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Rapid and specific detection of thiabendazole: Enzymatic digestion-enabled fluorescent aptasensor

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Abstract

Thiabendazole, a widely used broad-spectrum fungicide in agriculture, poses risks to human health. To monitor its presence in water, we propose a fluorescent aptasensor utilizing *E. coli* exonuclease I (Exo I). The findings demonstrate a linear correlation between thiabendazole concentrations and digestion percentage, with a detection limit (LOD) exceeding 1 μM and a determination coefficient (R^2) of 0.959. This aptamer-based fluorescence spectroscopy detection system holds promise for a rapid, specific, and sensitive analysis of thiabendazole in environmental waters and food matrices.

Introduction

Pesticides play a crucial role in agriculture by enhancing crop yields and ensuring adequate food production levels. Since their introduction, they have yielded significant economic advantages and are thus extensively utilized¹. Nevertheless, their extensive utilization has resulted in various toxicities and risks to both human well-being²⁻⁵ and to the environment^{6,7}. Thiabendazole is a widely used fungicide to prevent fruits, such as citrus, apples, and pears, from being affected by mold, rot, and blight, thus keeping them fresh before the waxing stage for storage⁸. Even though thiabendazole is of low toxicity in comparison to other pesticides, it has been associated with a range of harmful effects, including nephrotoxicity, hepatotoxicity, carcinogenicity, and teratogenicity⁹. As a result, the U.S. Environmental Protection Agency (EPA) has classified thiabendazole as potentially carcinogenic, particularly at doses that disrupt the balance of thyroid hormones¹⁰. The use of thiabendazole is also regulated by the European directive (98/93/EC) in drinking water¹¹, and its concentration should not exceed 0.1 $\mu\text{g L}^{-1}$, which corresponds to 0.5 nM. Consequently, there is a need to develop rapid and sensitive methods for detecting thiabendazole in environmental water to safeguard consumer's health.

Generally, identification and quantification of pesticides are based on chromatographic methods, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) coupled with mass spectrometry (MS)¹²⁻¹⁴. Thiabendazole detection in water bodies has mainly been performed using liquid chromatography coupled with different detectors (**Table 1**). Its concentration in environmental waters can vary significantly according to several factors, such as proximity to agricultural areas, as well as geographic locations, which depends on the regulation of this pesticide in the concerned regions. For example, thiabendazole's concentration varied between 20 nM – 8.5 μM in fruit-processing industry, wastewater treatment plants, and in sewage water belonging to the Castelló area (Spain)¹⁵.

Table 1 Chromatographic techniques used for the analysis of thiabendazole and their application to different matrices of water bodies.

Technique	Range (nM)	LOD (nM)	Validity for water bodies
HPLC ¹⁶	0.05 – 79	0.02	River and underground water
LC-MS/MS ¹⁷	0.05 – 5	Not defined (ND)	Treated waste water
LC-Fluorescence spectroscopy ¹⁸	25 – 4 × 10 ³	2.5 – 50	Lake, rain and well waters
LC-Fluorescence spectroscopy ¹⁹	50 – 5 × 10 ³	14.4 – 37	ND
HPLC ²⁰	5 – 500	0.4 – 0.64	Natural water, sea water and purified waste water
HPLC ²¹	2.5 – 1.5 × 10 ³	0.2	Seawater, groundwater and waste water

These techniques are highly sensitive and specific. However, they do not allow real-time analysis as they require lengthy sample preparation steps, in addition to highly skilled technical labor, which makes the analysis expensive.

To meet the requirements of selectivity and sensitivity for thiabendazole detection, there have been increasing efforts to explore alternative measures. Biosensors are promising tools that could potentially replace conventional methods, as they enable the real-time detection of analytes, while minimizing the need for tedious sample pretreatments.

