTA in liquid samples. The modified detection aptamer, annealed to a circularized probe, was then used to detect the toxin capture event. Indeed, in the presence of OTA and an isothermal enzyme, the circular DNA was amplified, producing a single-stranded and tandem repeated long homologous copy of its sequence. In the DNA strand, a self-catalytic structure was formed with hemin as the catalytic core, inducing the development of blue colour in the presence of ABTS and hydrogen peroxide. The results showed that the biosensor has high sensitivity and selectivity for the detection of OTA, as low as 1.09×10^{-12} ng/mL. Moreover, the proposed biosensor was successfully used for the detection of OTA in naturally contaminated rat urine. Accuracy and repeatability data obtained in recovery experiments were satisfying, being recoveries >95% with relative standard deviations in the range 3.6–15%. For the first time, an aptasensor was successfully applied to detect OTA in biological fluids. It can be used for mycotoxin biomonitoring and assessment of individual exposure.

* Corresponding author.
E-mail address: elisa.santovito@ispa.cnr.it (E. Santovito).

https://doi.org/10.1016/j.aca.2020.07.078
0003-2670/

Abbreviations

OTA	Ochratoxin A
RCA	Rolling circle amplification
AFB_1	Aflatoxin B_1
ZEN	Zearalenone
FB_1	Fumonisin B_1
DON	Deoxynivalenol
OTα	Ochratoxin α
CssDNA	Circular ssDNA
CS	Capture system
DS	Detection system

1. Introduction

Many fungal species of the genera *Penicillium* and *Aspergillus* produce the mycotoxin ochratoxin A (OTA) in their secondary metabolism [1]. OTA contamination can occur in the field and/or during storage in a variety of food and feed [2–4]. OTA has hepatotoxic, immunotoxic, neurotoxic, nephrotoxic, and teratogenic effects on several animal species. After ingestion, OTA is absorbed in the small intestine and excreted into bile and urine. OTA absorption is species-dependent, and in humans it occurs with high levels (62%–100%) [2,5]. When ingested, OTA can be metabolized resulting in the presence of different metabolites, including ochratoxin α (OTα), formed by the cleavage of the phenylalanine moiety of OTA [2–5]. Several *in vivo* studies showed that free OTA is more prevalent in urine and feces than OTA metabolites due to low level of metabolization [2–5]. Thereof, determination of OTA levels in biological fluids reported in many studies has demonstrated its capacity to be used as a biomarker and a good indication of OTA exposure [6]. Because of the severe dose-dependent effects of OTA on human health, it is of paramount importance to collect information on exposure to this mycotoxin for a risk evaluation. As such, its analysis in foods and biomonitoring in human biological samples play a very important role and are also recommended [6,7].

Assessment of mycotoxin exposure by biomonitoring a biomarker in biological fluids or tissue (internal exposure assessment) is considered the best way to assess individual exposure to mycotoxins rather than external exposure assessment (determination of mycotoxin occurrence in foods and combining with information on food consumption) [5]. Of course, measurement of internal exposure to mycotoxins needs validated biomarkers, validated analytical methods and easily accessible biological samples, such as biological fluids. Urine is considered the best matrix to estimate mycotoxin exposure as sampling is non-invasive, and it contains biomarkers for most mycotoxins.

Taking into account the literature, free OTA in biological fluids is one of the most widely studied mycotoxin biomarker for human biomonitoring [8–11]. Most of these studies analysed OTA in biological samples from humans or animals by liquid chromatography techniques, often coupled with tandem mass spectrometry (MS) or high resolution mass spectrometry. These techniques allowed the detection of OTA in urine samples from adults and infants with a limit of detection (LOD) of 0.03 ng/mL [12]. However, although chromatographic techniques offer high sensitivity and specificity, they require expensive analytical equipment and trained operators. Furthermore, they cannot be used for on-site detection. Biosensors, on the other hand, represent a valid alternative for mycotoxin detection in a wide range of matrices due to their high portability potential and ease of use [13].

To the best of our knowledge, few biosensors have been used to analyse mycotoxins in biological fluids in the last years, and a few of them achieved the determination of mycotoxins in real samples [11]. For OTA detection, different types of biosensors have been developed, based on the specific recognition of OTA by aptamers [14–18]. None of these aptasensors have been applied to detect OTA in biological fluids. Therefore, there is a need to develop and research new biosensors for mycotoxin determination in biological samples, and new advances are required to enhance their selectivity and sensitivity.

Aptamers are nucleic acids oligomers selected *in vitro* by the SELEX process [19] that can recognize different analytes. Aptamers recognizing low-molecular-weight substances as cocaine [20], flavin and NAD^+ cofactors [21] or adenosine triphosphate (ATP) [22] are available. Furthermore, aptamers against various mycotoxins such as aflatoxin B_1 and B_2, OTA and fumonisin B_1 were obtained [23]. The aptamer functions can benefit from complexing with other molecules as catalytic nucleic acids, with the possibility to obtain bifunctional nucleic acid complexes that include a capture and a reporter sites that can eventually provide a measurable signal.

Catalytic nucleic acids are deoxyribonucleic (DNAzymes) or ribonucleic (ribozymes) acid structures that have catalytic functions. While ribozymes are used as catalysts by living cells, DNAzymes have been obtained synthetically [24–26]. A DNAzyme frequently used for biosensing is an oligonucleotide with peroxidase-like catalytic activity [27], e.g. for the detection of nucleic acids [28,29]. To exert their catalytic activity, these DNAzymes have to fold in a G-quadruplex structure, thus binding hemin molecules and catalysing a redox reaction between ABTS and hydrogen peroxide, with the production of oxidized ABTS and consequently of a colorimetric reaction. These peculiar properties have been used to develop "DNA-based nano-machines" able to catalyse reactions in response to specific recognition or capture events associated to aptamers [30]. These detection systems are highly sensitive and can be valid substitutes of the PCR amplification processes [31]. Indeed, with appropriate engineering of the template DNA sequences, it is possible to synthesize large quantities of DNAzymes during an isothermal reactions as Rolling Circle Amplification (RCA) reactions, thus producing high throughput and high sensitivity systems [32]. On these basis, DNA-based biosensors appear to be promising in the detection of mycotoxins, since biodegradable, highly selective and easy to obtain.

The direct amplification of specific nucleotide sequences without the use of thermal cycling is possible because of the strand-displacement activity of some DNA polymerases that catalyse amplification in very high yields, without the need of a denaturing step at high temperature [33–35]. A commonly used enzyme with strand displacement activity is the ϕ29 DNA polymerase, a highly processive one whose error rate is in the range of 2.2×10^{-5} to 4×10^{-6} [34,36,37]. This enzyme is routinely used in the RCA reactions. RCA allows the extension of an oligonucleotide annealed to complementary sequences on a circular single-stranded DNA (ssDNA) template, thus triggering the synthesis of a long DNA molecule. This ability is useful for the detection of low amounts of target sequences [38]. For example, Lagunavicius et al. [39] applied this technique for the visualization of individual RNA targets *in situ* in cell cytoplasm. Taking into account all these factors, RCA can be used for the analysis of individual molecules [40].

In this research, we developed a DNAzyme-based biosensor for the detection of OTA, based on the use of magnetic microbeads specifically functionalized for the selective capture of OTA (Fig. 1). This system allows OTA to be concentrated in the samples, and its presence in positive samples to be visualized as a colour change of the solution. A quantitative detection is also possible by a spectrophotometer. A specific aptamer for the capture of the toxin was

