Glyphosate–Exonuclease Interactions: Reduced Enzymatic Activity as a Route to Glyphosate Biosensing

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ABSTRACT: N-phosphonomethyle-glycine (glyphosate) is the most widely used pesticide worldwide due to its effectiveness in killing weeds at a moderate cost, bringing significant economic benefits. However, owing to its massive use, glyphosate and its residues contaminate surface waters. On site, fast monitoring of contamination is therefore urgently needed to alert local authorities and raise population awareness. We report here the hindrance of the activity of two enzymes, the exonuclease I (Exo I) and the T5 exonuclease (T5 Exo) by glyphosate. These two enzymes digest oligonucleotides into shorter sequences, down to single nucleotides. The presence of glyphosate in the reaction medium hampers the activity of both enzymes, slowing down enzymatic digestion. We show by fluorescence spectroscopy that the inhibition of ExoI enzymatic activity is specific to glyphosate, paving the way for the development of a biosensor to detect this pollutant in drinking water at suitable detection limits, i. e. 0.6 nM.

1. INTRODUCTION

Pesticides and herbicides are essential to agriculture to increase crop yields and to provide sufficient food. Since their launch in the last century, they enabled substantial economic benefits and are thus widely used.[1] Organophosphates are particularly used worldwide since they target a wide variety of pests and weeds, enhancing agricultural production.[2] N-phosphonomethyle-glycine (glyphosate) is the most widely used organophosphorus pesticide due to its efficiency in killing weeds. It was introduced by Monsanto in 1970 under the name Roundup.[3] Glyphosate inhibits the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSPS), which is responsible for the biosynthesis of aromatic amino-acids, causing cessation of growth and plant death.[4] Due to its high solubility in water, glyphosate residues accumulate in surface waters causing serious

problems in the environment and human health, e. g. the irreversible inhibition of the activity of the acetylcholinesterase enzyme has detrimental effects on the central nervous system.[5] Besides, several studies demonstrated that glyphosate could affect cell cycle regulation, inhibit steroid hormone secretion in men, and also cause effects adverse to animals and to aquatic vegetation.[6,7] The carcinogenicity of glyphosate remains however under debate. An International Agency for Research on Cancer (IARC) report in 2015 classified glyphosate in category 2A, i.e. "probably carcinogenic for humans".[8] Two years later (2017), the European Food Safety Authority (EFSA) published a report concluding that glyphosate is not likely to be carcinogenic to humans.[9] Since glyphosate carcinogenicity remains to be confirmed, it is therefore regularly evaluated by national and international regulatory agencies. European Directive 98/83/EC set a maximum residue limit (MRL) for each pesticide in drinking water, including glyphosate, at 0.1 µg L^{-1}.[10] Furthermore, the tolerable daily intake (TDI) of glyphosate under chronic oral exposure and the acute reference dose are estimated at 0.5 mg kg^{-1} body weight per day.[11] Although the presence of glyphosate in different waters is already regulated, its low molecular weight, high polarity, and lack of fluorophore or chromophore groups rendered its detection difficult up to now.[12,13]

The main means of analysis of glyphosate are therefore chromatographic methods coupled with mass spectrometry (MS), including high pressure liquid chromatography (HPLC), ion exchange chromatography (IEC), ion chromatography (IC) and gas chromatography (GC).[14–16] These conventional techniques are sensitive and highly specific, allowing trace analysis of pesticides in environmental samples. However, they require highly qualified operators. Besides that, samples need to be pre-treated and then analyzed in sophisticated off-site laboratories offering these expensive techniques. This mode of detection is thus unsuitable for the survey of flowing waters. They require on site analysis at a suitable frequency to detect a possible pollution peak to rapidly alert the authorities and population. Most of the technologies used for the detection of glyphosate

require high-end low throughput equipment and resources. None of them are adequate for field detection.[17] Increasing efforts are thus devoted to finding sensitive and selective alternative ways to detect glyphosate on site. Biosensors, combining of a biological sensing element and a transducer, are an appealing alternative to conventional laboratory-based methods. Specificity and selectivity are ensured by the biological sensing elements, which could be antibodies, enzymes, aptamers or even cells,[18] while sensitivity is provided by the transducer, which converts the biological interaction into an electrical signal allowing detection of the analyte.

The potential selectivity and sensitivity of optical and electrochemical based biosensors make them attractive for the detection of pesticides.[19–22] Methods include absorption (UV-Vis) spectroscopy,[23],[24] fluorescence spectroscopy,[25],[26] photoluminescence assay,[27],[28] chemiluminescence assay,[29],[30] surface-enhanced Raman scattering (SERS),[31],[32] potentiometric sensing,[33] impedence sensing,[34],[35] and amperometry.[36],[37] Only a few of these biosensors developed to detect pesticides, have been applied to glyphosate (**Table S1**) [38–42]. Moreover, none had sensing elements suitable for in situ detection of glyphosate.