Biosensors, which combine a biological sensing element and a transducer, are a promising alternative to traditional laboratory-based methods. Biological sensing elements such as antibodies, enzymes, aptamers, or cells ensure specificity and selectivity²², whereas the transducer provides sensitivity by converting the biointeraction into a measurable signal for analyte detection. Optical and electrochemical-based biosensors are particularly attractive for pesticide detection due to their potential selectivity and sensitivity²³⁻²⁷. These biosensors employ various methods including UV-Vis absorption spectroscopy^{28,29}, fluorescence spectroscopy³⁰⁻³², photoluminescence assay^{33,34}, chemiluminescence assay^{35,36}, dynamic light scattering³⁷, surface-enhanced Raman scattering (SERS)^{38,39}, potentiometric sensing⁴⁰, impedance sensing^{41,42}, and amperometry^{43,26}. However, only a few biosensors developed for pesticide detection have been applied to thiabendazole⁴⁴⁻⁴⁷ (**Table 2**), and none of them are currently suitable for in situ detection.

Table 2 Immunosensors developed for the detection of thiabendazole.

Transduction	LOD	Validity of detection in real matrices
White Light Reflectance Spectroscopy (WLRs) ⁴⁴	4 nM	Wine: 4.7 nM
Surface Plasmon Resonance (SPR) ⁴⁵	250 nM	PBS: ND
SPR ⁴⁶	0.64 nM	Orange: 1.43 nM
Strip-based assay (colorimetry) ⁴⁷	0.4 nM	Fruit juices: 0.25 nM

We present here a novel approach for the specific detection of thiabendazole using a fluorometric aptasensor based on Exo I enzymatic digestion. The biosensor is composed of an aptamer sensing element and a fluorescence detection platform, offering high specificity and sensitivity. Aptamers, which are oligonucleotide switching structures, form a complex with the target with high affinity and specificity^{48,49}. In our study, we tested the interaction between thiabendazole and its aptamer BOL009 developed by Novaptech, using Exo I digestion method. The activity of Exo I is commonly used to demonstrate the specific interaction between a ligand and an oligonucleotide switching structure^{50–54}. The Exo I digest BOL009 into mononucleotides that do not fluoresce upon interaction with the fluorescent probe, Sybr Gold. However, in the presence of thiabendazole, a BOL009/thiabendazole complex is formed which hinders the enzymatic activity, resulting in a high fluorescence signal upon the interaction of not digested BOL009 with Sybr Gold. This method has allowed for the specific detection of thiabendazole in the range of 1 – 100 μM with an R^2 value of 0.959. These results not only demonstrate the potential of our biosensor for the accurate and rapid detection of thiabendazole in environmental waters, but also contribute to the immediate advancement of scientific research within the community, given the potential of this method to detect and analyze all types of targets for which the specific aptamers have been selected and optimized.

Material and methods

All chemicals and reagents used in this study were of analytical grade (> 99%) and used without further purification. Tris(hydroxymethyl)aminomethane (Tris-HCl), sodium chloride (NaCl), sodium acetate ($\text{CH}_3\text{CO}_2\text{Na}$), potassium chloride (KCl), potassium acetate ($\text{CH}_3\text{CO}_2\text{K}$), magnesium acetate tetrahydrate ($\text{CH}_3\text{CO}_2\text{Mg}_4\text{H}_2\text{O}$), hydrochloric acid (HCl), sodium hydroxide (NaOH), magnesium chloride hexahydrate ($\text{MgCl}_2\cdot 6\text{H}_2\text{O}$), ethylenediaminetetraacetic acid (EDTA), and sodium dodecyl sulfate 4X (SDS) were purchased from Euromedex (Strasbourg, France). Ethanol (EtOH) and methanol (MeOH) were purchased from VWR Chemicals (Radnor, Pennsylvania, USA). Thiabendazole, glyphosate, aminomethylphosphonic acid (AMPA), simazine, carbendazim, isoproturon, atrazine-desethyl and atrazine were purchased from Sigma Aldrich (St. Louis, MO, USA). SYBR Gold was purchased from Invitrogen (Thermo Fischer Scientific, Waltham, Massachusetts, USA). 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) was purchased from Pan Reac Application (ITW Reagents, Castellar del Vallès, Spain). E. coli Exo I (20 000 U mL^{-1}) was purchased from New England Biolabs (Ipswich, Massachusetts, USA).