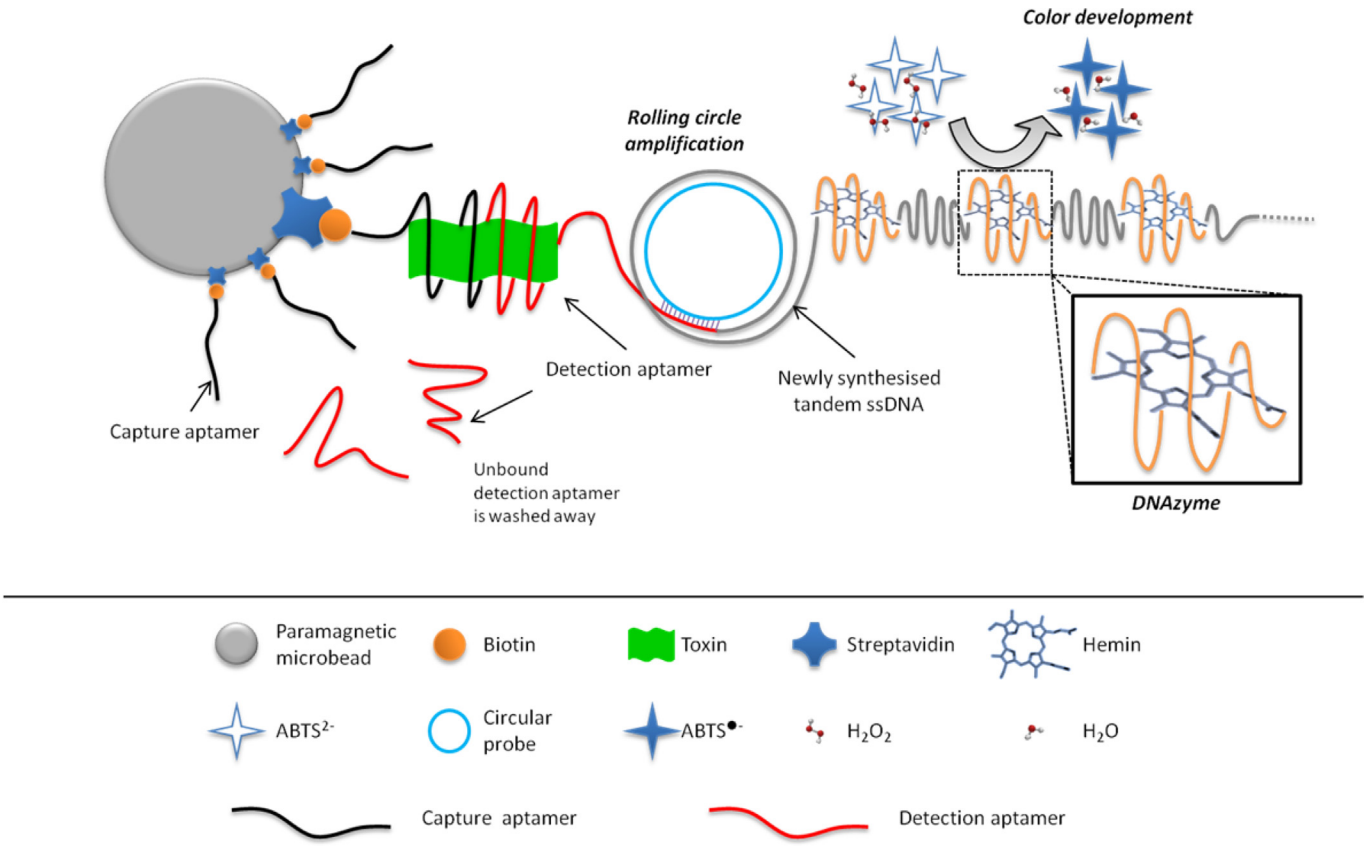


Fig. 1. Schematic diagram of aptamer-based biosensor for OTA detection, based on rolling circle amplification (RCA) and an auto-catalytic DNAzyme structure. Capture aptamers are linked to paramagnetic beads by biotin-streptavidin interaction. OTA is captured specifically and a second aptamer is used for its detection. This latter contains a DNAzyme generating sequence and an RCA priming sequence for the isothermal DNA amplification triggered by a circular ssDNA. In the presence of hemin and ABTS a blue colour is generated when OTA is captured. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

used to coat paramagnetic microbeads. Once in the sample, these beads captured the target toxin and were recovered by a magnet. A further detection aptamer was used as a reporter for the presence of the captured toxin on the microbeads. This second aptamer was modified with a priming sequence that triggers a solid-phase isothermal RCA amplification when the toxin is present.

In the RCA reaction, a high number of self-catalytic DNAzyme structures are generated causing a blue-coloured tone as a visual sign of a positive reaction, which is also quantifiable. This biosensor was in-house validated and applied for OTA detection in rat urine.

2. Materials and methods

2.1. Reagents and DNA oligomers

The sequences of the oligomers used in this study are reported in Table 1. They were synthesized by ThermoFisher Scientific (Milan, Italy) with the required terminal modifications and with HPLC purification grade. The padlock probe was designed to incorporate the template sequence for the synthesis of the PS2.M DNAzyme [27] with a peroxidase-mimicking activity in the presence of hemin. The OTA aptamer reported by Cruz-Aguado and Penner [41] was used for the study.

Oligonucleotides were diluted in ultrapure water (18.25 MΩ cm) at the concentration of 100 μM. Tubes were incubated at 95 °C for 5 min and cooled down to room temperature for 2 h before use. These oligonucleotides stock solutions were aliquoted and stored at −20 °C, and then kept on ice during the experiments.

ABTS and hemin were purchased from Sigma Aldrich (Milan, Italy). Φ29 DNA polymerase, Exonuclease I, Exonuclease III, T4 DNA ligase and T4 polynucleotide kinase were purchased from ThermoFisher Scientific.

2.2. Mycotoxins and chemicals

Solid standards of mycotoxins were purchased from Sigma-Aldrich. Unless otherwise stated, all chemicals used were of analytical grade.

Table 1
Sequences of the oligonucleotides used in this study.

Oligo name	Sequence (5' - 3')	Terminal modification
Ligation template	cttagcgccttagaatctggtttagctac	None
Padlock probe	ctaaggcgctaagaaacatcgaaaaaCCCAACCCGCCCTACCCAaaaaccacggggaaggcgtagctaaaccagatt[a]	5′ phosphate
OTA capture aptamer	gttgggcacgtgttgtctctctgtgtctcgtgcccttcgctaggccc	3′ biotin
Detection OTA aptamer (modified)	gttgggcacgtgttgtctctctgtgtctcgtgcccttcgctaggcccccgtggCGATGTTTCTTAGCGCCTTAC[b]	None

[a] Capital letters in the padlock probe sequence indicate the PS2.M DNAzyme scaffold template.
[b] capital letters in the detection OTA aptamer indicate the target sequence for the circular RCA probe.

A 1 mg/mL stock solution of OTA was prepared in 1% acetic acid in toluene. A 10 μg/mL OTA standard in methanol was also prepared and spectrophotometrically analysed at 332 nm (ε = 6330 cm^2/mmol). For HPLC calibration, the 500 μg/mL OTA solution in acetonitrile was dried under a nitrogen stream and dissolved in 15% methanol in water. OTA standard solutions for the calibration of the biosensor and for urine spiking in recovery tests were prepared in PBS buffer diluting the 500 μg/mL OTA solution in acetonitrile.

Multi-toxin standard solutions containing or not OTA in addition to aflatoxin B_1 (AFB_1), deoxynivalenol (DON), T2 and HT-2, fumonisin B_1 (FB_1), OTα, and zearalenone (ZEN), at the concentration of 10 ng/mL, were prepared from 10 × stock solutions of the single mycotoxins. Preparation of these standard solutions and analysis by Ultra-Performance Liquid chromatography, coupled with Fluorometric or UV detection (UPLC-FLD/PDA) was performed according to Avantaggiato et al. [42].

2.3. Circular probe synthesis

Since required for the ligation of 3′ and 5′ termini, the single-stranded padlock probe DNA had to be phosphorylated at 5′-hydroxyl terminus before circularization. A T4 polynucleotide kinase was used to transfer the γ-phosphate in the 5′ terminus ATP of the padlock probe. In the phosphorylation reaction, 300 pM of the padlock probe, 1 mM of ATP, 10 U of T4 polynucleotide kinase and 1 × of the specific reaction buffer were added, and the tubes were incubated at 37 °C for 30 min and then at 65 °C for 20 min.

The procedure for the synthesis of the circular probe is detailed in Fig. 2. The ligation template and the phosphorylated padlock probe were annealed in 1:1 ratio by incubating 300 pM of each oligonucleotide at 25 °C for 2 h. Subsequently, the padlock probe was circularized by linking its 5′ phosphorylated end to the 3′ terminus, using T4 DNA ligase. For the ligation reaction 100 pM of the phosphorylated padlock probe, 100 pM of ligation template and 10 U of T4 DNA ligase, were added in a final volume of 100 μL. Tubes were incubated at room temperature for 30 min. In the presence of a dsDNA portion (Fig. 2a), the nick repair activity of T4 DNA ligase closed the linear padlock probe thus generating a single-stranded circular DNA, annealed with the ligation template.

