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Amperometric nanobiosensor for quantitative determination of glyphosate and glufosinate residues in corn samples*

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Abstract: This study presents a simple, sensitive, rapid, and low-cost amperometric method for direct and quantitative determination of glyphosate and glufosinate herbicides. Electrochemical synthesis and characterization of poly(2,5-dimethoxyaniline)-poly(4-styrenesulfonic acid) (PDMA-PSS) nanoparticles was achieved by cyclic voltammetry (CV) and scanning electron microscopy (SEM). The nanobiosensor was constructed by immobilizing the enzyme horseradish peroxidase (HRP) electrostatically onto the surface of a rotating gold disk electrode modified with PDMA-PSS nanoparticles. The biosensing principle was based on determination of the sensor response to glyphosate and glufosinate by amperometric methods. Hydrogen peroxide (H_2O_2) was used to measure activity of the enzyme before injection of the herbicides into the electrolyte solution. The enzyme electrode was stable for a long period of time and was used for over 60 measurements. Glyphosate and glufosinate analyses were realized on spiked corn samples within a concentration range of 2.0–78.0 μg L^{-1}, corroborating that the nanobiosensor is sensitive enough to detect herbicides in these matrices. Based on a 20-μL sample injection volume, the detection limits were 0.1 μg L^{-1} (10^{-10} M) for both glyphosate and glufosinate without sample clean-up or preconcentration.

Keywords: electrochemical nanobiosensor; glyphosate; glufosinate; herbicides; poly(2,5-dimethoxyaniline)-poly(4-styrenesulfonic acid); horseradish peroxidase.

INTRODUCTION

Herbicides are a heterogeneous group of chemicals used to kill or inhibit the growth of undesirable plants that might cause damage, present fire hazards, or impede work crews. In recent years, the public has become more concerned about the extensive use of herbicides and their effects on the environment on a global scale. Among the herbicides used, glyphosate [*N*-(phosphonomethyl)glycine] and glufosinate [DL-homoalanine-4-yl-(methyl)phosphonic acid] are two important examples and are broad-spectrum, nonselective herbicides for control of long grasses and broad-leaved weeds. The phosphorus-containing herbicides interfere with the formation of amino acids and other chemicals in plants [1,2]. As they are of comparatively low acute and chronic toxicity to human and animal health, these herbicides

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have become the most extensively used worldwide. Some studies have reported that if they are ingested over a period of time, they may affect the central nervous system, resulting in respiratory, myocardial, and neuromuscular malfunctions, which can even lead to death [3–5]. However, because their effects on non-target organisms and overall environmental impact have not been fully investigated, questions regarding the environmental safety with their increasing use have to be addressed. The increase in the use of these compounds has led to their residues being found in soil, atmosphere, agricultural products, as well as in ground water.

The United Nations Food and Agricultural Organization (FAO) has set maximum residue limits (MRLs) for residues of glyphosate and glufosinate on most crops at 0.1–5 and 0.05 mg kg^{-1}, respectively [6]. Currently, glyphosate is in the list of the U.S. national primary drinking water contaminants with a maximum contaminant level goal (MCLG) of 0.7 mg L^{-1}. The European Union (EU) limit of any pesticide in drinking water has been set at 0.1 μg L^{-1} irrespective of their toxicological effects [7]. Therefore, the monitoring of trace levels of these compounds in environmental and biological samples has gained increasing importance. For these reasons, there is a need to develop rapid, easy, and sensitive methods that are capable of detection and quantification of these herbicides at low concentrations, such as those that exist in foodstuffs and drinking water.

A simple analytical method for the determination of these herbicides at the sub μg L^{-1} level has proven to be very difficult to obtain, mainly due to their ionic character, low volatility, high solubility in water, insolubility in organic solvents, low mass, and favored complexing behavior [8]. Additionally, the absence of chromophore or fluorophore groups in their structures disables the photometric and fluorometric detection of these substances in liquid chromatography (LC) techniques. The reported methods for the determination of glyphosate mainly consisted of gas chromatography (GC), LC, capillary electrophoresis (CE), and enzyme-linked immunosorbent assay (ELISA). Most standard analytical methods developed until now require pre- or post-column derivatization procedures to improve both the chromatographic behavior and the detection ability by GC or high-performance liquid chromatography (HPLC). GC detectors have been used to improve the sensitivity, and furthermore, tandem mass spectrometry (MS/MS) and GC/MS have been employed. Normally, HPLC has been used in combination with fluorescence and UV/vis detection after derivatization, although in a few cases glyphosate has been determined directly by ion chromatography (IC) with UV detection [9] or suppressed conductivity detection, but with limited sensitivity. GC/MS methods involved derivatization with different reagents [9] to confer volatility to the analytes. Normally, there is quite a lot of sample manipulation, and the methods are time-consuming and tedious. The detection of glyphosate derivatives in LC and GC exhibited high sensitivity and selectivity; however, these derivatization procedures are quite complicated and require special equipment [10]. CE methods for glyphosate [11,12] provided high resolutions and efficiency, but some of them suffered low sensitivity owing to the limited sample injection volume. These reasons have given new impulse toward the development of alternative analytical devices and methods, to be applied in the screening of herbicides in environmental matrices, minimizing the pretreatment of sample and reducing the cost and time of analysis. Detection methods for glyphosate and glufosinate without derivatization, such as electrogenerated chemiluminescence detection [13], conductivity detection [14], inductively coupled plasma/mass spectrometry (ICP/MS) [11], and integrated pulsed amperometric detection (IPAD) at gold electrode [10], have been reported.

Biosensors and electrochemical methods appear well suited to complement standard analytical methods for a number of environmental monitoring applications since they do not require derivatization. Their application to environmental monitoring has been continuously growing in the last few years [15–17], though biosensor application for determination of glyphosate and glufosinate herbicides has not been previously reported. These methods have advantages such as low cost, easy operation, and high sensitivity and selectivity. Several kinds of electrochemical sensors for the determination of herbicides have been reported [18–22]. Most of them were based on ELISA, in which herbicides were detected by competitive reactions with labeled antibodies. Although the immunoassay permits one to detect herbicide with high sensitivity and selectivity, the procedure is complicated, and it is difficult to

monitor mixed herbicides because of the specificity of antibodies. An obvious drawback was the high cost and currently difficult commercial availability of the test kit. There have been a few potentiometric sensors (ion-selective electrodes) reported [23]. However, only herbicides that are positively charged could be determined by this kind of sensor.