Our main objective is thus the identification of a suitable sensing element to develop in the future an electrochemical aptasensor.[43] This biosensor would thus be composed of an aptamer sensing element and of an electrochemical detection platform. Aptamers are oligonucleotide switching structures that form a complex with the target, e.g. small organic molecules, proteins or even cells, with very high affinity and specificity.[44,45] The interaction between glyphosate and its aptamers described in the literature[41,46,47] was thus tested using enzymatic digestion by the T5 exonuclease (T5 Exo) and exonuclease I (Exo I).[48] The activity of these enzymes is widely used to demonstrate the specific interaction between a ligand and an oligonucleotide switching structure.[49–53] The enzymes digest the aptamer into shorter oligonucleotides and/or mononucleotides. In the presence

of the target, an aptamer/target complex is formed which leads to a decrease of enzymatic activity allowing detection of the interaction.

As reported below, we demonstrate the specific inhibition of Exo I and T5 Exo enzymatic activity in a specific buffer by glyphosate, with high potential for the development of an efficient and specific biosensor of glyphosate in the future.

2. Results and Discussion

2.1. Interaction of Aptamer Candidates with Glyphosate as Assessed by T5 Exo Enzymatic Digestion followed by Polyacrylamide Gel Electrophoresis (PAGE)

Conventional PAGE[48] subsequent to T5 Exo digestion was used to assess the interaction between glyphosate and its aptamer candidates. The interaction between glyphosate and potential aptamer candidates reported in the literature, GLY1,[41] GLY2[46] and GLY3,[47] was first tested. In parallel to the use of GLY2 and GLY3 glyphosate specific aptamer candidates, two negative controls, SCR GLY2 and SCR GLY3, were designed in order to further validate the specificity of GLY2 and GLY3 against glyphosate. The main conformations of the aptamers and negative controls are shown in **Figure S1**. No negative control was designed for the GLY1 aptamer since it is specific not to glyphosate but to two other pesticides, i.e. trichlorfon and malathion.[41] The negative control sequences have the same number of nucleic acid bases and the same GC content as GLY2 and GLY3, but were designed with a random nucleic acid order resulting in a new conformation that prevents target recognition.

Reduced enzymatic activity in the presence of glyphosate was observed with the GLY1 and GLY3 candidates, as well as for the SCR GLY3 negative control (**Figure 1**).