To degrade the annealed ligation template 10 U of Exonuclease I and 10 U of Exonuclease III were added and tubes incubated at 37 °C for 30 min and then at 95 °C for 10 min (Fig. 2b). By the end of the procedure, a circular ssDNA (CssDNA) was obtained.

2.4. Biosensor preparation

The workflow for the preparation of the biosensor is detailed in Fig. 3. Paramagnetic streptavidin-coated beads were obtained by Thermo Fisher Scientific. Beads were washed 5 times with 1 mL of a buffer solution (namely OTA buffer) containing 120 mM NaCl, 20 mM $CaCl_2$, 10 mM Tris pH 8.5, and 5 mM KCl [18,43], to remove any residual of the storage buffer in which the magnetic particles were supplied. Washing was performed by placing the particles solution on a magnetic stand (DynaMag™-2, Thermo Scientific) for 3 min and removing the solution with a pipette.

Different amounts (10-10^{-12} ng/mL) of OTA were tested in the capture reaction, in the presence of 0.1 μM OTA-capture aptamer and in a final volume of 10 μL of OTA buffer. The OTA-capture reaction was conducted at 25 °C. Then, the capture mix was supplemented with 100 μg of paramagnetic beads in a total volume of 100 μL of OTA buffer. The reaction mixture was then incubated at room temperature for 30 min. In this reaction, the complex was captured by the streptavidin on the surface of the magnetic beads, thus forming the capture system (CS) of the biosensor. The solution was gently mixed and placed on a magnetic stand to collect the magnetic beads on a side of the tubes. The beads were then washed three times with 200 μL of clean OTA buffer to remove eventually unbound aptamers. Finally, the apta-beads were resuspended in 10 μL of OTA buffer.

The detection system (DS) was prepared by annealing the detection aptamer with the circular DNA. In this reaction, 0.1 μM of OTA detection aptamer were mixed with the circular DNA in 1:1 ratio. The reaction mix was incubated at 95 °C for 5 min then slowly cooled to room temperature for 2 h to allow the annealing of the complementary sequences on the two oligomers. The structure of the detection system is detailed in Fig. 4.

In the OTA detection reaction, 10 μL of the CS were incubated with 0.1 μM of OTA DS in a total volume of 20 μL. Unbound DS was

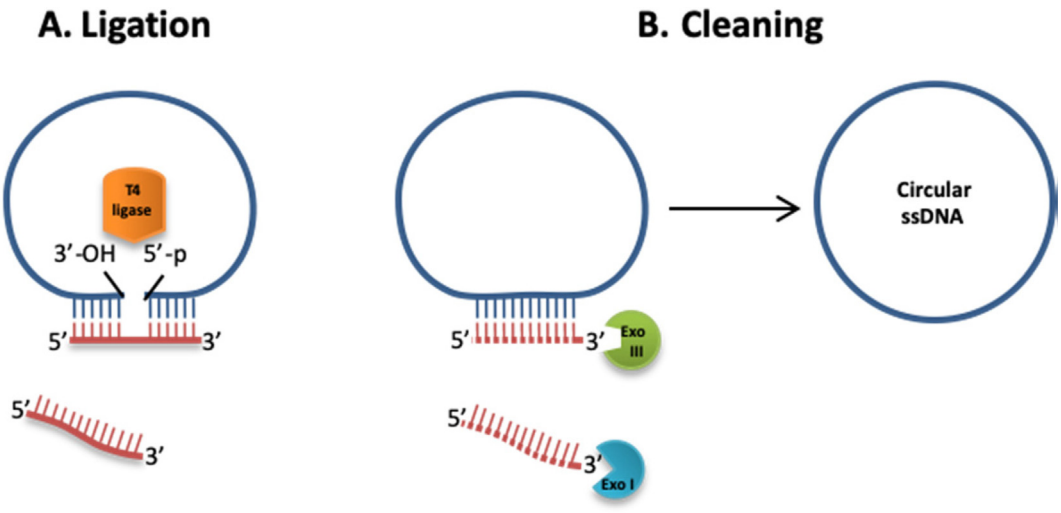


Fig. 2. Preparation of the circular ssDNA probe.
A. The phosphorylated padlock probe is annealed to the ligation template to bring near the 5′(P) to the 3′(OH) terminus. This creates a dsDNA portion with a nick on the padlock probe strand. The nick repair activity of the T4DNA ligase joins the 5′(P) to the 3′(OH) terminus on the padlock probe strand thus generating a circular ssDNA annealed with the ligation template. B. Exonuclease III (ExoIII) catalyses the removal of mononucleotides from 3 terminus of the double-stranded DNA portion. ExoIII also acts at nicks in non-ligated ds DNA to produce single-strand gaps and thus degrading the extremities of the non-ligated padlock probe. At the same time, Exonuclease I (ExoI) hydrolyses single-stranded DNA in the 3′→5′ direction, thus removing linear ssDNA still available in the reaction. The products remaining after the cleaning step is the circular ssDNA and mononucleotides.

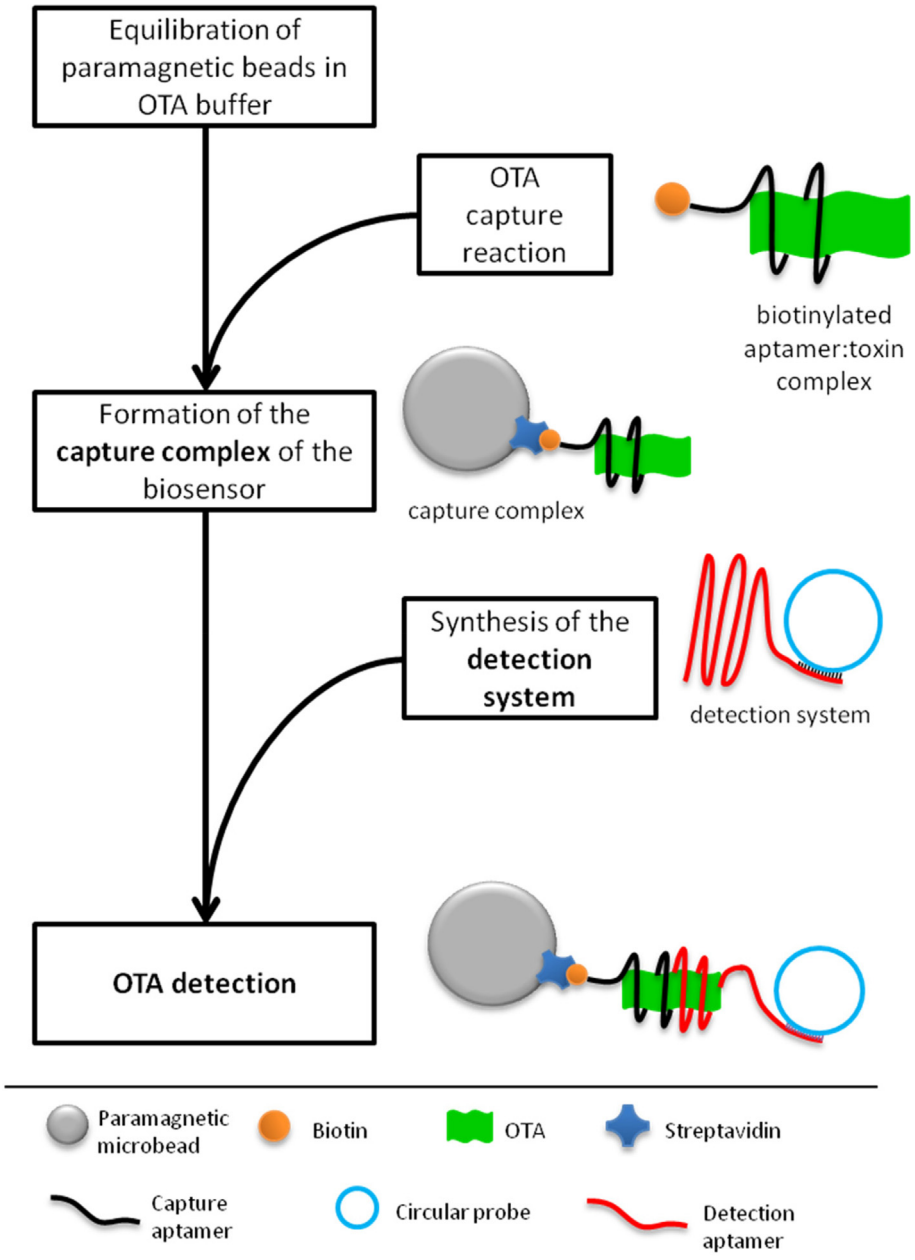


Fig. 3. Experimental workflow for the construction of the OTA biosensor.
The main steps for the construction of the OTA biosensor include the build-up of a capture system and a detection system in two separate reactions. The final detection is performed when the final biosensor is fully assembled.

removed by placing the tubes on a magnetic stand following three washes with OTA buffer.