Different biosensor formats have been developed for single-target analytes and for broad-spectrum monitoring. From a general point of view, all biosensors are based on the coupling of a biochemical agent with a physicochemical signal transducer. A biochemical component (i.e., an enzyme or biological material with the enzyme activity such as microorganisms, plant [24] or animal tissues and cells [25], etc.) is chosen for its selectivity toward the substrate or inhibitor to be determined. The signal-transducing element (e.g., electrode, optical detector, piezo crystal, etc.) converts the biochemical response into electric and optic signals that are amplified, measured, or decoded by an appropriate electronic unit.

One important step in biosensor development is immobilization of the biological recognition element to the sensor surface. A number of innovative immobilization techniques have been reported using enzymes. Approaches for these techniques include the use of new materials and incorporation of oxidation–reduction (redox) mediators into the immobilization process. In this configuration, the electroactive mediator acts as electron shuttle between the redox center of the enzyme and the electrode surface. Nanomaterials have also been used to improve the operational characteristics of the enzyme-based biosensors. This improvement results from both increased surface area and increased catalytic activity.

Among the different biosensors employed in environmental analysis, inhibition-based biosensors are fairly common. The basic principle of operation of these biosensors is based on the interaction that occurs between specific chemical and biological agents (inhibitors), present in the sample, and the active site of the biochemical component immobilized on the biosensor itself. Inhibitors block the active site of the enzymes by modifying the key amino acid residues needed for enzymatic activity, leading to decrease in enzyme activity and signal production. The response of the biosensor is, therefore, proportional to the reduction rate of the enzymatic reaction that takes place at the sensor's interface. Inhibition-based biosensors have been used for analysis of a few compounds including pesticides and heavy metals. Thus, there is a need to fill the gap left in application of biosensors for glyphosate and glufosinate analyses.

In order to fill this gap, we present a unique approach for the development of a novel HRP/PDMA-PSS-based nanobiosensor for herbicide analysis. The aim of this work is to study the viability of a new, sensitive, simple, direct, and low-cost amperometric detection method for glyphosate and glufosinate analysis. Rotating disk electrode (RDE) operating at 400 rotations per minute (rpm) was used to add convection to the cell in order to increase current and sensitivity. The act of rotation brings material to the electrode surface where reaction takes place. Electroactive nanofilms of PDMA-PSS have been used as electron-transfer redox mediators, shuttling electrons between the immobilized enzyme and the rotating gold disk electrode surface. They also served as a point of electrostatic attachment for the heme protein HRP. The active site of HRP contains iron (Fe) which is capable of undergoing oxidation and reduction. Glyphosate has three groups (amine, carboxylate, and phosphonate) that should coordinate strongly to metal ions, particularly to transition metals [26–29]. Glufosinate, like glyphosate, has a reactive amine functional group that can coordinate strongly with transition-metal ions. Glyphosate has been reported to possess a high affinity and chelating capacity for Fe and other metals, resulting in the formation of poorly soluble glyphosate-metal complexes or insoluble precipitates [30,31]. This ability has been utilized for the detection of these herbicides by the developed nanobiosensor. The sensing principle was based on selective inhibition of the redox center of HRP by the herbicides. The herbicides have the ability to bind strongly to the Fe in the active site of the enzyme. H_2O_2 has been used as the substrate for HRP to study its activity before inhibition by the herbicides.

EXPERIMENTAL

Reagents

Horseradish peroxidase (HRP, E.C. 1.11.1.7, 169 U/mg powder), hydrogen peroxide, sodium hydrogen phosphate, potassium dihydrogen phosphate, hydrochloric acid, sulfuric acid, 2,5-dimethoxyaniline (DMA), and PSS were all obtained from Sigma-Aldrich (South Africa). Glyphosate and glufosinate reference standard solutions were also obtained from Sigma-Aldrich (South Africa). All chemicals were of analytical grade and were used as received. All solutions were prepared with doubly distilled water. Stock standard solutions of glyphosate, glufosinate, and dilutions were prepared in doubly distilled water.

Instrumentation

All voltammetric and amperometric measurements were performed using BAS 100B electrochemical analyzer from Bioanalytical Systems, Inc. (West Lafayette, IN). The RDE experiments were carried out using a gold disk electrode with a geometric area of 0.071 cm^2 and rotation speed of 400 rpm. A 20-ml cell was used in a conventional three-electrode system consisting of a rotating gold disk electrode as the working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl (saturated 3 M NaCl) electrode as the reference electrode (BAS Technicol). Electrolyte solutions were purged with high-purity nitrogen gas prior to and blanketed with nitrogen during electrochemical experiments. All electrochemical measurements were carried out in phosphate buffer solution (PBS, 0.1 M, pH 6.10) at 20 °C.

The scanning electron microscopy (SEM) image was taken with the Gemini LEO 1525 Model. Screen-printed carbon electrodes were used for polymer deposition for SEM analysis. Electrodes were cut from the printed sheet, then pretreated in 0.2 M H_2SO_4 solution before use.

Construction of the biosensor

Prior to the experiment, the bare gold disk electrode was polished with aqueous slurries of 1.0, 0.3, and 0.05 μm alumina powder, rinsing with distilled water after polishing with each grade of alumina. The polished electrode was sonicated in water and absolute ethanol. The counter electrode was cleaned by burning in a flame for several minutes. Ag/AgCl electrode was rinsed with copious amounts of distilled water. The PDMA-PSS composite film was prepared by electrochemical polymerization of DMA in 1.0 M HCl in the presence of a dopant PSS (DMA-PSS ratio of 2:1), followed by electrodeposition of the film on gold electrode surface. This was obtained by cycling the potential repeatedly from –0.2 to +0.8 V, at a potential scan rate of 40 mV s^{-1}.

Following deposition of PDMA-PSS onto the electrode surface, the electrode was transferred to a batch cell containing 10 ml PBS, and the polymer surface was reduced at a constant potential of –0.5 V until the reduction attained steady state. 0.6 ml of 2.0 mg/ml buffer solution of HRP was then added to the cell containing 10 ml of fresh PBS, and the PDMA-PSS film was oxidized for 20 min at +0.65 V. During this oxidation process, the heme protein HRP became electrostatically attached onto the polymer surface. The biosensor was stored in PBS at 4 °C in a refrigerator until required.

Electrochemical measurements

Electrochemical behavior of PDMA-PSS-modified electrode was investigated by cyclic voltammetry (CV) in the potential range of –0.2 to +0.8 V in 1.0 M HCl solution. The electrochemical behavior of HRP/PDMA-PSS/Au modified electrode (biosensor) was investigated by differential pulse voltammetry (DPV) in the potential range between –0.6 and +0.2 V in PBS. This was carried out at a scan rate of 5 mV s^{-1}.