All oligonucleotides used in this work were synthesized by Eurogentec (Seraing, Belgium) with polyacrylamide gel electrophoresis (PAGE) or HPLC purification grade. The oligonucleotides were dissolved in MilliQ water and the concentrations were measured by UV-Vis spectroscopy using the Nanodrop 2000 spectrometer (Mettler Toledo, Columbus, Ohio, USA).

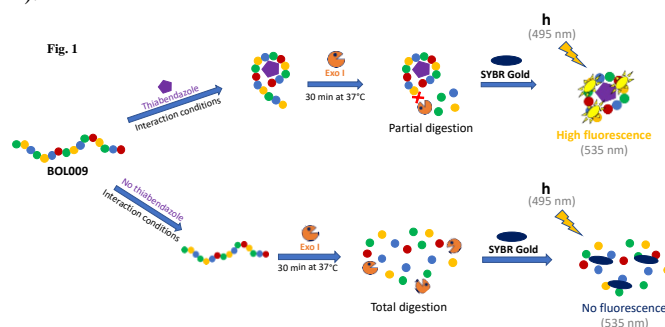
Enzymatic digestion was performed at 37 °C with gentle stirring at 240 rounds per minute (rpm) using a thermomixer (Eppendorf, Hamburg, Germany). All experiments of interaction between

BOL009 and thiabendazole (or other pesticides) were performed in the HEPES buffer according to the appropriate interaction conditions (Table 3). All experiments were performed in duplicate or triplicate. The standard deviations of fluorescence values, obtained in triplicate and converted into digestion percentages according to Equation 1, were calculated and shown on graphs as error bars. The pH of all buffers was measured with a Seveneasy pH-meter (Mettler Toledo, Columbus, Ohio, USA) and was adjusted using either a 1 M NaOH solution or a 0.1 M HCl solution.

Table 3 Interaction conditions of thiabendazole with BOL009.

HEPES buffer composition	Interaction conditions
20 mM HEPES, 20 mM $\text{CH}_3\text{CO}_2\text{Na}$, 140 mM $\text{CH}_3\text{CO}_2\text{K}$, 3 mM $\text{CH}_3\text{CO}_2\text{Mg}_4\text{H}_2\text{O}$ and 17% MeOH	BOL009 is incubated with thiabendazole in HEPES buffer for 30 min at room temperature

Enzymatic digestion prior to fluorescence spectroscopy is widely used to analyze the interaction between an aptamer and its target^{50–54}. The described interaction leads to a delayed digestion of the oligonucleotide as a result of the formation of an aptamer/target complex, which hinders the enzymatic digestion^{50–54}. Building upon this principle, we have developed a fluorescent aptasensor for detecting thiabendazole using its specific aptamer, BOL009 (Figure 1).



Schematic illustration of the fluorescence aptasensor developed for the detection of thiabendazole.

The assessment of the interaction between BOL009 and thiabendazole was performed using the following experimental procedure mainly developed in our previous work³¹: initially, 10 μM of BOL009 solution was heated at 95 °C for 3 minutes and subsequently cooled on ice for 3 minutes. Next, 10 μL of 10 μM BOL009 were withdrawn and mixed with 70 μL of the HEPES buffer without MeOH. The mixture was then incubated at room temperature for 30 minutes in order to obtain the functional conformation of BOL009 aptamer. Afterward, 17 μL of thiabendazole solubilized in MeOH at different concentrations were added to the reaction medium, followed by another 30-minutes incubation at room temperature. As a control, a sample without thiabendazole was prepared as-well, using HEPES buffer only. Upon completion of the incubation period, 3 μL of Exo I at a concentration of 20 U μL^{-1} (which corresponds to a final concentration of 0.6 U μL^{-1} , as optimized) were added to the reaction medium and the digestion was performed as described before. Subsequently, 5 μL of the samples were withdrawn and loaded into the wells of a black 384-well microplate (Corning black, Thermo Fisher Scientific) containing 25 μL of a stop solution (1.2X SYBR Gold, 12 mM Tris-HCl, pH 7.4, 48% formamide v/v, 3.75 mM EDTA). Fluorescence measurements were performed at 535 nm after excitation at 495 nm using a Tecan Infinite M1000 Pro instrument (Männedorf, Switzerland). The obtained fluorescence intensity was converted into digestion yield using the following equation:

$$\% \text{ of digestion} = \frac{(F_0 - F)}{F_0} \times 100 \quad (\text{Equation 1})$$

where F_0 represents the fluorescence signal without digestion, and F represents the fluorescence signal after digestion.

Results and discussion

In order to analyze the interaction between BOL009 and thiabendazole, the digestion test of BOL009 was performed in the absence and presence of thiabendazole in excess (1 mM). Initially, the optimization of Exo I concentration was conducted to achieve the maximum yield of BOL009 digestion (**Figure S1**). Notably, the optimal percentage of digestion was achieved at a concentration of $0.6 \text{ U } \mu\text{L}^{-1}$. Consequently, this concentration value was chosen to perform the interaction test. **Figure 2a** shows the percentage of digestion obtained upon enzymatic digestion of BOL009 by Exo I in the presence and absence of thiabendazole according to the conditions described above.

The experiment revealed significant findings regarding the impact of thiabendazole on the activity of Exo I digestion. In the absence of thiabendazole, Exo I achieved a digestion rate of 92% for the BOL009 aptamer. However, in the presence of thiabendazole, the digestion rate decreased to 56%. These results provide strong evidence that the presence of thiabendazole significantly affects the activity of Exo I digestion. The reduction in enzymatic activity is directly attributed to the formation of the BOL009/thiabendazole complex. Therefore, these findings confirm the existence of an interaction between BOL009 and thiabendazole, highlighting its critical role in modulating the activity of Exo I digestion.

It is well-established that pesticides can inhibit the activity of some enzymes, including acetylcholinesterase (AChE)⁵⁵, butyrylcholinesterase (BChE)⁵⁶, tyrosinase^{57,58}, alkaline phosphatase^{59,60}, peroxidase⁶¹, acid phosphatase⁶², urease⁶³ and most recently Exonuclease I in particular conditions³¹. Therefore, it is crucial to verify that thiabendazole does not inhibit the activity of Exo I. To accomplish this, a validation test was conducted under the same experimental conditions as the BOL009/thiabendazole interaction test, employing the glyphosate aptamer⁶⁴ (GLY3) instead of BOL009 (**Table 4**).

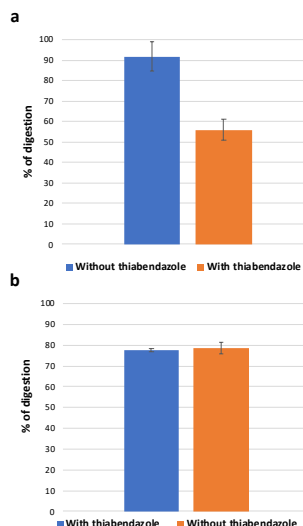
Table 4 The glyphosate aptamer sequence used to study the inhibition of Exo I activity by thiabendazole.

Aptamer name	Sequence
GLY3 ⁶⁴	5'-TGC-TAG-ACG-ATA-TTC-GTC-CAT-CCG-AGC-CCG-TGG-CGG-GCT-TTA-GGA-CTC-TGC-GGG-CIT-CGCGGC-GCT-GTC-AGA-CTG-AAT-ATG-TCA-3'

Since GLY3 is not expected to interact with thiabendazole, we performed GLY3 digestion by Exo I in the presence and absence of thiabendazole (**Figure 2b**). Similarly to the optimization process conducted for BOL009, an optimization of Exo I concentration was carried out for the GLY3 before analyzing its interaction with thiabendazole (**Figure S2**). The optimal concentration was determined to be $0.6 \text{ U } \mu\text{L}^{-1}$ and it is therefore this concentration value that is selected for the interaction assessment.