2.5. OTA detection and colorimetric reaction

The capture of OTA was detected by a solid-phase RCA, according to the protocol used by Gomez et al. [44]. The reaction volume was 10 μL and contained the biosensor particles, 1 × of ϕ29 DNA polymerase buffer, 1 mM of dNTPs, 2 mg/mL of BSA, 5 U of ϕ29 DNA polymerase and 200 nM of CssDNA. Tubes were incubated for 4 h at 30 °C. For colour development, the protocol suggested by Travascio et al. [45] was used with some modifications. Hemin was added to the RCA product to reach a final concentration of 0.5 μM. The mixture was incubated at room temperature for 30 min to allow the hemin to complex with DNA, forming the self-catalytic G-quadruplex DNAzyme structure. Then, 5 mM of both ABTS and stabilized H_2O_2 were added to the RCA product solution. Absorbance data were obtained at 420 nm by a spectrophotometer (Multiskan EX, Labsystem).

2.6. Intra-laboratory validation of the biosensor as a quantitative method

To assess cross-reactivity and selectivity of the system, the biosensor was tested against OTα and other six mycotoxins commonly occurring in biological samples, *i.e.* AFB_1, ZEN, FB_1, DON, T2, HT-2. Two multi-mycotoxin solutions, containing (mix OTA+) or not OTA (mix OTA-), at the concentration of 10 ng/mL for each

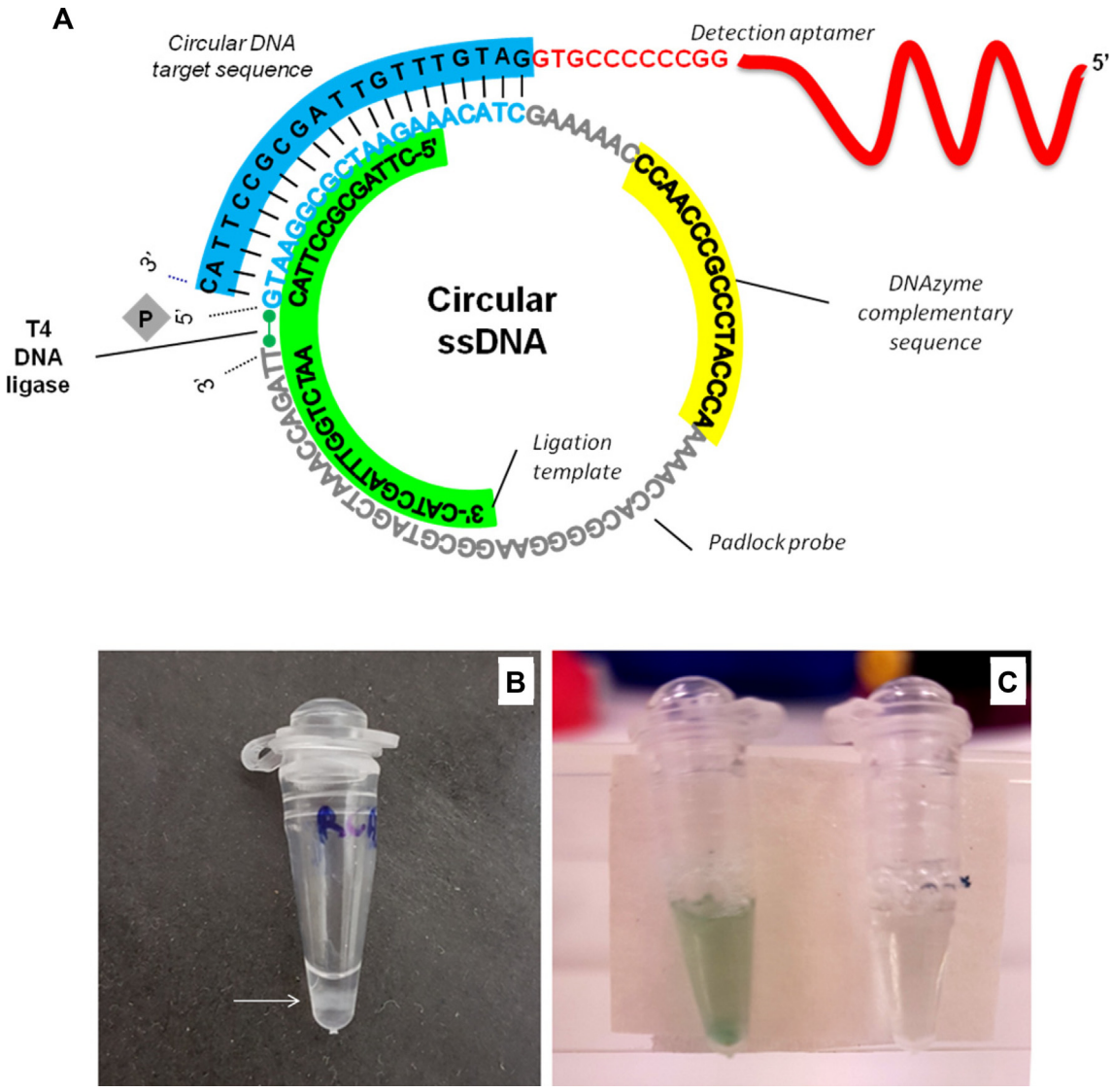


Fig. 4. Structure of the detection system (DS) and product of the RCA biosensor reaction.
A. The circular ssDNA is generated by annealing of the ligation template (green), that joins the 5′ phosphate and the 3′ termini of the padlock probe. The T4 DNA ligase repairs the nick, thus closing the padlock probe into a circular single strand DNA molecule. The detection aptamer is modified by adding a 21 nt tail which anneals to its complementary sequence on the circular ssDNA (CssDNA), thus forming the DS. **B.** In presence of OTA, RCA reaction generated high amounts of long branched DNA that precipitates and is visible at naked eye. The arrow indicates the DNA precipitate in the reaction tube. **C.** In the presence of the CS:OTA:DS, the DNAzyme-hemin catalysed the peroxidation of $ABTS^{2-}$ generating a coloured product ($ABTS^{\cdot-}$) that stained the solution in blue; whereas in the absence of CssDNA, the RCA reaction did not take place, and the solution remained colourless. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

toxin, were analysed as reported above. Absorbance values recorded for the mycotoxin mixtures were compared to those obtained for OTA standard solutions standing alone and containing the same amount of toxin. Experiments were performed in triplicate and repeated three times on different days.

Standard curves in PBS buffer, determination coefficients, and linear equations were determined by plotting OD_{420} values *versus* OTA concentration. The experiment was conducted in triplicate and repeated three times on different days. Results were expressed as mean ± standard deviation ($n = 9$). OTA concentrations ranged from 10 to 10^{-12} ng/mL. Limit of blank (LOB) was calculated as $LOB = \text{mean}_{\text{blank}} + 1.645(\sigma_{\text{blank}})$, where σ_{blank} is the standard deviation of blank absorbance measurements at 420 nm [46]. Limit of quantification (LOQ) and LOD were calculated according to Shrivastava and Gupta (2015) [47] as: $LOD = 3(\sigma/s)$, $LOQ = 10(\sigma/s)$. In these formulas, s is the slope of the calibration curve, and σ is the standard deviation of the response. The relative standard deviation (RSD_r), a measure of relative variability among tests, was calculated as $(\sigma/\text{mean}) \times 100$.

For recovery experiments, the procedure described above was applied to blank urine samples of rats that were spiked in triplicate with OTA at final concentrations of 0.001, 0.01, 0.1, 1, and 10 ng/mL. Recovery experiments were performed in triplicate and repeated in three different days. Results were reported as mean values ± standard deviations ($n = 9$). To analyse urine samples by the biosensor, no pre-treatment was required except an initial 1:4 dilution with 100 mM PBS, pH 7.2. For spiked urine samples, the determination of OTA concentration was carried out by interpolation of the $OD_{420\ nm}$ values for the samples into the calibration plot constructed with OTA in the buffer. Also, parameters of OTA calibration curves in buffer and urine were compared.