Sample preparation

Dry corn sample was ground to fine powder (<200 mesh) and 1.0 g extracted with 20 ml of water: chloroform mixture (5:3) overnight. The content was transferred to centrifuge tubes and centrifuged (10 000 rpm, 25 °C, 10 min) to remove solid particles, then filtered in a 0.22-μm membrane and stored at 4 °C. The sample was spiked with an appropriate volume of a stock solution of glyphosate or glufosinate and topped up to mark with PBS before injection.

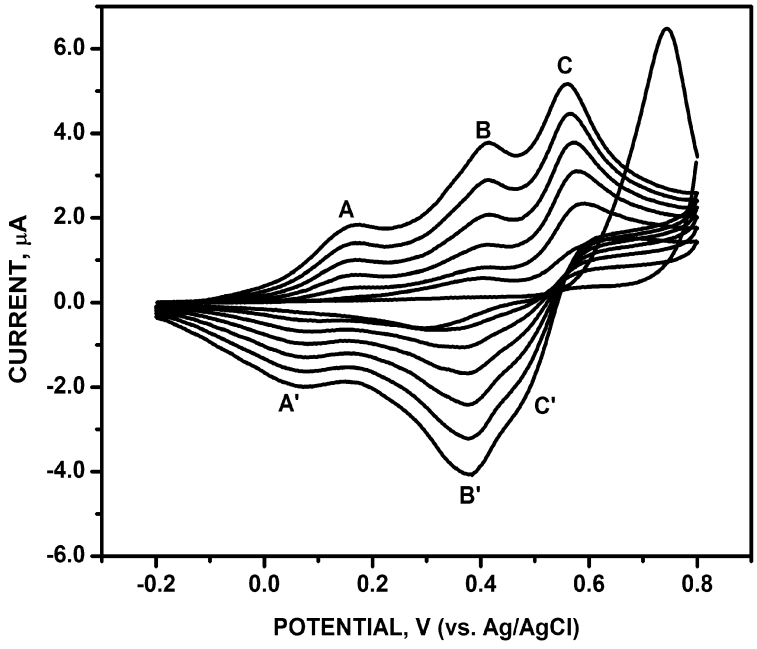
Detection of glyphosate and glufosinate

Amperometric experiments were carried out in PBS using RDE at 400 rpm and applied potential of –0.1 V. After the background current reached a considerably steady value, standard solutions of H_2O_2, glyphosate, and glufosinate were injected into the detection solution, and the steady-state currents produced were recorded as response. The biosensor was used for analysis of one herbicide at a time. H_2O_2 was first injected in order to measure activity of the enzyme; this was followed by detection of the blank solution, then the standard herbicide solution. For the detection of glyphosate and glufosinate in samples, the spiked corn sample solutions were injected, then the currents produced were recorded as response.

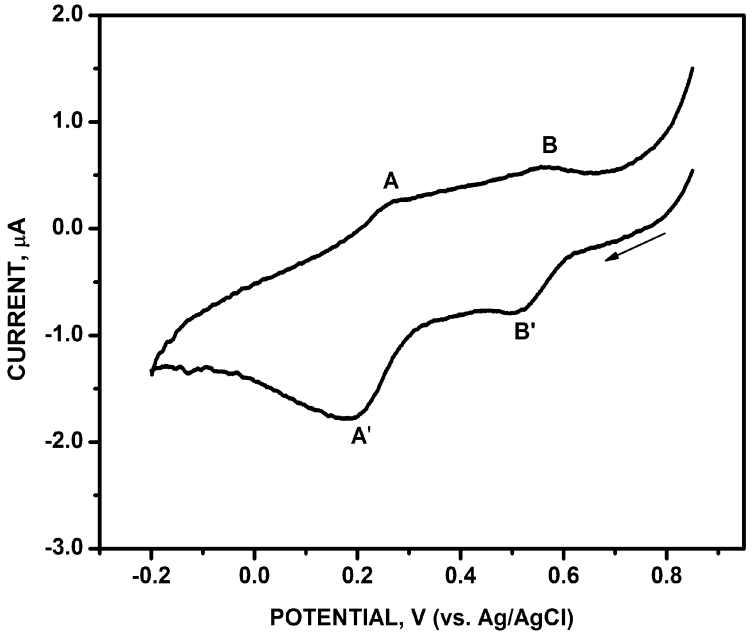
RESULTS AND DISCUSSION

Electrochemical synthesis and behavior of PDMA-PSS nanoparticles

Electrochemical synthesis of PDMA-PSS resulted in a dark-green polymer film on the surface of the electrode. CV for electrochemical synthesis of PDMA-PSS nanoparticles (Fig. 1a) showed two main redox processes corresponding to transition from leucoemeraldine to emeraldine and also emeraldine to pernigraniline states. Also, another redox process corresponding to incorporation of oligomers into the polymer matrix or degradation products of the polymer was noticed. The electrochemical behavior of the PDMA-PSS/Au electrode was studied in 1.0 M HCl solution by CV, and the results are shown in Fig. 1b. The number of electrons taking part in the reaction was calculated to be one. Two pairs of redox peaks centered at around 0.20 and 0.56 V corresponding to the transformation of leucoemeraldine base to emeraldine salt and emeraldine salt to pernigraniline salt [32], respectively, can be observed for the modified electrode. This implies that the redox peak centered at around 0.4 V (Fig. 1a) is due to incorporation of oligomers into the polymer matrix or degradation products of the polymer.



(a)



(b)

Fig. 1 CVs for (a) electropolymerization of PDMA-PSS; (b) electrochemical behavior of PDMA-PSS/Au electrode.

Micrographs of PDMA and PDMA-PSS nanoparticles

SEM images of the bulk PDMA and PDMA-PSS films electrodeposited on screen-printed electrodes are shown in Fig. 2. The SEM image of PDMA film (Fig. 2a) shows a tubular morphology expected for substituted polymers, while that of PDMA-PSS film (Fig. 2b) shows buds or nuclei of polymer dominating the surface of the electrode, giving the polymer a "cauliflower-like" appearance. The buds appear to be more orderly and uniformly aligned compared to the tubular structures making up the PDMA film. The change in morphology when the dopant PSS was incorporated indicates the presence of PSS in the polymer matrix. It suggests that PSS played a role in aligning the DMA monomers, promoting a more ordered *para*-linked reaction. The bud sizes are less than 1000 nm, they vary from approximately 100 to 1000 nm. The image indicates that the sizes of PDMA-PSS particles are in the nanometer range. Considering a bud size of about 200 nm consisting of a number of small particles (5–10), each particle in the bud would be 20–40 nm. This assists in classifying them as "nanoparticles". The "nanoparticles" were, therefore, applied as a platform for biosensor construction.