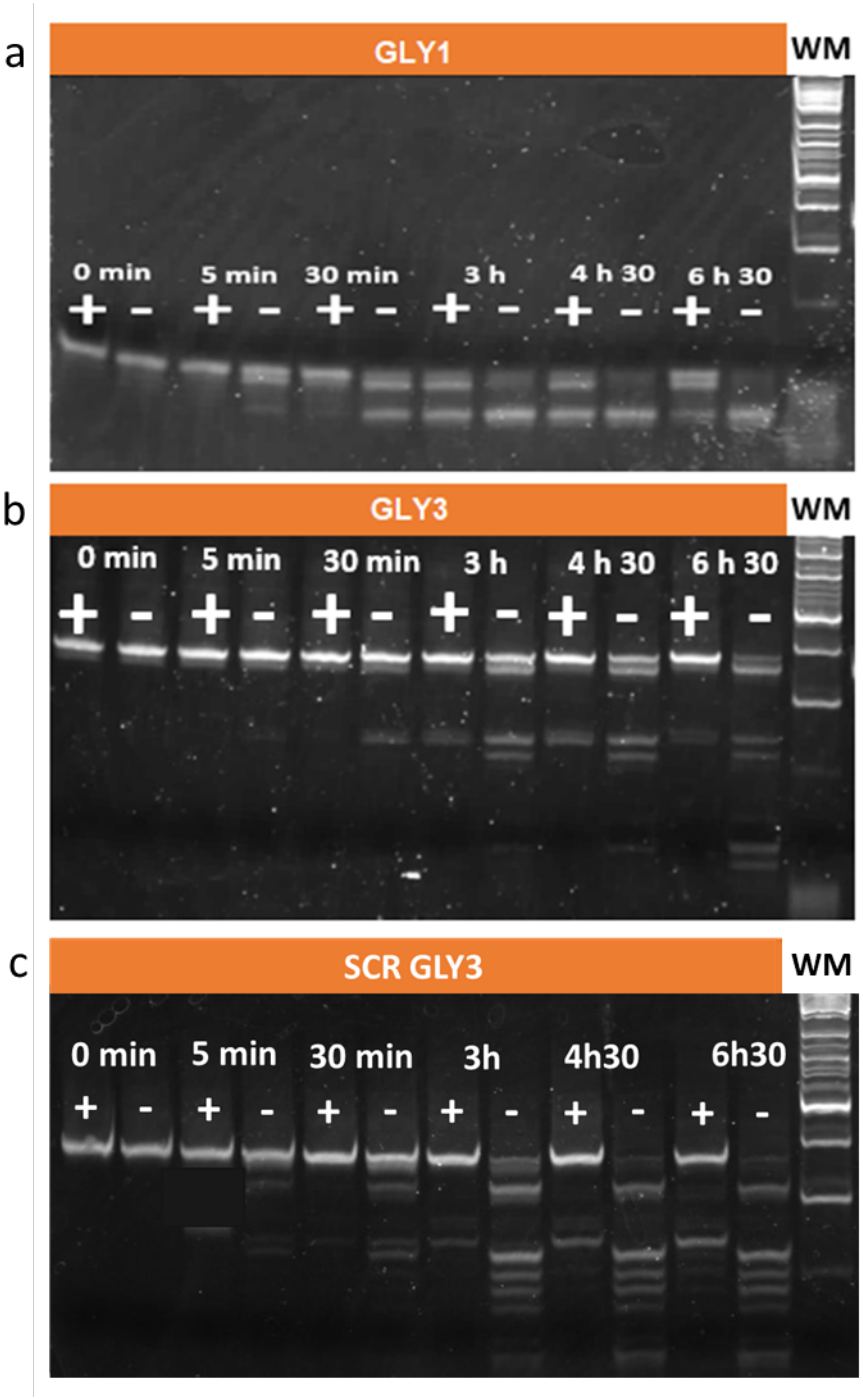

Figure 1. Digestion of the GLY1 candidate (a), GLY3 candidate (b) and SCR GLY3 (c) at 1 µM each by T5 Exo (0.2 U/µL) at room temperature in GLY1 and GLY3 buffers, respectively, after 2 h incubation at 37°C with (+) or without (-) glyphosate (1 mM).

GLY2 and SCR GLY2 (**Figure S2**) yielded no reduction in enzymatic activity in the presence of glyphosate, throughout the digestion time tested. These results suggest that GLY1 and GLY3 are probably aptamers of glyphosate since a reduction in enzymatic activity is observed, potentially due to the formation of a complex between the aptamer and glyphosate, whereas with this method GLY2 showed no interaction with glyphosate. As expected the SCR GLY2 negative control showed no interaction with glyphosate. However, the negative control SCR GLY3, which should not interact with glyphosate, showed the opposite. To conclude on a potential interaction between glyphosate and SCR GLY3, a rapid detection approach was thus needed to replace PAGE which is a tedious method as it requires several cumbersome steps, namely the preparation of the gel plate, the migration step, the disassembly of the gel plate, the staining step and finally reading of the fluorescence. In addition, enzymatic digestion by T5 Exo is relatively slow (complete digestion of SCR GLY3 is achieved in about ten hours or more, **Figure S3**) and doesn't allow quantification.

2.2.Quantitative Analysis of the Interaction between Glyphosate and its Aptamer Candidates as Assessed by Fluorescence Spectroscopy

In order to quantify the percentage of digestion and to shorten the analysis time, we resorted to fluorescence spectroscopy. Assays were carried out first with both GLY3 and SCR GLY3 which clearly demonstrated a decrease in T5 Exo activity in the presence of glyphosate by PAGE. Different conditions of incubation and digestion were tested using both enzymes, T5 Exo and Exo I (**Figure S4**). The reduction of the enzymatic activity was confirmed with both GLY3 and SCR GLY3 regardless of the incubation time or of the temperature of incubation and/or digestion tested. As expected, digestion at 37 °C is more efficient than digestion at room temperature.[48,54–56] Digestion at 37 °C with two hours of incubation between GLY3 or SCR GLY3 and glyphosate at 37 °C was thus selected as the reference condition for subsequent studies of interaction specificity

as it provides the best digestion efficiency while enabling differentiation between the two conditions, i.e. with or without glyphosate.

In order to shorten the digestion time needed to clearly exhibit enzymatic activity, the digestion kinetics of GLY1, GLY3 and SCR GLY3 were measured (**Figure S5**). Near-total digestion (in the absence of glyphosate) was achieved with all candidates in only 30 min, demonstrating the acceleration of enzymatic digestion when combining both enzymes. In what follows, we fix the digestion time to 30 min.

Figure 2 shows the percentage of digestion obtained with GLY1, GLY3 and SCR GLY3, measured by fluorescence spectroscopy, in the presence or absence of glyphosate.

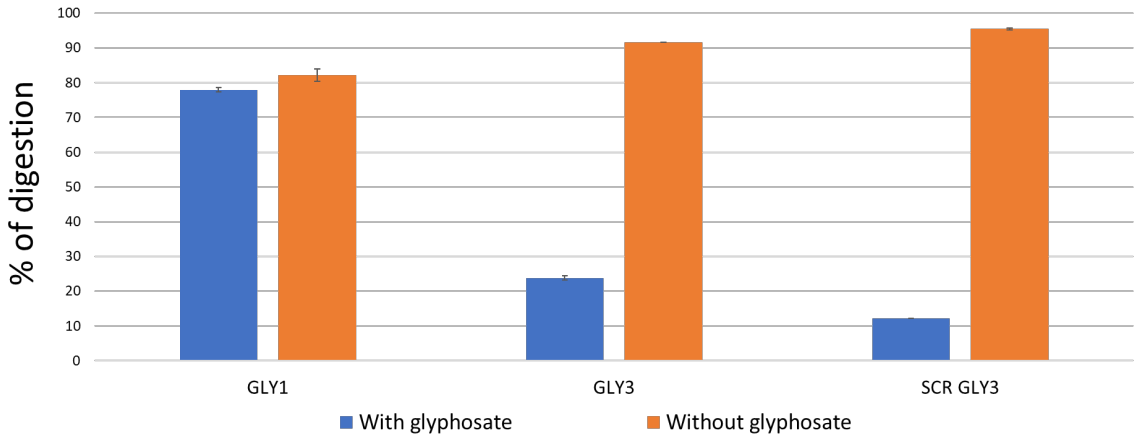

Figure 2. Digestion of GLY1, GLY3 and SCR GLY3 at 1 µM by both enzymes, T5 Exo and Exo I, at 37 °C for 30 min, after 2 hours of incubation at 37 °C with or without glyphosate (1 mM) in GLY3 buffer.