For validation purposes, naturally contaminated urine samples

were analysed. These samples were collected from rats fed by naturally contaminated feed, and provided by University of Bari Aldo Moro. Samples were obtained from previous studies performed according to EU Dir. 2010/63 and Italian law on the care and use of experimental animals (D. Lgs March 4th, 2014 nr. 26), and approved by the local Ethical Committee (nr. 39161-X10, 31.05.2017). For naturally contaminated samples, the determination of OTA concentration was carried out by interpolation of the $OD_{420\ nm}$ values for the samples into the calibration plot constructed with OTA spiked urines. To validate the system as a quantitative method, naturally contaminated samples of rat urine were analysed by the RCA biosensor and an in-house validated HPLC-FLD method detailed below.

2.7. HPLC-FLD analysis of OTA in urine samples

HPLC-FLD analysis of OTA in rat urine was performed according to Akdemir et al. [48] with minor changes, which was in-house validated. Validation studies included the determination of mycotoxin linearity dynamic ranges by standard calibration curves (in mobile phase) and matrix-assisted calibration curves, the signal suppression/enhancement value (SSE), the LOD and LOQ, the accuracy and the precision. To measure OTA in the urine of rats exposed to the toxin, 6 mL of ammonium acetate buffer were added to 0.5 mL of the urine sample. Then, the suspension was incubated with 25 μL of β-glucuronidase/arylsulfatase (30/60, Merck Millipore, Vimodrone, IT) and shaken at controlled temperature of 37 °C (±0.5 °C), at a speed of 70 rpm for 16 h. After the incubation period, the sample was diluted with 6 mL of PBS and 1 mL of methanol. Prior clean-up step, the sample was clarified by centrifugation (4500 rpm for 15 min), and the whole supernatant was passed through an immunoaffinity (IMA) column previously attached to a vacuum manifold.

Ochratest™ IMA columns (VICAM Science Technology, Watertown, MA, USA) were used to clean-up the samples. Elution of samples was performed at a flow rate of about 1 drop per s. Washing with 5 mL of saline solution containing tween 20 (0.01%) and 5 mL of water was followed before eluting OTA with 2 mL of methanol. The eluate was collected in an amber silanized vial and dried at 50 °C. OTA was reconstituted adding 250 μL of methanol/water (15/85). OTA was analysed using an Agilent 1100 HPLC system (Germany) with degasser (G1379A), quatpump (G1311A), autosampler (ALS) (G1313A), column oven (G1316A), and an Agilent 1200 fluorescence detector (FLD) (G1321A). The software Chem Station (Rev A 10.2, Agilent Technologies) was used.

Chromatographic elution was achieved through a Kinetex PFP analytical column, 50 × 4.6 mm i.d., 2.6 μm particle sizes (Phenomenex, Bologna, Italy) at 35 °C. Isocratic mobile phase containing a mixture of water/methanol/acetonitrile (50/25/25) and acetic acid (1%) was eluted at 0.8 mL/min flow rate for 12 min. The injected volume was 100 μL (corresponding to 0.2 mL urine injected). The fluorescence detector was set at 333 nm (λ_{ex}) and 460 nm (λ_{em}). OTA retention time was 7.0 min. For the external matrix-assisted calibration, calibrant solutions were prepared in blank sample extracts and cleaned up using IMA columns. The standard solution was then added to the eluate (methanol) and the final solution was dried down. The residue obtained was dissolved in 250 μL of methanol/water (15/85).

Matrix-matched and standard calibration curves in the mobile phase showed good linearity ($r = 0.999$) over the concentration range tested (1–500 ng/mL, 6 mycotoxin levels). Considering that 100 μL calibrant solution or purified sample extract (equivalent to 0.2 mL urine) was injected for HPLC analysis, the equivalent toxin concentrations in urine ranged between 0.5 and 250 ng/mL urine. Linear equations for OTA standard calibration curve in the mobile phase and OTA matrix-assisted calibration curve were $y = 1371x + 3777$ and $y = 1283x - 3.3$, respectively, where y is the area of OTA peak and x is OTA concentration (μg/mL).

To assess any effect of matrix on mycotoxin quantification, the SSE% index (signal suppression/enhancement) was calculated as the ratio (%) of the slope of the matrix-matched curve to that of the standard calibration curve. Negligible suppression of the signal due to the matrix was recorded, being the value of SSE% of 94%. LOD and LOQ in urine were 0.1 and 0.5 ng/mL respectively. Recovery experiments were performed by spiking blank urines with OTA at 1.0, 10, 50 and 100 ng/mL. For each spiking level, six different replicates were analysed. A volume of 0.5 mL of each spiked sample was purified by IMA columns as described above. The amount of recovered toxin was calculated by using external matrix-assisted calibration. The results for the within-day precision, expressed as relative standard deviation (RSD_r), were satisfying being ≤ 4%. Mean recoveries ranged from 99 to 108%. This method was successfully used to quantitatively determine OTA in rat urine and to validate the RCA biosensor.

3. Results and discussion

3.1. Design strategy

In our study, we used engineered DNA to combine aptamer chemistry with an isothermal DNA amplification, and to obtain a system with high throughput and selectivity. A cheap and easy biosensor for the on-site detection of OTA was developed, which resulted in more easily affordable tool than traditional chromatographic techniques, since it does not require special costly equipment for the analysis.

The working principle of the signal amplification strategy is represented in Fig. 1. It foresaw a capture system (CS) and a detection system (DS). OTA-capture aptamer (CA) included a 3′ biotinylated terminus that allowed its link to paramagnetic streptavidin-coated beads, thus forming the CS. In the presence of OTA, CA captured the toxin. A second aptamer, the OTA-detection aptamer (DA) included a 3' primer sequence of 21 nucleotides length, which was complementary to a target sequence in a circular ssDNA (CssDNA) probe. This latter contained also a sequence that was complementary to the functional site of an auto-catalytic DNAzyme (PS2.M). The CssDNA and the annealed DA formed the DS, which selectively binded the OTA captured by the CS. The formation of the complex CS:OTA:DS allowed, in presence of the enzyme ϕ29 DNA polymerase, the RCA reaction of the CssDNA. The ϕ29 is a highly processive enzyme from bacteriophage Φ29 of *Bacillus subtilis* [49]. This enzyme catalyses the synthetic reaction of 5′-to-3′ polymerization, and the degradative reaction of 3′-to-5′ ssDNA exonucleolysis and has a strand displacement activity [50–52]. The 5′–3′ polymerization produces tandem repeats of a sequence that is complementary to the CssDNA template. On the other side, the degradative 3′–5′ ssDNA exonucleolytic activity ensures highly accurate DNA synthesis, giving to ϕ29 a high proofreading activity [53]. Furthermore, this DNA polymerase can synthesize DNA chains greater 10 kilobases, from each priming event, thus producing about an order of magnitude more DNA than PCR-based methods [54].

The result of the amplification was the formation of a DNA precipitate that was visible at naked eye (Fig. 4B). The formation of the precipitate was due to the presence of the DS bound to OTA, since the washing steps guaranteed the elimination of unbounded complexes. Therefore, no RCA reaction occurred in absence of OTA, as reported in Figs. 4C and 5A. In the presence of hemin, the PS2.M sequences formed a G-quadruplex DNAzyme, with a peroxidase-mimicking activity [55]. With the addition of H_2O_2, the DNAzyme

induced the oxidation of ABTS, giving a blue colour to the reaction mix measurable at about 420 nm. Furthermore, no white precipitate, no color reaction, and no absorbance was visible or recorded in the absence of OTA (Figs. 4C and 5A-B).

3.2. Intra-laboratory validation of the biosensor as a quantitative method

The DNAzyme biosensor was validated to determine selectivity, linearity, repeatability, accuracy, LOQ and LOD.

A positive signal was obtained either when OTA was analysed alone or included in a multi-mycotoxins mixture (Fig. 5A). The absorbance values recorded for the multi-mycotoxin standard solutions containing OTA at 10 ng/mL were similar to those recorded for the single mycotoxin standard solutions containing the same amount of OTA. No signal was recorded when OTA was excluded from the multi-mycotoxins mixture (Fig. 5A). These results showed that the biosensor was selective for the detection of OTA, and it does not cross-reacted with mycotoxins other than OTA, including its metabolite OTα.