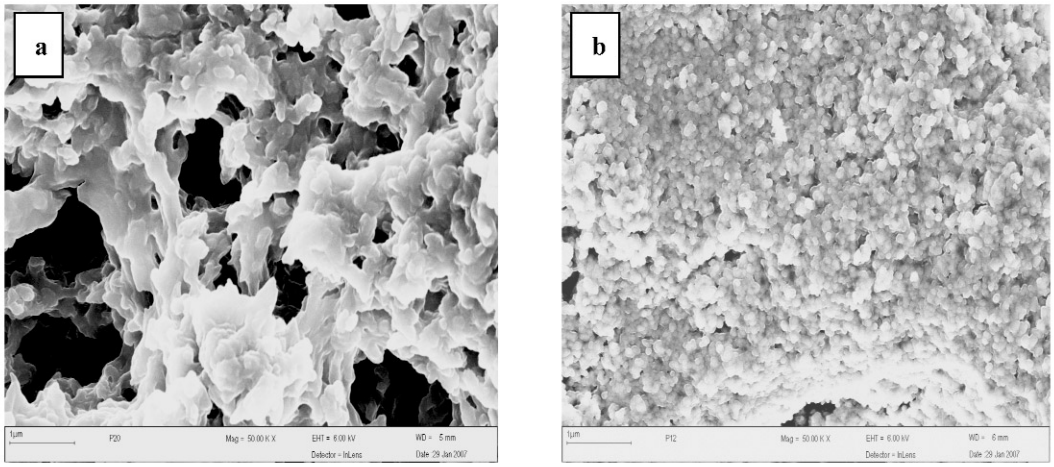


Fig. 2 SEM images of (a) PDMA and (b) PDMA-PSS nanoparticles (magnification of 50 000×).

Electrochemical behavior and application of the nanobiosensor

Electrochemical behavior of the nanobiosensor was investigated in PBS by DPV. The DPV results (Fig. 3) showed that the biosensor exhibits a quasi-reversible behavior with one redox couple (reduction peak at –0.106 V and oxidation peak at –0.023 V) and a peak separation of 0.083 V. Characterization of the nanobiosensor at various scan rates (data not shown) indicated that the reduction and oxidation peak currents increased with square root of scan rates ($v^{1/2}$) but were not proportional to it. The peak potentials shifted negatively with increasing scan rates. The results of these tests confirmed quasi-reversibility of this system. The peak at –0.106 V is characteristic of the enzyme HRP, and it confirms that HRP was electrostatically attached onto the electrode surface.

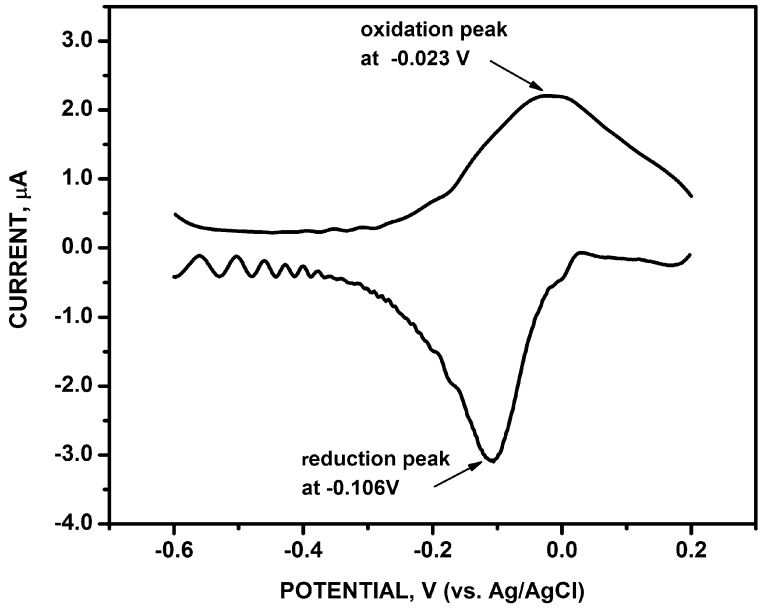


Fig. 3 DPVs for the biosensor.

Detection of H_2O_2

The steady-state current response of the optimized HRP/PDMA-PSS/Au (biosensor) electrode toward addition of H_2O_2 was studied. Figure 4 illustrates the catalytic cycle of HRP in a mediated anaerobic system. HRP enzyme has been applied as the biological component of the biosensor. Peroxidases are ubiquitous oxidative heme-containing enzymes that are usually isolated from plants (e.g., horseradish roots), fungi, and bacteria but also increasingly from mammalian sources. The Fe in the heme is capable of undergoing oxidation and reduction (usually to +2 and +3, though stabilized ferryl [Fe^{4+}] compounds are well known in the peroxidases). HRP provides selectivity by virtue of its biological affinity for its substrate H_2O_2 molecule. An early step in the catalytic cycle following the binding of H_2O_2 to the heme in the Fe(III) state (active form), is the heterolytic cleavage of the O–O bond of H_2O_2. A water molecule is released during this reaction with the concomitant two-electron oxidation of the heme to form an intermediate (compound I) comprising a ferryl species [Fe(IV) = O] and a porphyrin radical cation. Compound I is then converted back to the resting enzyme via two successive single-electron transfers from reducing substrate molecules. The first reduction, of the porphyrin radical cation, yields a second enzyme intermediate, compound II, which retains the heme in the ferryl [Fe(IV) = O] state [33]. In this study, electroactive PDMA-PSS nanoparticles have been used as electron-transfer mediators in the biosensor. Mediators are molecules that can shuttle electrons between the redox center of the enzyme and the electrodes. PDMA-PSS nanoparticles also served as points of attachment for the heme protein enzyme HRP. In Fig. 4, the $PDMA^0$ redox sites in the biosensor are the electron donors in the anaerobic reaction medium. The reduction charge is propagated along the polymer chain to the electrode surface where $PDMA^+$ species accept electrons to give the reduction current, which is then amplified, measured, and decoded.