Contrary to when PAGE was used, no decrease of the enzymatic activity of GLY1 was observed in the presence of glyphosate. This is explained by the fact that fluorescence spectroscopy measures the total fluorescence in a sample. However, it is the shortest sequence hence low fluorescence. GLY1 led to the same low fluorescence signal and thus to the same percentage of digestion even in the presence of glyphosate (78% and 82% of digestion, **Figure 2**). On the contrary, with GLY3 and SCR GLY3, the fluorescence intensity after digestion in the presence of glyphosate is clearly

higher (24% vs. 12% digestion) than in its absence (92% vs. 95% digestion, **Figure 2**) because they are protected against digestion.

As observed in **Figure 2**, for both GLY3 and SCR GLY3 candidates, decreased enzymatic activity in the presence of glyphosate is observed. Similar to PAGE, fluorescence spectroscopy shows reduced activity of the negative control SCR GLY3 in the presence of glyphosate.

Glyphosate inhibits the activity of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS),[4] and inhibition of both Exo I and T5 Exo enzymes activity cannot be ruled out. EPSPS synthase catalyzes the transfer of the carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P), forming 5-enolpyruvoylshikimate 3-phosphate (EPSP) and inorganic phosphate[57] (**Figure S6a**). Glyphosate however mimics the PEP substrate of the enzyme (**Figure S6b**) which results in a stable complex of EPSPS.S3P.glyphosate leading to the inhibition of EPSPS.[58–62] A similar mechanism could be proposed to understand the inhibition of Exo I by glyphosate. ExoI breaks PEP phosphodiester bonds (**Figure S6c**).[63] In the presence of glyphosate, a more stable complex of the enzyme with glyphosate instead of one with its substrate, i. e. oligonucleotides, might preferentially form leading to enzymatic inhibition.

Before concluding on the interaction between glyphosate and SCR GLY3, we however investigated the role of sequence composition and of the buffer on the reduction of enzymatic activity by glyphosate.

2.3.Sequence Composition and Reduction of Enzymatic Activity in the Presence of Glyphosate

By definition, an aptamer is a unique sequence that recognizes a specific target. If SCR GLY3 is an aptamer specific to glyphosate, it must be unique and none of the sequences selected for this assay should interact with glyphosate.

Several oligonucleotides were therefore tested in the same conditions of digestion than as GLY3 and SCR GLY3, namely GLY1 and GLY2, the AA40 library (which contains about 10^{15} unique sequences), and ATZ1,[64] ATZ2[65] which are aptamers developed against atrazine (**Figure 3**).