The quantification of OTA by the biosensing complex was performed using a standard curve in PBS buffer obtained by serial dilutions of the toxin from 10^{-12} to 10 ng/mL (Fig. 5B). The equation of the standard curve in buffer was *Log[Absorbance$_{420nm}$] = 0.075 Log [OTA concentration (ng/mL)]+0.14*, with $R^2 = 0.99$. The LOB was 0.19, corresponding to 1.46×10^{-13} ng/mL of OTA. LOD and LOQ were calculated in a log-log linear regression and were 1.03×10^{-12} and 1.09×10^{-12} ng/mL, respectively. The method was selective, sensitive and fast, with the possibility to detect and determine very low levels of OTA.

The suitability of the biosensor for the determination of OTA in complex matrices as urine samples, its accuracy and the repeatability were evaluated by analysing spiked samples of rat urine. A calibration curve was obtained by spiking rat urine with different concentrations of OTA (from 0.001 to 10 ng/mL) (Fig. 5C). The standard curve, linear equation, and R^2 coefficient were determined by plotting Log [Absorbance$_{420nm}$] (*y*-axis) values against Log[OTA concentration] (*x*-axis). The equation for the spiked urine calibration curve was: *Log[Absorbance$_{420nm}$] = 0.079 Log[OTA concentration (ng/mL)]+ 0.17*. For linearity, the determination factor R^2 was >0.99. The slope of the calibration curve obtained with spiked urines was not different ($p > 0.05$) from that obtained with OTA standards in the buffer (Fig. 5B and C). Negligible enhancement of the signal due to the matrix was recorded, being the value of SSE% of 105%. Thereof, no matrix effect on the biosensor was observed and urine samples did not require further pre-cleaning treatments before OTA detection. This reduced either cost of OTA analysis in urine or detection time. Besides, a matrix-assisted calibration curve is not

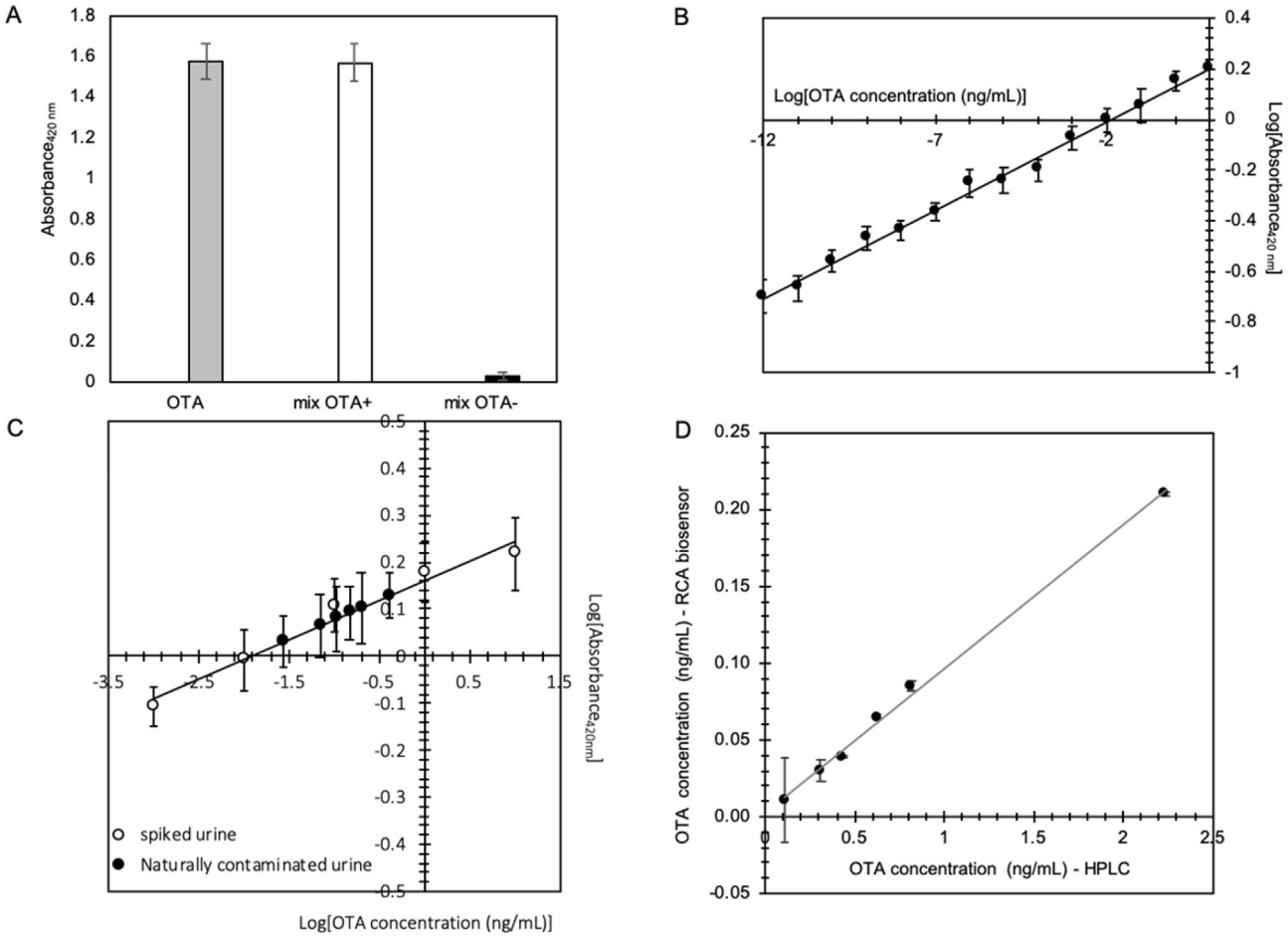


Fig. 5. OTA detection and quantification with RCA biosensor.
A. OTA detection in the presence of other mycotoxins. The absorbance values were reported for OTA alone and in the mix with 8 different mycotoxins. In the absence of OTA, no signal was detected in the mycotoxins mix. The concentration of each toxin was 10 ng/mL. B. Standard curve obtained by analysing different concentrations of OTA in PBS buffer. The absorbance values were reported for different OTA concentrations in the range 10^{-12} to 10 ng/mL. C. OTA quantification by HPLC-FLD and OTA RCA-biosensor. OTA standard curve obtained by analysing spiked urine with the DNA biosensor. D. OTA analysis in naturally contaminated urine samples by HPLC and DNA biosensor. Data are means and error bars represent standard deviations ($n = 9$).

required to measure OTA in urine samples. Indeed, the determination of OTA in urine samples can be carried out by interpolation of the absorbance measured with the DNA sensor from the calibration plot prepared with OTA standards in PBS buffer. Accuracy and repeatability data obtained in recovery experiments were satisfying, being overall average recoveries >95%, with relative standard deviations in the range 3.6–15%. The values of accuracy and precision obtained for the OTA biosensor herein developed fulfil the criteria of acceptability of an analytical method for OTA determination fixed by the European Commission [56].

Comparison of data obtained by measuring OTA in urine samples of rats fed OTA contaminated feed, by RCA biosensor and HPLC-FLD validated method was performed by *t*-Test analysis. Differences between the two methods were not significant ($p < 0.05$) (Fig. 5D).

4. Conclusions

Unlike the PCR, the RCA can be ran at a constant temperature in solution or on a solid support such as paramagnetic microbeads. The possibility to generate long DNA chains from a single molecular binding event using RCA allows the detection of single molecules of targets [57,58]. By appropriate sequence engineering in the padlock probe, the RCA allows the formation of highly processive DNA based nanomachines with auto-catalytic detection of target molecules in liquid samples, in the presence of cofactors such as hemin. Aptamer based sandwich assays have been successfully used for the detection of OTA [59] and other small molecules, such as 2,4,6-trinitrotoluene (TT)N [60]. Furthermore, the portability of this system is guaranteed by the chance to perform the RCA at a temperature range of 30–37 °C and to generate a visual signal in the presence of a positive reaction. Furthermore, the system can be made quantitative using a portable photometer.

The engineering versatility of DNA makes this polymer a powerful and programmable element for the construction of nano- and micro-scale systems that are useful for a broad range of applications in biosensing [61]. On the other hand, some features of the RCA system for practical applications can still be improved. The main practical challenges are the need of large quantities of high-purified circular templates, and the few companies that provide this synthesis service. Additionally, long RCA products aggregate in solution, therefore it is not advisable to store the amplification products for subsequent analysis and colour reaction must be promptly performed. However, these drawbacks will reasonably have a future solution with the technological advance.