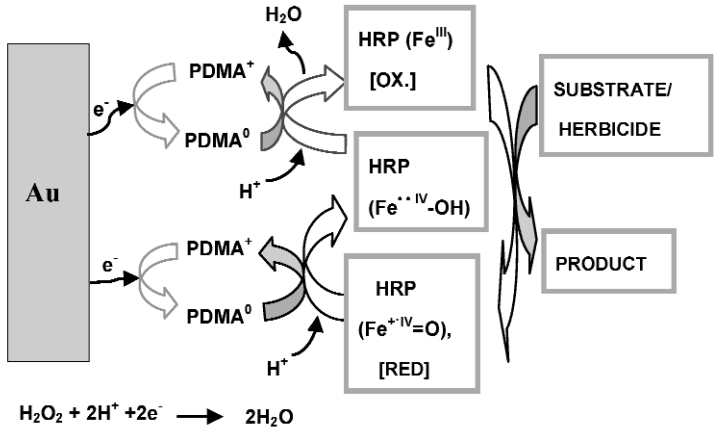


Fig. 4 Mechanism of HRP/PDMA-PSS/Au biosensor in anaerobic medium. $PDMA^{0/+}$ are the polymer-bound redox sites.

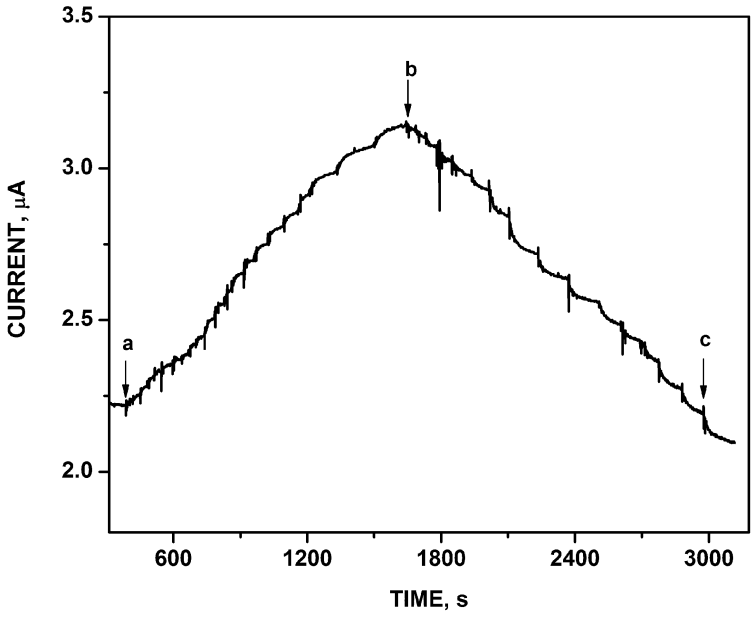
Figure 5 shows the typical amperometric response of the biosensor to successive injections of H_2O_2 into PBS (optimum pH of 6.10). The steady-state responses of the biosensor to H_2O_2 were studied in order to establish the linear range of the biosensor to H_2O_2 (data not shown). The biosensor exhibited a linear range from 0.01 to 0.06 mM. Points a–b (Figs. 5A and 5B) correspond to successive additions of 0.01–0.06 mM H_2O_2 into PBS. H_2O_2 was added in order to check the activity of HRP before addition of the herbicide. It was added dropwise to avoid inhibition of the enzyme due to excess amount of peroxide. The amount of H_2O_2 added must be within the linear range of the biosensor to avoid inhibition of the enzyme at high concentrations of H_2O_2. Catalytic characteristics of the biosensor and increase in reduction current were observed for successive injections of H_2O_2 into the electrolyte solution. This is characteristic of HRP-H_2O_2 reactions and confirms the activity of HRP. The biosensor exhibited a fast response time to H_2O_2 (10 s), which may be due to the nanoparticles formed on the electrode surface.

The biosensor consisted of the enzyme attached to the surface of RDE operating at 400 rpm. RDE was used to add convection to the cell in order to increase current and sensitivity. RDE is a hydrodynamic device that uses convection to enhance the rate of mass transport to the electrode and can offer advantages over techniques that operate in stagnant solution. The addition of convection to the cell usually results in increased current and sensitivity in comparison to voltammetric measurements performed in stagnant solution. Also, the introduction of convection (usually in a manner that is predictable) helps to remove the small random contribution from natural convection, which can complicate measurements performed in stagnant solution. Finally, it is possible to vary the rate of reaction at the electrode surface by altering the convection rate in the solution, and this can be usefully exploited in mechanistic analysis and electroanalytical applications. 400 rpm was selected as the optimum rotation speed for the biosensor. The flow was laminar at this selected speed. In this arrangement, the injected analytes are brought to the surface by the gold disk electrode, which rotates in solution.

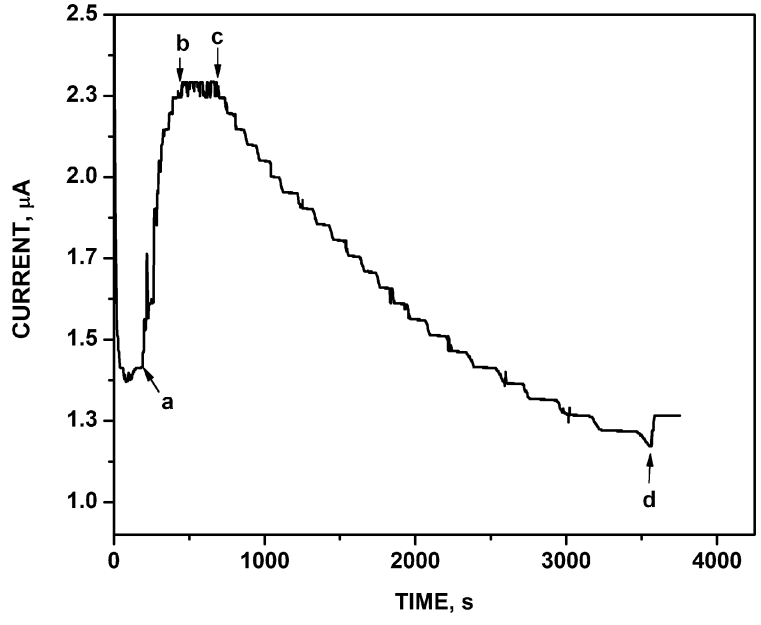
Detection of glyphosate and glufosinate

Glyphosate and glufosinate standard solutions

HRP/PDMA-PSS-based nanobiosensor has been used for the determination of glyphosate and glufosinate. Initial response of the biosensor and its decay after contact with the standard herbicide solutions were measured. 0.06 mM H_2O_2 was used to study the initial response of the biosensor before addition of glyphosate and glufosinate standard solutions. High H_2O_2 concentrations may lead to inhibition of