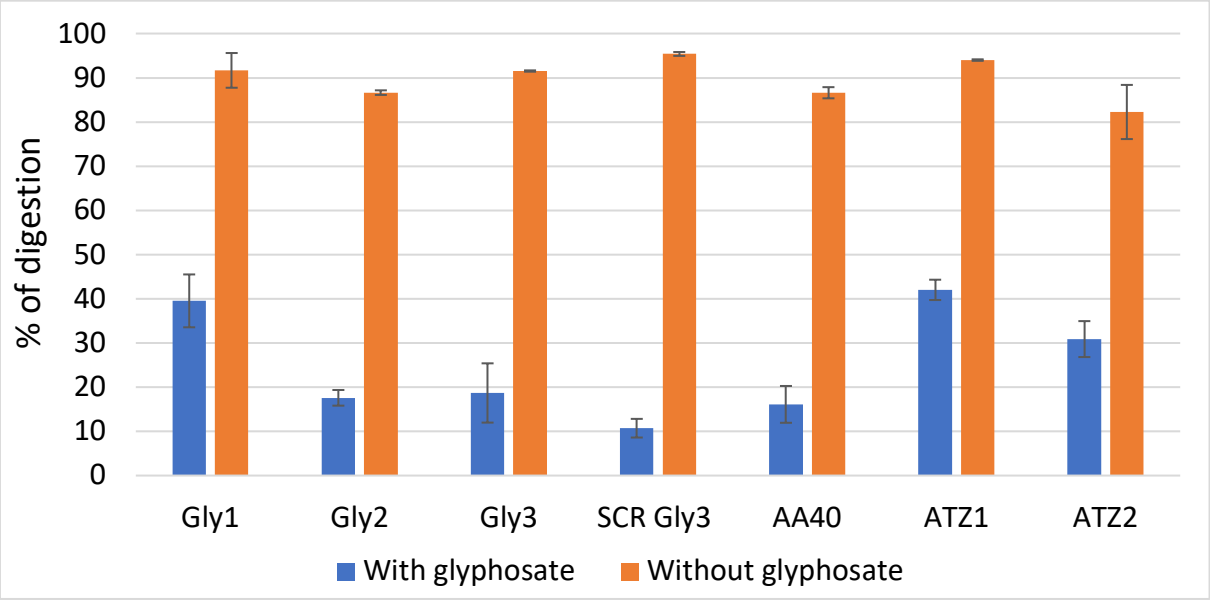

Figure 3. Digestion of different oligonucleotides at 1 µM by both enzymes, T5 Exo and Exo I, at 37 °C for 30 min after 2 hours of incubation at 37 °C with or without glyphosate (1 mM) in GLY3 buffer.

Decreased enzymatic activity in presence of glyphosate is found with all these oligonucleotides tested but before concluding non-specificity, we should check if reduced activity is related to the GLY3 buffer or to enzyme inhibition by glyphosate.

2.4.Role of the GLY3 Interaction Buffer in Reduction of Enzymatic Activity

We therefore assessed the role of the GLY3 interaction buffer in enzymatic inhibition by the digestion of both the GLY3 and the AA40 library sequences in HEPES buffer, which is used frequently in the development of aptamers at Novaptech (**Figure S7**).

Neither showed reduction of enzymatic activity in the presence of glyphosate, and both enzymes are active in the HEPES buffer (90 % digestion). These results clearly confirm that the GLY3 interaction buffer is crucial to the reduction of enzymatic activity.

Although it contains few divalent cations (1 mM of Mg^{2+}) it is essential to enzymatic activity. Indeed, as reported earlier, Mg^{2+} concentrations as low as 10^{-2} to 10^{-3} mM reduce the activity of Exo I by 10 to 20%.[66] Besides, according to the literature, glyphosate has a chelating effect strongly complexing divalent cations, including Mg^{2+}.[67] Thus 1 mM of Mg^{2+} could be complexed by 1 mM of glyphosate and reduce enzymatic activity. To rule out this effect, GLY3 was digested in its own buffer with or without glyphosate, but with excess of Mg^{2+} cations, i. e. 5 mM (instead of 1 mM) (**Figure S8**). Enzymatic activity was nevertheless reduced implying that enzymatic inhibition is not related to the cation concentration.

To rule out the effect of monovalent cations (K^+ and Na^+) on enzymatic activity, different concentration ratios were tested in the GLY3 buffer in the same conditions of digestion (**Figure S9a**). The first is the same as in which enzymatic activity is not reduced. The same test was performed with the HEPES buffer (**Figure S9b**).

Thus, monovalent cations do not significantly affect enzymatic digestion. It is rather the Tris buffer component, contrary to HEPES, which plays the main role in the reduction of enzymatic activity.

2.5.Glyphosate as an Inhibitor of Enzymatic Activity

We probed separately the inhibition of either Exo I and T5 Exo by the digestion of a random sequence, SUP000, of comparable length to GLY3 (81 nucleotides *vs.* 84 nucleotides), in GLY3

and HEPES buffers, but without the incubation step of SUP000 with glyphosate. The enzymes were tested separately in order to evaluate their individual behavior in the two buffers.

Being a random sequence, SUP000 is not expected to interact with glyphosate. Therefore, each enzyme solution was prepared with and without glyphosate and the SUP000 sequence was added at the last stage. The whole set was incubated for 30 min at 37 °C.

As observed in **Figure 4**, and in the HEPES buffer, neither enzyme reduces activity in the HEPES buffer, with or without glyphosate. In the GLY3 buffer, T5 Exo is already inhibited (20% digestion without glyphosate) compared to the HEPES buffer (90% of digestion without glyphosate). Exo I is active in both buffers. However, this test clearly shows that the presence of glyphosate in the GLY3 buffer inhibits the activity of Exo I.