As observed in **Figure 2b**, Exo I performs over 80% of digestion both in presence and absence of thiabendazole which proves that Exo I is not inhibited by thiabendazole.

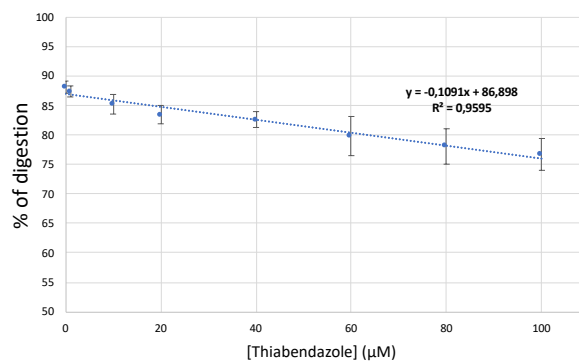
Fig. 2



Digestion of (a) BOL009 and (b) GLY3 at $1 \mu\text{M}$ by Exo I ($0.6 \text{ U } \mu\text{L}^{-1}$) for 30 min at $37 \text{ }^\circ\text{C}$ (240 rpm) in HEPES buffer in the presence and absence of 1 mM thiabendazole.

Following the confirmation of the interaction between BOL009 and thiabendazole, our proposed fluorescence aptasensor successfully detected thiabendazole. The reduction in enzymatic activity was found to be directly proportional to the number of complexes formed between BOL009 and thiabendazole. Thus, an increase in the concentration of complexes was expected to result in a greater reduction of Exo I activity. Leveraging these findings, we conducted an experiment wherein BOL009 was subjected to digestion at various concentrations of thiabendazole (0, 1, 10, 20, 40, 60, 80, and $100 \mu\text{M}$), under the same previously described conditions (**Figure 3**).

Fig. 3



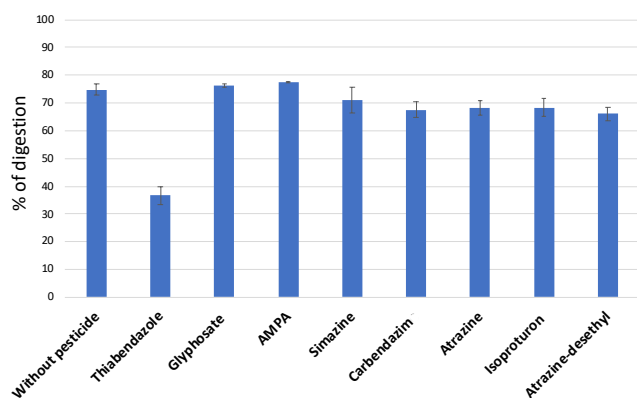
Digestion of BOL009 at $1 \mu\text{M}$ by Exo I ($0.6 \text{ U } \mu\text{L}^{-1}$) at $37 \text{ }^\circ\text{C}$ for 30 min (240 rpm) in HEPES buffer in the presence of different concentrations of thiabendazole ($1 - 100 \mu\text{M}$).

The results shown in **Figure 3** demonstrate that the concentration of thiabendazole has a direct impact on the activity of Exo I, resulting in a decrease of digestion efficiency. Our findings show that the reduction of enzymatic activity occurs within a linear range of 0 to $100 \mu\text{M}$ with a determination coefficient of 0.959 and a limit of detection (LOD) over $1 \mu\text{M}$. We observed however notable variations in the error, more particularly at high concentrations of thiabendazole. This can be attributed to the high number of BOL009/thiabendazole complexes formed, leading to the inhibition of the enzymatic activity and resulting in oligonucleotides of different size at the end of digestion. These interact differently with the fluorescence probe, Sybr Gold, which results in variations in the fluorescence signal.