(A)



(B)

Fig. 5 Amperometric responses of the biosensor to successive injections of (A) H_2O_2 (a–b) and glyphosate standard solutions (b–c) (B) H_2O_2 (a–b, d), blank solutions (b–c) and glufosinate standard solutions (c–d) into PBS under RDE rotation of 400 rpm.

the enzyme and the inability to precisely monitor herbicide concentrations. Typical amperometric responses of the biosensor to successive injections of glyphosate and glufosinate standard solutions, under RDE rotation of 400 rpm, are illustrated in Fig. 5. Points b–c (Fig. 5A) correspond to additions of 2.0–78.0 μg L^{-1} glyphosate standards into PBS, while points c–d (Fig. 5B) correspond to additions of 2.0–70.0 μg L^{-1} glufosinate standards. Points b–c in Fig. 5B correspond to addition of blank solutions prepared the same way as the standard solutions. No significant change in current was observed when blank solutions were added. This shows that the decrease in current or the inhibition of HRP was entirely due to the herbicides. After addition of H_2O_2 or H_2O_2 and blank, different concentrations of glyphosate and glufosinate standards were injected successively into PBS and their steady-state currents recorded as response. It was observed that the biosensor response reduced after injection of the herbicide standards into the solution leading to a decrease in signal production. This shows that glyphosate and glufosinate reduced the activity of the enzyme, therefore reducing the response of the biosensor. The active site of HRP contains Fe, which is capable of undergoing oxidation and reduction. Since glyphosate has three reactive functional groups (amine, carboxylate, and phosphonate) that can coordinate strongly to transition-metal ions and has high affinity for Fe, it could have bound strongly to the Fe in the active site of HRP blocking the active sites hence a decrease in biosensor response. The rapid reduction in HRP activity following the glyphosate addition (Fig. 5A) suggests that glyphosate or its degradation products may form insoluble stable Fe-complexes or insoluble precipitates that are not useful. Glyphosate has been reported to posses a high affinity and chelating capacity for Fe and other metals, resulting in the formation of poorly soluble glyphosate-metal complexes or insoluble precipitates [30,31]. The report suggests that glyphosate or its degradation product(s) can diminish the availability of Fe(III), the active form of HRP, by forming insoluble complexes. Alternatively, glyphosate may inhibit HRP activity directly through an unknown mechanism. Since HRP was electrostatically attached to the RDE surface, no visible complexes were observed in the electrolyte solution. Glufosinate has a reactive amine functional group similar to glyphosate and may also have a high affinity for Fe in the active site of HRP. It was observed that glufosinate reacted in a similar way to glyphosate and inhibited the activity of HRP.

Glyphosate depressed the HRP activity by 30 % within 22 min after glyphosate addition to the PBS. Glufosinate depressed the HRP activity by 46 % within 50 min. Inhibition of HRP increased as the concentrations of the herbicides were increased. After contact of the biosensor with glyphosate for 22 min, the enzyme electrode was removed from the solution, rinsed with PBS, and used for other sets of measurements. It was observed that the biosensor recovered its activity after rinsing with PBS, this was investigated with H_2O_2. When H_2O_2 was injected into fresh PBS under RDE rotation of 400 rpm, an increase in reduction current was again observed. After addition of H_2O_2 to check the activity of the enzyme, the same procedure for addition of glyphosate was repeated as before and measurements were taken. It was observed that the same biosensor could be used for over 60 measurements. After contact of the biosensor with glufosinate for 50 min, H_2O_2 was immediately injected into the solution, and suddenly an increase in current was observed. This is illustrated at point d in Fig. 5B. This shows that not all of the active sites of HRP had been blocked by glufosinate even after contact for 50 min. The sensor to sensor reproducibility for glyphosate and glufosinate was very good with relative standard deviation (RSD) values less than 10 % (triplicate measurements).

The results from this study indicate that both glyphosate and glufosinate can be classified as reversible inhibitors. This is because the biosensor was reactivated by transferring from the inhibitor solution and rinsing with PBS, and one biosensor could be used for several measurements. Reversible inhibitors bind to enzymes with noncovalent interactions such as hydrogen bonds, hydrophobic interactions, and ionic bonds. Multiple weak bonds between the inhibitor and the active site combine to produce strong and specific binding. Reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis. This explains why no insoluble precipitates were observed in the electrolyte solutions after contact of the biosensor with the herbicides. The strong and specific binding of the protein-bound Fe with glyphosate explains the high

sensitivity of such Fe-containing enzyme to glyphosate. Glufosinate also bound strongly to the Fe in the protein, reducing its activity. Glufosinate demonstrated a particular kind of reversible inhibition known as noncompetitive inhibition. This is a form of inhibition where the binding of the inhibitor to the enzyme reduces its activity but does not affect the binding of substrate. As a result, the extent of inhibition depends only on the concentration of the inhibitor. The development of this method is a step forward in the analysis of glyphosate without formation of insoluble complexes that would cause interference at sub-μg L^{-1} analysis.

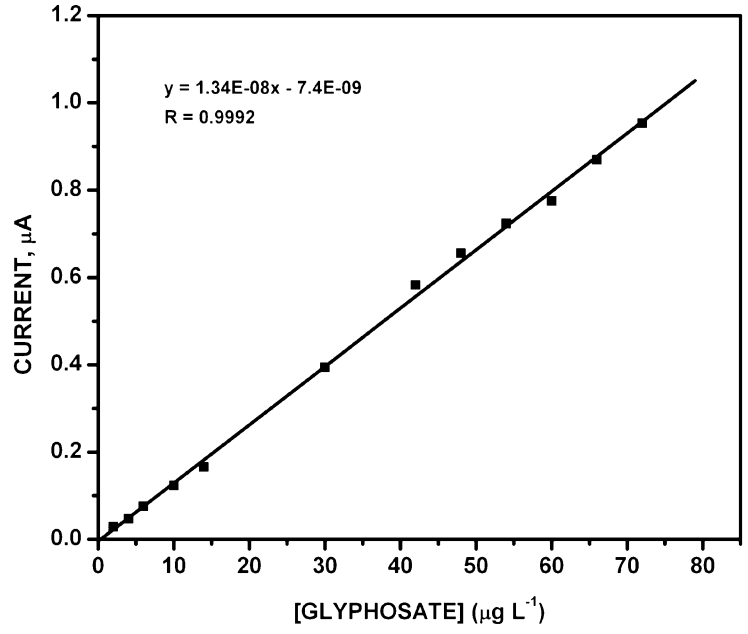
The reduced response of the biosensor due to inhibition of the enzyme was then related to the concentration of glyphosate and glufosinate in the test solution. Calibration curves were constructed by injection of glyphosate standard solutions with concentrations ranging from 2.0 to 78.0 μg L^{-1} and glufosinate standard solutions with concentrations ranging from 2.0 to 10.0 μg L^{-1}. Figure 6 shows the calibration curves obtained for glyphosate and glufosinate by amperometric detection under optimized RDE conditions. The calibration curve for glyphosate has been found to be linear in all the concentration ranges tested and with a correlation coefficient, $r = 0.9992$. The calibration curve for glufosinate has been found to have a narrow linear range but with an excellent correlation coefficient, $r = 0.9998$. Application of glyphosate and glufosinate as low as 2.0 μg L^{-1} appeared to inhibit HRP activity, but this effect was not significant.