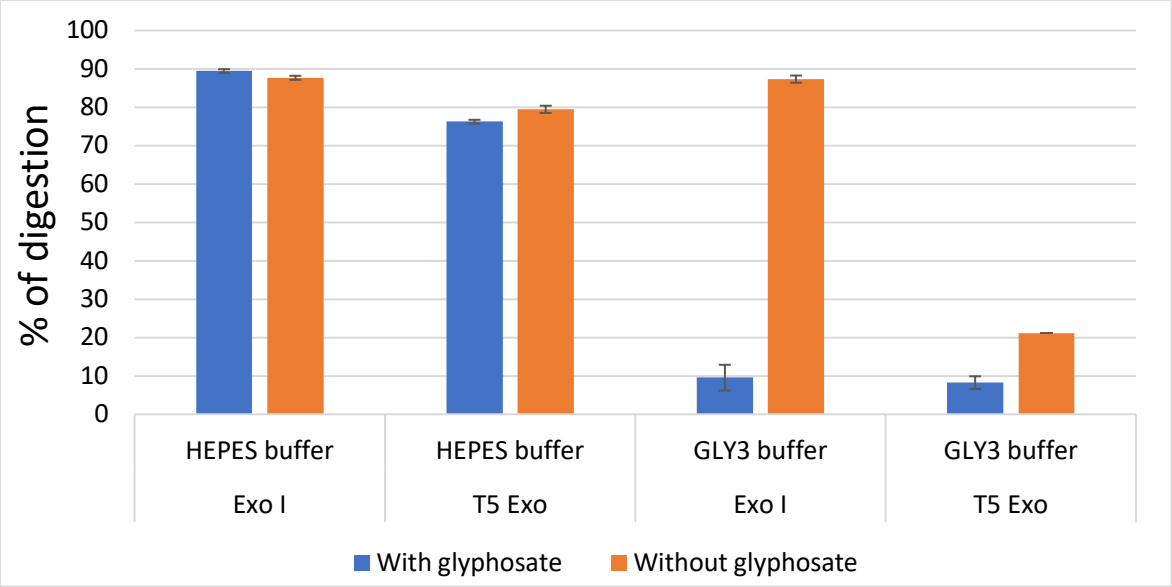

Figure 4. Digestion of SUP000 (1 µM), Exo I and T5 Exo, for 30 min at 37 °C in HEPES and GLY3 buffers with and or glyphosate (1 mM).

2.6.Specificity of the Reduction of the Enzymatic Activity

Since its activity is inhibited in the GLY3 buffer, the expected enhancement of digestion by T5 Exo is ruled out and therefore its use discarded from studies reported below. Is the reduction of enzymatic activity specific to glyphosate? Other pesticide molecules, especially the main

metabolite of glyphosate (AMPA), were tested using SUP000 as the substrate for Exo I: simazine, alachlor, atrazine, atrazine-desethyl, and isoproturon (**Figure 5**).

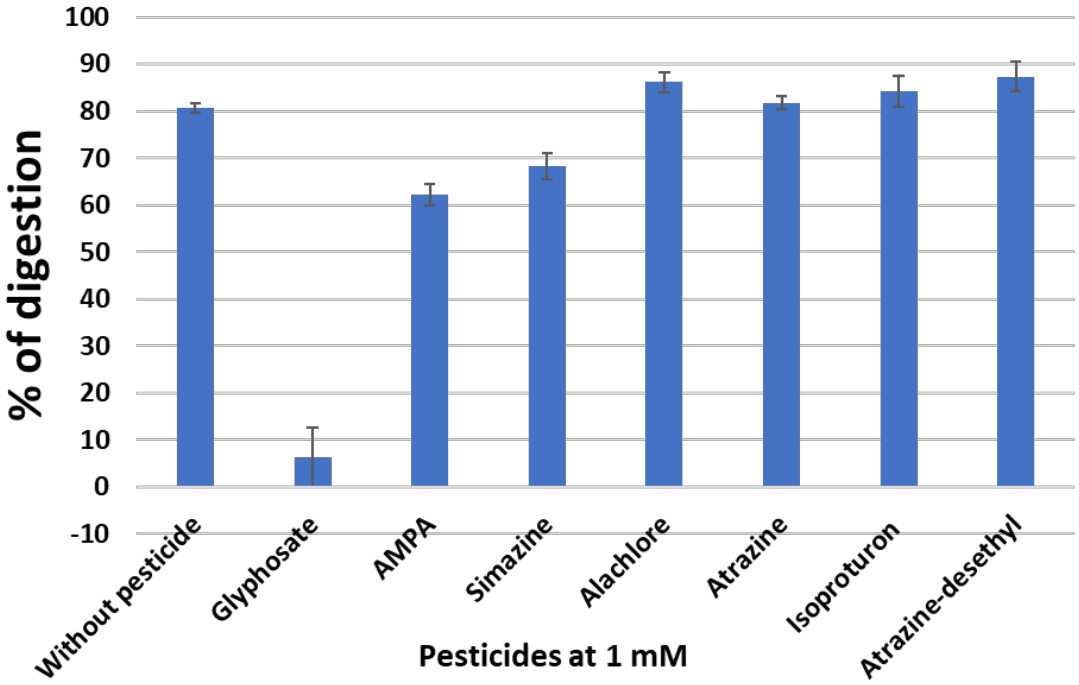

Figure 5. Digestion of SUP000 at 1 µM by Exo I (0.15 U μL^{-1}) at 37 °C for 30 min in GLY3 buffer in the absence or in the presence of different pesticide molecules at 1 mM.

Slight inhibition is observed in presence of AMPA and simazine, (62% and 68% digestion compared to 80% without pesticide). But only glyphosate induces strong inhibition (≈ 6% of digestion), which means that inhibition is specific to glyphosate.

3. Conclusion

Here we show that glyphosate inhibits the T5 Exo and Exo I, as quantified by fluorescence spectroscopy. Identification of this oligonucleotide digesting enzyme sensing element paves the way to the future development of a biosensor since the activity is specifically inhibited by glyphosate. The outcomes of this study are of high relevance since exonucleases are human enzymes, especially the Exo I enzyme that is required for 5' and 3' mismatch repair in human,[68]

further demonstrating that the mechanisms, consequences and danger to human health of this pesticide should be urgently elucidated.