The biosensor herein developed can be considered selective, sensitive and accurate for the detection and quantification of OTA in complex matrices as urine. Further ongoing studies are focused on the improvement of the preparation steps, as well as on the validation of the OTA biosensor on other matrices, including biological samples other than urine, and foodstuffs. Furthermore, the system proposed in this research can be putatively translated for the detection of other target molecules, by simply modifying the sequence of the detection aptamer. For example the system can be applied to the rapid and early detection of plant or human pathogens, which is of paramount importance to apply effective control means [61–63].

Funding

This research was funded by CNR-ISPA internal funding and by research agreement between CNR-ISPA and New Feed Team (NFT) Srl (CNR Prot. Nr.184 on 29/01/2018).

CRediT authorship contribution statement

Elisa Santovito: Conceptualization, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Donato Greco:** Investigation, Data curation. **Vito D'Ascanio:** Formal analysis, Validation. **Simona Marianna Sanzani:** Methodology, Investigation, Writing - review & editing. **Giuseppina Avantaggiato:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge Prof. Domenico Tricarico from University of Bari, the MycoKey project (EU Horizon2020 Grant Agreement Nr.678781) for providing urine samples for the validation study, and Prof. Antonio Ippolito from University of Bari for reviewing the manuscript.

References

[1] J.D. Miller, H.L. Trenholm, Mycotoxins in Grain: Compounds Other than Aflatoxin, Eagan Press, 1994.
[2] A.H. Heussner, L.E.H. Bingle, Comparative ochratoxin toxicity: a review of the available data, Toxins 7 (2015) 4253–4282, https://doi.org/10.3390/toxins7104253.
[3] J. Wolff, H. Bresch, C. Cholmakov-Bodechtel, G. Engel, M. Garais, P. Majerus, H. Rosner, R. Scheuer, Ochratoxin A: contamination of foods and consumer exposure, Arch Leb. Hyg. 51 (2000) 2.
[4] J. Alexander, H. Autrup, D. Bard, D. Benford, A. Carere, L.G. Costa, J. Cravedi, A. di Domenico, R. Fanelli, J. Fink-Gremmels, Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to ochratoxin A in food, EFSA J 365 (2006) 1–56.
[5] A. Vidal, M. Mengelers, S. Yang, S. De Saeger, M. De Boevre, Mycotoxin biomarkers of exposure: a comprehensive review, Compr. Rev. Food Sci. Food Saf. 17 (2018) 1127–1155.
[6] J.B. Soto, M. Ruiz, L. Manyes, A. Juan-García, Blood, breast milk and urine: potential biomarkers of exposure and estimated daily intake of ochratoxin A: a review, Food Addit. Contam. 33 (2016) 313–328.
[7] S.M. Sanzani, M. Reverberi, R. Geisen, Mycotoxins in harvested fruits and vegetables: insights in producing fungi, biological role, conducive conditions, and tools to manage postharvest contamination, Postharvest Biol. Technol. 122 (2016) 95–105.
[8] FAO/WHO, Ochratoxin A (Addendum), WHO Food Addit. Ser. 59, 68th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), WHO, Geneva, Switzerland, 2008, pp. 281–387.
[9] FAO/WHO, A. Ochratoxin, WHO Food Addit. Ser. 47, 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JEFCA), WHO, Geneva, Switzerland, 2001, pp. 281–387. Appendix A 388–415.
[10] J. Gilbert, P. Brereton, S. MacDonald, Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples, Food Addit. Contam. 18 (2001) 1088–1093.
[11] Beatriz Arce-López, Elena Lizarraga, Ariane Vettorazzi, Elena González-Peñas, Human biomonitoring of mycotoxins in blood, plasma and serum in recent years: a review, Toxins 12 (2020) 147, https://doi.org/10.3390/toxins12030147, 2020.
[12] K. Muñoz, B. Cramer, J. Dopstadt, H.-U. Humpf, G.H. Degen, Evidence of ochratoxin A conjugates in urine samples from infants and adults, Mycotoxin Res. 33 (2017) 39–47, https://doi.org/10.1007/s12550-016-0261-y.
[13] M. Puiu, C. Bala, N. Jaffrezic-Renault, Progress in biosensor for mycotoxins assay, Chem. Senses 1 (2014) 4–11.
[14] B. Prieto-Simón, M. Campàs, J.-L. Marty, T. Noguer, Novel highly-performing immunosensor-based strategy for ochratoxin A detection in wine samples, Biosens. Bioelectron. 23 (2008) 995–1002.
[15] J.C. Vidal, P. Duato, L. Bonel, J.R. Castillo, Use of polyclonal antibodies to ochratoxin A with a quartz–crystal microbalance for developing real-time mycotoxin piezoelectric immunosensors, Anal. Bioanal. Chem. 394 (2009) 575–582.
[16] J. Yang, P. Gao, Y. Liu, R. Li, H. Ma, B. Du, Q. Wei, Label-free photoelectrochemical immunosensor for sensitive detection of Ochratoxin A, Biosens. Bioelectron. 64 (2015) 13–18.
[17] Z. Zhu, M. Feng, L. Zuo, Z. Zhu, F. Wang, L. Chen, J. Li, G. Shan, S.-Z. Luo, An