Glyphosate and glufosinate in samples

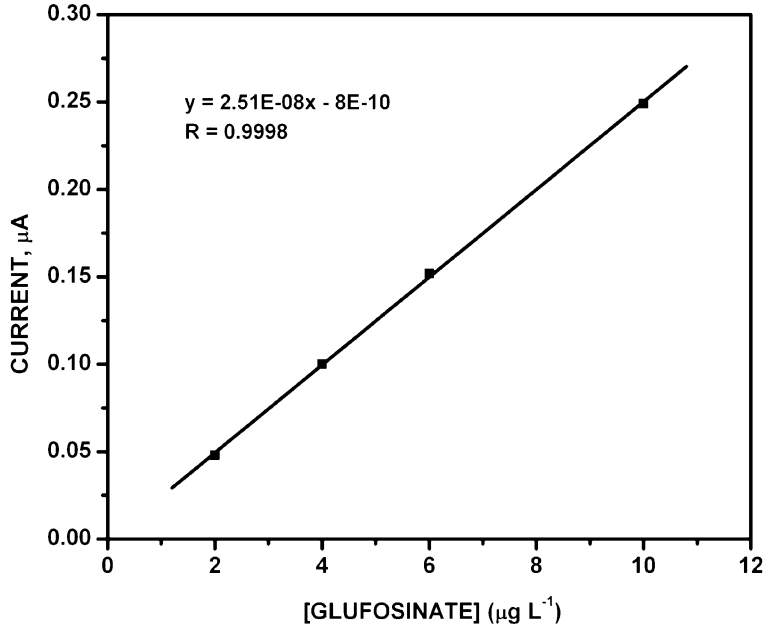
To evaluate its applicability in a complex sample, the HRP/PDMA-PSS-based nanobiosensor was applied for the determination of glyphosate and glufosinate in a spiked corn sample. It has been estimated that two-thirds of the total time required for analysis is spent in sample preparation and pretreatment steps before the final determination. Furthermore, these preparation steps are the main sources of errors in an analytical procedure [34]. Therefore, the development of a method that does not require a tedious and complex sample preparation is always very attractive. In our work, the unique procedure in sample preparation was the removal of the solid particles of the sample by centrifugation and filtration, without any kind of extraction, clean-up, derivatization, or preconcentration step.

The method of standard addition was used to determine the concentration of glyphosate and glufosinate in the samples. Standard addition is applied to most analytical techniques and is used instead of a calibration curve to solve the matrix effect problem. The matrix effect problem occurs when the sample contains many impurities. In the extraction procedure for corn, other water-soluble components of foods, like amino acids, amino sugars, etc. may also have been extracted. These compounds interfere in the determination of glyphosate and glufosinate, making the spiking of the sample necessary. If impurities present in the sample interact with the analyte to change the instrumental response or themselves produce an instrumental response, then a calibration curve based on pure analyte samples will give an incorrect determination.

One way to solve this problem is to use standard addition. The standard solutions (solution of known concentration of analyte) were added to the extracted corn sample solutions so any impurities in the extracted samples were accounted for in the calibration. The initial concentration of glyphosate and glufosinate in the samples were then determined by extrapolation. Figures 7a and 7b show the amperometric responses of the biosensor to corn samples spiked with glyphosate and glufosinate, respectively. H_2O_2 was first added followed by addition of blank solutions extracted using the same extraction procedure for the sample. Blank solutions were injected at points b–c (Fig. 7a), a–b, and a'–b' (Fig. 7b). It was observed that the addition of blank into the solution did not cause any significant change in current. This indicated that the reduction in current after injection of spiked samples was entirely due to inhibition of the enzyme by glyphosate and glufosinate present in the samples. A decrease in current was observed when corn samples spiked with 0–10.0 μg L^{-1} glyphosate were injected into the electrolyte solution at points c–d, while an increase in current was observed when H_2O_2 was injected into the solution at point d (Fig. 7a). In Fig. 7b, a decrease in current was observed when corn samples spiked with 0–40.0 μg L^{-1} glufosinate were injected at points b–c and b'–c'. The reduced response of

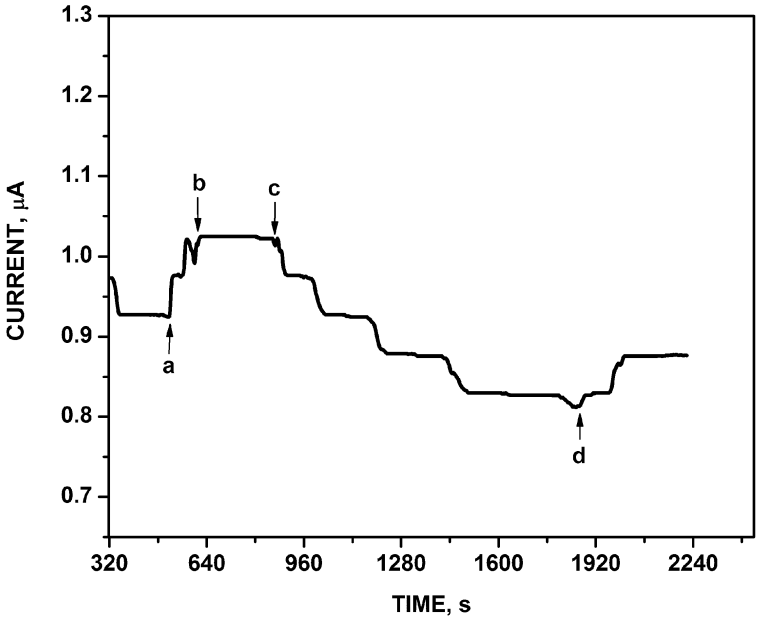


(a)