To assess the specificity of the interaction between BOL009 aptamer and thiabendazole, we investigated the binding affinity with other

pesticide molecules that have similar weight and structure, specifically carbendazim analogs containing a benzimidazole group. The following pesticide molecules were tested: thiabendazole, glyphosate, AMPA, simazine, carbendazim, atrazine, isoproturon and atrazine-desethyl (**Figure 4**).

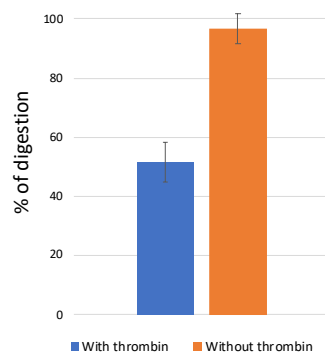
Fig. 4



Digestion of BOL009 at 1 μM by Exo I ($0.6 \text{ U } \mu\text{L}^{-1}$) at 37 $^{\circ}\text{C}$ for 30 min (240 rpm) in HEPES buffer in the absence and presence of different pesticide molecules at 1 mM.

As shown in **Figure 4**, only thiabendazole induces a strong reduction in Exo I activity, indicating that its interaction is specific to BOL009. In order to verify whether the developed enzymatic digestion method is applicable for the detection of other targets having their aptamers, the detection of thrombin molecule was tested. It should be recalled that the thrombin aptamer is a short 20-mer sequence, whereas thrombin is a large molecule (33.7 kDa). **Figure 5** shows the result of the interaction test performed in the following thrombin interaction buffer: 20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 1 mM MgCl_2 and 10 mM KCl.

Fig. 5



Digestion of thrombin aptamer (1 μM) by Exo I ($0.6 \text{ U } \mu\text{L}^{-1}$) for 30 min at 37 $^{\circ}\text{C}$ in the appropriate interaction buffer in the absence and presence of 0.1 mM thrombin.

In the presence of thrombin, enzymatic digestion is reduced (52% of digestion) compared to the same condition without thrombin in which the enzymatic digestion is optimal (97% of digestion).

This result shows clearly that the enzymatic digestion method is applicable to any target for which a specific aptamer has been selected and optimized.

Conclusion

In conclusion, the findings of this study demonstrate the successful interaction of BOL009 with thiabendazole, resulting in a specific reduction of Exo I activity, as determined by fluorescence spectroscopy. This decrease in enzymatic activity is attributed to the

formation of the BOL009/thiabendazole complex, indicating the high specificity of our method in detecting thiabendazole. The developed method offers various advantages as it enables simple, rapid, and specific detection of thiabendazole, with potential applications for the detection of other targets for which specific aptamers have been developed as demonstrated for thrombin, a large molecule recognized by a 20-mer short sequence aptamer. Nevertheless, this method provides an LOD that exceeds current EU recommendations. To address this limitation, a preconcentration step, using for example a dedicated Solid Phase Extraction (SPE) column, would enable to both concentrate and extract thiabendazole from environmental matrices. Besides, additional studies are ongoing to assess the analytical properties of the method, including robustness, batch-to-batch and day-to-day variability tests, its validation for water bodies, and exploration of miniaturization techniques to enable on-site and real-time analysis of thiabendazole in environmental waters.

Conflicts of interest

There are no conflicts to declare.

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Declarations

Dr. Mohamed Amine Berkal conducted the experimental work reported in this manuscript in the frame of his PhD thesis. Prof. Jean-Jacques Toulmé is the chief scientific officer and cofounder of the Novaptech company, who develops and optimize the aptamers used in the study. Prof. Corinne Nardin was the supervisor of the PhD thesis of Dr. Mohamed Amine Berkal. All coauthors contributed to the preparation of the manuscript.

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