4. Experimental Section

4.1.Chemicals and Reagents

All the chemicals and reagents were of analytical grade (> 99%) and used without further purification. Tris(hydroxymethyl)aminomethane (Tris-HCl), sodium chloride (NaCl), potassium chloride (KCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), magnesium chloride hexahydrate ($MgCl_26H_2O$), calcium chloride tetrahydrate ($CaCl_24H_2O$), formamide, ethylenediaminetetraacetic acid (EDTA), glycerol and sodium dodecyl sulfate 4X (SDS) were purchased from Euromedex (Strasbourg, France). Ethanol (EtOH), magnesium acetate, sodium acetate, potassium acetate, tris(hydroxymethyl)aminomethane, boric acid, EDTA, tris(hydroxymethyl)aminomethane and boric acid (TBE), urea and tetramethyl ethylenediamine (TEMED) were purchased from VWR Chemicals (Radnor, Pennsylvania, USA). Glyphosate, aminomethylphosphonic acid (AMPA), atrazine, xylene cyanol and ammonium persulfate (APS) 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). Exo I (20000 U mL^{-1}) and T5 Exo (10000 U mL^{-1}) were purchased from New England Biolabs (Ipswich, Massachusetts, USA).

4.2.Oligonucleotides

All oligonucleotides used in this work were synthesized by Eurogentec (Seraing, Belgium) with polyacrylamide gel electrophoresis (PAGE) purification grade. The oligonucleotides were dissolved in MilliQ water and the concentrations were measured by UV-Vis spectroscopy using

1 the Nanodrop 2000 spectrometer (Mettler Toledo, Columbus, Ohio, USA). The sequences of the
2 glyphosate aptamer candidates are listed in **Table 1**.
3

Table 1. Sequences of glyphosate aptamer candidates

Aptamer name	Sequence
GLY1 aptamer[41]	5'-AGC-TTG-CTG-CAG-CGA-TTC-TTG-ATC-GCC-ACA-GAG-CT-3'
GLY2 aptamer[46]	5'-CGT-ACG-GAA-TTC-GCT-AGC-AGA-GGG-ATG-GTG-TGG-GTG-GCT-GCG-GCT-ATA-GGA-GCG-TAC-CGGATC-CGA-GCT-CCA-CGT-G-3'
GLY3 aptamer[47]	5'-TGC-TAG-ACG-ATA-TTC-GTC-CAT-CCG-AGC-CCG-TGG-CGG-GCT-TTA-GGA-CTC-TGC-GGG-CTT-CGCGGC-GCT-GTC-AGA-CTG-AAT-ATG-TCA-3'

4.3.Design of Negative Controls (DNA Oligonucleotides)

In order to assess the specificity of interaction of the GLY2 and GLY3 aptamer candidates towards glyphosate, two DNA oligonucleotide negative controls, scrambled GLY2 and scramble GLY3 (SCR GLY2, SCR GLY3), were designed and used with the same buffers as GLY2 and GLY3. Two aptamers developed against atrazine, ATZ1[64] and ATZ2,[65] the thrombin aptamer,[69] the arsenic aptamer,[70] ssDNA, and the AA40 library sequences (which contains about 10^{15} unique sequences) were also used as negative controls (**Table S2**).

4.4.Experimental Conditions

All incubations between glyphosate and its aptamer candidates, as well as enzymatic digestion, were performed using a thermomixer (Eppendorf, Hamburg, Germany).

Enzymatic digestion was performed at room temperature (~20 °C) and at 37 °C with stirring (240 rpm). In each experiment, the buffers described in **Table S3** were used with their corresponding aptamer. All experiments were done in duplicate or triplicate. The standard deviations are presented in the form of 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.

4.5.T5 Exonuclease Digestion and Gel Electrophoresis

The interaction between glyphosate and its aptamer candidates described in the literature was done by T5 Exo digestion prior to PAGE.[48] The T5 Exo enzyme digests double-stranded and single-stranded DNA sequences in the 5' to 3' direction, down to single nucleotides (**Figure S10**). The working principle of this method is described in **Figure S11**. Interaction between the aptamer and its target results in delayed digestion of the oligonucleotide due to the formation of an aptamer/target complex impeding the enzymatic digestion.[49–53]

For all digestion assays, 1 µL of a 25 µM aptamer solution is added to 22 µL of interaction buffer containing glyphosate at a final concentration of 1 mM. After incubation between glyphosate and their aptamer candidates in the appropriate conditions (**Table S4**), 0.5 µL of the T5 Exo enzyme at 10 U μL^{-1} is added to the reaction mixture (at a final concentration of 0.2 U μL^{-1}), and the digestion is started at room temperature under gentle stirring. After each digestion period, 5 µL of the reaction mixture are drawn off and added to 15 µL of loading buffer (75% formamide (v/v), 10% glycerol (v/v), 0. 125% SDS (v/v), 10 mM EDTA and a pinch of xylene cyanol) and then loaded into the wells of a previously prepared denaturing polyacrylamide gel plate (final gel concentration = 15%, see preparation in electronic supplementary information ESI). The samples are separated by gel migration using an EPS-300X electric generator (CBS, Paris, France), under the following conditions: applied voltage U = 750 V, intensity I = 300 mA, power P = 20 W and 1 h10 min of migration time.