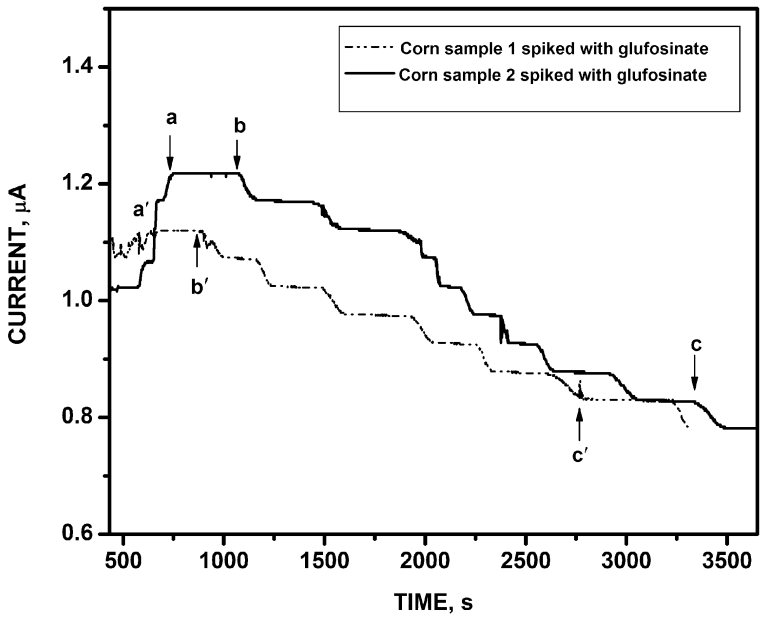


(b)

Fig. 6 Calibration curves for biosensor response to (a) glyphosate and (b) glufosinate standards.

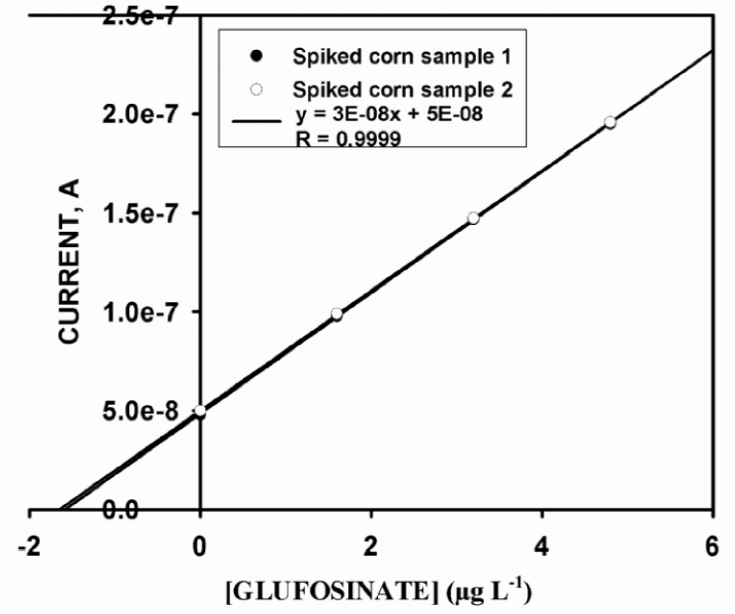


(a)



(b)

Fig. 7 (a) Amperometric response of the biosensor to successive injections of corn samples spiked with glyphosate; (b) response of the biosensor to injections of corn samples spiked with glufosinate; (c) extrapolated linear curve for glufosinate in samples. (*continues on next page*)



(c)

Fig. 7 (*Continued*).

the biosensor due to inhibition of the enzyme was related to the concentration of glyphosate and glufosinate in the test solutions and used to obtain an extrapolated plot shown in Fig. 7c. The extrapolated curve was found to be linear within a concentration range of 0–4.8 µg L^{-1} and with a perfect correlation coefficient (r = 0.9999, n = 4).

The detection limits for glyphosate and glufosinate achieved by the procedure described above were both 0.1 µg L^{-1} (three times the standard deviation of replicate analyses of the blank divided by sensitivity of the biosensor toward the analyte). The sensitivity of the biosensor is defined as the slope of the linear calibration plot. The sensor reproducibility for successive blank measurements was very good with RSD value of 1 %. The sensitivity for glyphosate and glufosinate of the proposed method was much high due to their high affinity for Fe in the protein heme. The concentration of glufosinate in the corn sample (the same sample analyzed twice) analyzed was found to be 1.70 µg L^{-1}. The concentration of glyphosate in the corn sample was found to be 1.50 µg L^{-1}. The absolute value of the x-intercept is the concentration of herbicide in the sample solutions, in this case 1.70 and 1.50 µg L^{-1}. The point at zero concentration added herbicide is the reading of the unknown in the sample; the other points are the readings after adding increasing amounts (spikes) of standard solution. In Fig. 7c, the reading of the unknown in the sample is 5.0 e^{-8} A, which corresponds to glufosinate concentration of 2.0 µg L^{-1} in Fig. 6b.

The Environmental Protection Agency (EPA) [35] has set an MRL of glyphosate in fruits and vegetables in the range of 0.2–5.0 mg kg^{-1}, in soybean at 20 mg kg^{-1}, and in drinking water at 0.7 mg L^{-1}. The FAO has set an MRL of glyphosate in the range of 0.1–5.0 mg kg^{-1} for fruits and grains and MRL of glufosinate at 0.05 mg kg^{-1} for grains [6]. It can be seen that all the MRL values of glyphosate and glufosinate in fruits, vegetable, grains, and water are higher than the detection limits obtained in this work. The nanobiosensor is, therefore, sufficient in providing high sensitivity for determination of glyphosate and glufosinate in real samples.

CONCLUSIONS

In this study, a novel HRP/PDMA-PSS-based nanobiosensor has been successfully developed and evaluated for the detection of glyphosate and glufosinate in corn sample. The limit of detection found for the nanobiosensor was 0.1 μg L^{-1}, providing, therefore, sufficient sensitivity for the determination of glyphosate and glufosinate in real samples without any preconcentration step. This method shows several advantages over other detection techniques presented in literature, including the optical methods and ICP/MS. The nanobiosensor demonstrates its fast response time, simplicity, rapidity, sensitivity, and low cost. Our research is presently directed toward analysis of more real samples.

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