aptamer based surface plasmon resonance biosensor for the detection of ochratoxin A in wine and peanut oil, Biosens. Bioelectron. 65 (2015) 320–326.
[18] S.M. Sanzani, M. Reverberi, C. Fanelli, A. Ippolito, Detection of ochratoxin a using molecular beacons and real-time PCR thermal cycler, Toxins 7 (2015) 812–820, https://doi.org/10.3390/toxins7030812.
[19] Z. Zhuo, Y. Yu, M. Wang, J. Li, Z. Zhang, J. Liu, X. Wu, A. Lu, G. Zhang, B. Zhang, Recent advances in SELEX technology and aptamer applications in biomedicine, Int. J. Mol. Sci. 18 (2017) 2142, https://doi.org/10.3390/ijms18102142.
[20] M.N. Stojanovic, P. de Prada, D.W. Landry, Fluorescent sensors based on aptamer self-assembly, J. Am. Chem. Soc. 122 (2000) 11547–11548.
[21] C.T. Lauhon, J.W. Szostak, RNA aptamers that bind flavin and nicotinamide redox cofactors, J. Am. Chem. Soc. 117 (1995) 1246–1257.
[22] D.E. Huizenga, J.W. Szostak, A DNA aptamer that binds adenosine and ATP, Biochemistry 34 (1995) 656–665.
[23] Y. Xi-Hui, K. Wei-Jun, Y. Mei-Hua, Z. Ming, Z. Ouyang, Application of aptamer identification technology in rapid analysis of mycotoxins, Chin. J. Anal. Chem. 41 (2013) 297–306.
[24] N.G. Walter, D.R. Engelke, Ribozymes: catalytic RNAs that cut things, make things, and do odd and useful jobs, Biologist 49 (2002) 199–203. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3770912/.
[25] R.R. Breaker, DNA enzymes, Nat. Biotechnol. 15 (1997) 427–431.
[26] G.M. Emilsson, R.R. Breaker, Deoxyribozymes: new activities and new applications, Cell. Mol. Life Sci. C. 59 (2002) 596–607.
[27] J. Kosman, B. Juskowiak, Optimization study of the catalytic activity of DNA-zymes based on telomeric G-quadruplexes, Open Chem 10 (2012) 368–372.
[28] V. Pavlov, Y. Xiao, R. Gill, A. Dishon, M. Kotler, I. Willner, Amplified chemiluminescence surface detection of DNA and telomerase activity using catalytic nucleic acid labels, Anal. Chem. 76 (2004) 2152–2156.
[29] Y. Xiao, V. Pavlov, R. Gill, T. Bourenko, I. Willner, Lighting up biochemiluminescence by the surface self-assembly of DNA–hemin complexes, Chembiochem 5 (2004) 374–379.
[30] M.K. Beissenhirtz, I. Willner, DNA-based machines, Org. Biomol. Chem. 4 (2006) 3392–3401.
[31] I. Willner, B. Shlyahovsky, M. Zayats, B. Willner, DNAzymes for sensing, nanobiotechnology and logic gate applications, Chem. Soc. Rev. 37 (2008) 1153–1165.
[32] J. Liu, Y. Lu, Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor, Anal. Chem. 76 (2004) 1627–1632, https://doi.org/10.1021/ac0351769.
[33] F.B. Dean, S. Hosono, L. Fang, X. Wu, A.F. Faruqi, P. Bray-Ward, Z. Sun, Q. Zong, Y. Du, J. Du, M. Driscoll, W. Song, S.F. Kingsmore, M. Egholm, R.S. Lasken, Comprehensive human genome amplification using multiple displacement amplification, Proc. Natl. Acad. Sci. U. S. A. 99 (2002), https://doi.org/10.1073/pnas.082089499.
[34] P.M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D.C. Thomas, D.C. Ward, Mutation detection and single-molecule counting using isothermal rolling-circle amplification, Nat. Genet. 19 (1998), https://doi.org/10.1038/898.
[35] G.T. Walker, M.S. Fraiser, J.L. Schram, M.C. Little, J.G. Nadeau, D.P. Malinowski, Strand displacement amplification—an isothermal, in vitro DNA amplification technique, Nucleic Acids Res. 20 (1992) 1691–1696.
[36] J.A. Esteban, M. Salas, L. Blanco, Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization, J. Biol. Chem. 268 (1993) 2719–2726.
[37] L. Blanco, M. Salas, Characterization of a 3′→ 5′ exonuclease activity in the phage ϕ29-encoded DNA polymerase, Nucleic Acids Res. 13 (1985) 1239–1249.
[38] F. Dean, J. Nelson, T. Giesler, R. Lasken, Rapid amplification of plasmid and phage DNA using Phi29 polymerase and a multiply-pimed rolling circle amplification, Genome Res. 11 (2001), https://doi.org/10.1101/gr.180501.
[39] A. Lagunavicius, E. Merkiene, Z. Kiveryte, A. Savaneviciute, V. Zimbaite-Ruskuliene, T. Radzvilavicius, A. Janulaitis, Novel application of Phi29 DNA polymerase: RNA detection and analysis in vitro and in situ by target RNA-primed RCA, RNA 15 (2009) 765–771, https://doi.org/10.1261/rna.1279909.
[40] C. Larsson, J. Koch, A. Nygren, G. Janssen, A.K. Raap, U. Landegren, M. Nilsson, In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes, Nat. Methods 1 (2004) 227–232.
[41] J.A. Cruz-Aguado, G. Penner, Fluorescence polarization based displacement assay for the determination of small molecules with aptamers, Anal. Chem. 80 (2008) 8853–8855, https://doi.org/10.1021/ac8017058.
[42] G. Avantaggiato, D. Greco, A. Damascelli, M. Solfrizzo, A. Visconti, Assessment of multi-mycotoxin adsorption efficacy of grape pomace, J. Agric. Food Chem. 62 (2014) 497–507, https://doi.org/10.1021/jf404179h.
[43] J. Chen, Z. Fang, J. Liu, L. Zeng, A simple and rapid biosensor for ochratoxin A based on a structure-switching signaling aptamer, Food Contr. 25 (2012) 555–560.
[44] A. Gomez, N.S. Miller, I. Smolina, Visual detection of bacterial pathogens via PNA-based padlock probe assembly and isothermal amplification of DNA-zymes, Anal. Chem. 86 (2014) 11992–11998, https://doi.org/10.1021/ac5018748.
[45] P. Travascio, Y. Li, D. Sen, DNA-enhanced peroxidase activity of a DNA-aptamer-hemin complex, Chem. Biol. 5 (1998) 505–517.
[46] D.A. Armbruster, T. Pry, Limit of blank, limit of detection and limit of quantitation, Clin. Biochem. Rev. 29 (2008) S49–S52. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2556583/.
[47] A. Shrivastava, V.B. Gupta, Methods for the determination of limit of detection and limit of quantitation of the analytical methods, Chronicles Young Sci. 2 (2011) 21.
[48] C. Akdemir, O.C. Ulker, A. Basaran, S. Ozkaya, A. Karakaya, Estimation of ochratoxin A in some Turkish populations: an analysis in urine as a simple, sensitive and reliable biomarker, Food Chem. Toxicol. 48 (2010) 877–882.
[49] Č. Vlček, V. Pačes, Nucleotide sequence of the late region of Bacillus phage ϕ29 completes the 19285-bp sequence of ϕ29 genome. Comparison with the homologous sequence of phage PZA, Gene 46 (1986) 215–225, https://doi.org/10.1016/0378-1119(86)90406-3.
[50] L. Blanco, A. Bernad, J.M. Lazaro, G. Martin, C. Garmendia, M. Salas, Highly efficient DNA synthesis by the phage Φ29 DNA polymerase. Symmetrical mode of DNA replication, J. Biol. Chem. 264 (1989) 8935–8940. https://www.scopus.com/inward/record.uri?eid=2-s2.0-0024335104&partnerID=40&md5=deaeece2485d976c0c25802cae1e26f2.
[51] L. Blanco, M. Salas, Relating structure to function in ϕ29 DNA polymerase, J. Biol. Chem. 271 (1996) 8509–8512, https://doi.org/10.1074/jbc.271.15.8509.
[52] S. Kamtekar, A.J. Berman, J. Wang, J.M. Lázaro, M. De Vega, L. Blanco, M. Salas, T.A. Steitz, Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage ϕ29, Mol. Cell. 16 (2004) 609–618, https://doi.org/10.1016/j.molcel.2004.10.019.
[53] T.J. Pugh, A.D. Delaney, N. Farnoud, S. Flibotte, M. Griffith, H.I. Li, H. Qian, P. Farinha, R.D. Gascoyne, M.A. Marra, Impact of whole genome amplification on analysis of copy number variants, Nucleic Acids Res. 36 (2008), https://doi.org/10.1093/nar/gkn378 e80–e80.
[54] R. Pinard, A. de Winter, G.J. Sarkis, M.B. Gerstein, K.R. Tartaro, R.N. Plant, M. Egholm, J.M. Rothberg, J.H. Leamon, Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing, BMC Genom. 7 (2006) 216, https://doi.org/10.1186/1471-2164-7-216.
[55] W. Li, Y. Li, Z. Liu, B. Lin, H. Yi, F. Xu, Z. Nie, S. Yao, Insight into G-quadruplex-hemin DNAzyme/RNAzyme: adjacent adenine as the intramolecular species for remarkable enhancement of enzymatic activity, Nucleic Acids Res. 44 (2016) 7373–7384, https://doi.org/10.1093/nar/gkw634.
[56] European Commission, Commission Regulation (EC), No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, Off. J. Eur. Union. L70 (2006) 12–34.
[57] T. Konry, I. Smolina, J.M. Yarmush, D. Irimia, M.L. Yarmush, Ultrasensitive detection of low-abundance surface-marker protein using isothermal rolling circle amplification in a microfluidic nanoliter platform, Small 7 (2011) 395–400.
[58] X.J. Zhou, G. Gibson, Cross-species comparison of genome-wide expression patterns, Genome Biol. 5 (2004) 232.
[59] F. Costantini, C. Sberna, G. Petrucci, M. Reverberi, F. Domenici, C. Fanelli, C. Manetti, G. de Cesare, M. DeRosa, A. Nascetti, D. Caputo, Aptamer-based sandwich assay for on chip detection of Ochratoxin A by an array of amorphous silicon photosensors, Sensor. Actuator. B Chem. 230 (2016) 31–39, https://doi.org/10.1016/j.snb.2016.02.036.
[60] M.Y. Ho, N. D'Souza, P. Migliorato, Electrochemical aptamer-based sandwich assays for the detection of explosives, Anal. Chem. 84 (2012) 4245–4247, https://doi.org/10.1021/ac300606n.
[61] M.M. Ali, F. Li, Z. Zhang, K. Zhang, D.-K. Kang, J.A. Ankrum, X.C. Le, W. Zhao, Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine, Chem. Soc. Rev. 43 (2014) 3324–3341.
[62] F. Fallanaj, S.M. Sanzani, K. Youssef, C. Zavanella, M.G. Salerno, A. Ippolito, A new perspective in controlling postharvest citrus rots: the use of electrolyzed water, Acta Hortic. 1065 (2015) 1599–1606.
[63] K. Youssef, A.F. Hashim, R. Margarita, M.A. Alghuthaymi, K.A. Abd-Elsalam, Fungicidal efficacy of chemically-produced copper nanoparticles against *Penicillium digitatum* and *Fusarium solani* on citrus fruit, Philippine Agric. Sci. 100 (2017) 69–78.