The gel plate is dismantled and the polyacrylamide gel is immersed in 100 mL of 0.5X TBE containing 1X SYBR Gold, which is an intercalator binding to DNA by insertion between the DNA bases[71] (the absorption spectrum of SYBR Gold as well as calculations of its molar concentration

are shown in **Figure S12** and **Table S5** respectively). The whole set is incubated for 25 min in the dark under gentle stirring (18 rpm) on a horizontal incubator (See-saw rocker, Stuart-equipment, Waltham, Massachusetts, USA). After incubation, the gel fluorescence is visualized with G-Box (SYNGENE, Synoptics, Cambridge, UK).

4.6.Fluorescence Spectroscopy

To achieve fast, quantitative analysis of the interaction between potential aptamer candidates and glyphosate, a second enzyme, exonuclease I, is combined with T5 Exo, and the analysis is done by measuring the fluorescence intensity of SYBR Gold. The activity of Exo I is indeed complementary to that of the T5 Exo as it digests single-stranded DNA into mononucleotides in the opposite direction to T5 Exo (from 3' to 5', **Figure S13**). In addition, it digests the products of T5 Exo enzymatic digestion, which accelerates the process. Protection of the aptamer by its target thus translates into a higher fluorescence intensity of SYBR gold than without. Fluorescence intensity is converted to the digestion yield via **Equation 1**. The yield of digestion is lower when the target protects its aptamer (**Figure 6**).

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

F_0: Fluorescence signal before digestion
F: Fluorescence signal after digestion

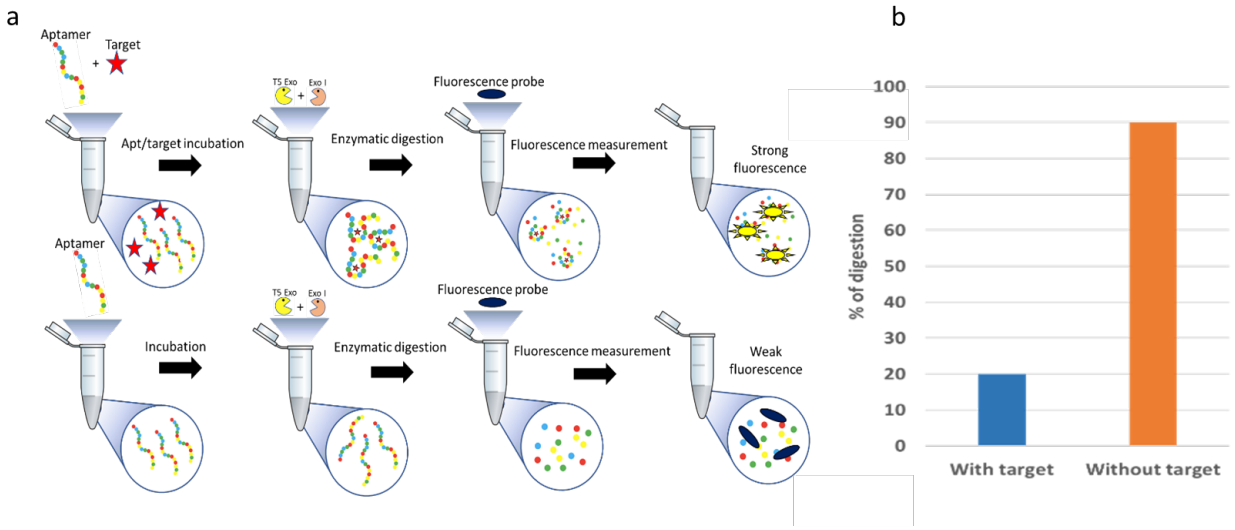

Figure 6. (a) Principle of analyzing aptamer/target interaction by T5 Exo and Exo I digestion, followed by fluorescence spectroscopy. (b) The expected percentage of enzymatic digestion in the presence and in the absence of the target.

We followed the protocol reported previously.[48] 1 µL of a 25 µM aptamer solution is added to 22 µL of interaction buffer containing glyphosate at a final concentration of 1 mM. After incubation between glyphosate and aptamer candidates in the appropriate conditions (**Table S4**), 1.5 µL of both T5 Exo and Exo I at a final concentration of 0.2 U μL^{-1} and 3.75 U μL^{-1} respectively were added to the reaction mixture, and the digestion started at 37 °C under gentle stirring. After each incubation period, 5 µL of the reaction mixture were drawn off and loaded into the wells of a black 384-well microplate (Corning black, Thermo Fischer Scientific) containing 25 µL of stop solution (1.2X SYBR Gold, 12 mM Tris-HCl pH = 7.4, 48% formamide (v/v), 3.75 mM EDTA). Fluorescence was measured at 545 nm (excitation at 495 nm) (Tecan Infinite M1000 Pro, Männedorf, Suisse).

Supporting information: Supporting Information is available from the Wiley Online Library.

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