Label-free disposable immunosensor for detection of atrazine

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ABSTRACT

This work reports the construction of a fast, disposable, and label-free immunosensor for the determination of atrazine. The immunosensor is based on a field effect transistor (FET) where a network of single-walled carbon nanotubes (SWCNTs) acts as the conductor channel, constituting carbon nanotubes field effect transistors (CNTFETs). Anti-atrazine antibodies were adsorbed onto the SWCNTs and subsequently the SWCNTs were protected with Tween 20 to prevent the non-specific binding of bacteria or proteins. The principle of the immunoreaction consists in the direct adsorption of atrazine specific antibodies (anti-atrazine) to SWCNTs networks. After exposed to increasing concentrations of atrazine, the CNTFETs could be used as useful label-free platforms to detect atrazine. Under the optimal conditions, a limit of detection as low as 0.001 ng mL⁻¹ was obtained, which is lower than that of other methods for the atrazine detection, and in a working range between 0.001 and 10 ng mL⁻¹. The average recoveries obtained for real water samples spiked with atrazine varied from 87.3% to 108.0%. The results show that the constructed sensors display a high sensitivity and could be useful tools for detecting pesticides like atrazine at low concentrations. They could be also applied to the

determination of atrazine in environmental aqueous samples, such as seawater and riverine water.

Keywords: Atrazine; Carbon nanotubes; Field effect transistor; Immunosensor.

1. Introduction

Due to the increasing worldwide use of pesticides, there is a perceived threat of environmental damage as well as health issues. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, ATZ) is one of the most used pesticides in agriculture, in order to optimize yields of crops such as corn, sugar cane, and sorghum [1]. It is considered a persistent environmental contaminant, occurring at trace levels in groundwater, and it has been recognized as mutagenic and teratogenic besides having effects on the reproductive, endocrine, central nervous, and immune systems [1]. According to ... Lasserre et al. [1], ATZ has effects on the expression of several proteins located in different cellular compartments (nucleus, cytosol, membrane) and it is involved in various processes such as oxidative stress, DNA damages, shape of the cell, gene expression regulation or spermatogenesis. In 2004, ATZ was banned in the European Union because of its persistent groundwater contamination [2,3].

Many analytical methods have been developed for the determination of ATZ based on high performance liquid chromatography (HPLC) coupled to diode array detection (HPLC-DAD), HPLC coupled to tandem mass spectrometry, LC coupled to mass spectrometry (LC-MS), and gas chromatography coupled also with mass spectrometry (GC-MS) [4-9]. Techniques, such as GC-MS requires extraction using organic solvents and expensive equipment. Moreover, these methods cannot be used for continuous and on-site analysis [5]. Recently, the enzyme-linked immunoassays, such as ELISA and other immunosensors to ATZ have also been reported [10-12]. The binding properties of an antibody to an antigen have been used for the development of a broad variety of analytical techniques and sensors for clinical and environmental monitoring [13-15]. Besides, other immunosensors namely electrochemical immunosensors [16-19], surface plasmon resonance (SPR) biosensors [20,21], and optical immunosensors [22] have been also constructed for ATZ detection.

The incorporation of nanomaterials in sensors for the development of a wide variety of nano-electronic systems with applications on environmental, food and clinical analysis has been reported, since such nanostructures display particular electrical, chemical and transport properties [23]. For example, SWCNTs are one-dimensional nanostructures which show high

electron transfer reactions due to the presence of all carbon atoms in their surface [24]. Thus, their large surface area together with the possibility of functionalization with different molecules allowed turning them into ideal sensors for the specific detection of various analytes and even viruses [24,25]. CNTFETs have become a suitable system for immunosensor applications because they combine the principles of molecular recognition through the recognition layer with the transduction capabilities of the carbon nanotubes and they have been used to detect various compounds such as proteins [26-28]. In this work, a simple disposable immunosensor based on CNTFETs principle was constructed for the label-free detection of ATZ with potential application in seawater and riverine water analysis.

2. Material and methods

2.1. Reagents

SWCNTs synthesized through cobalt-molybdenum catalysis (CoMoCAT, Southwest Nanotechnologies), sodium cholate (SC), Dulbecco's saline phosphate buffer (PBS, pH 7.4), Tween 20, acetone, 1-propanol, desethylatrazine, hydroxyatrazine, and ATZ (purity: 99.1%) were purchased from Sigma-Aldrich. Anti-atrazine (Anti-ATZ) was purchased from Antibodies-online (1.17 mg mL⁻¹). All chemicals and solvents were of commercially available analytical reagent grade. Anti-ATZ was dissolved with 0.01 M phosphate buffer solution (PBS, pH 7.4) and stored at 4°C. ATZ was dissolved with PBS containing 25µl of Tween 20 (PBST).

2.2 Preparation and characterisation of SWCNTs dispersion

SWCNTs (approximately 4 mg) were dispersed in 14 mL of aqueous solution of SC (0.2 % w/v). Based on the work of Justino et al. [29], where the experimental conditions for the dispersion of SWCNTs were optimized through the application of an experimental design, the SWCNT dispersion in this work was obtained after 60 minutes of sonication (Branson 2510) and then 7 minutes of centrifugation (Benchtop centrifuges K2015) at a relative centrifugal force of 2000×g at 20°C. UV-Visible spectra (Shimadzu UV-2101PC) were obtained were obtained between 400 and 800 nm.

2.3. FET fabrication

The final FET architecture is shown in Fig. 1.

< Fig. 1 >

The FET were fabricated in three inch silicon wafer (in black, Fig. 1a) and in the final configuration, they were based on ten interdigitated electrodes of 1.5 μm of width and 1000 μm of length (in blue, Fig. 1b). For that, various steps were followed: a) passivation of wafer with SiO_2 (400 nm-thick layer, in grey, Fig. 1a) by plasma enhanced chemical vapour deposition (Electrotech Delta CVD System); b) deposition of Ti (10 nm-thick, in red, Fig. 1a) and Au (100 nm-thick, in blue, Fig. 1a) on Si/ SiO_2 substrate through physical vapour deposition by sputtering (Alcatel Magnetron Sputtering System); c) definition of source and drain metal electrodes through optical lithography (Direct Write Laser 2.0, DWL, Heidelberg Instrument), using a 1.5 μm -thick photoresist layer (PFR 7790G-27cP, JSR Corporation) coated in a standard spin coating system; d) upon development, photoresist layer defines the mask for the ion beam etching (Nordiko 3000 Series) of the Au film; e) removal of the photoresist film through immersion of the wafer in a stripper solution (Microstrip® 2001, Fujifilm) at 65°C; f) coating of a photoresist film (1.5 μm) on the wafer and definition of a second mask (non-inverted) by optical lithography (DWL system) in order to define the back gate electrode (at green, Fig. 1b), with definition of an opening in the resist (as shown in Fig. 1a); g) removal of 500 nm of SiO_2 by reactive ion etching (LAM Rainbow 440 System); h) deposition of Cr/Au (50 nm-thick/100 nm-thick) films by ion beam deposition (Nordiko 3000 Series) immediately after ion beam etching (without vacuum break) - the photoresist and the Cr/Au films were removed by liftoff, immersing the wafers in a microstrip solution (Microstrip® 2001, Fujifilm) at 65°C. After such processing steps, the wafers were cleaned (isopropanol and distilled water), dried with N_2 , and the silicon wafer was diced (DISCO DAD 321) in order to promote individualized FET ($\sim 3 \times 2 \text{ mm}^2$). Each FET was then mounted into a printed circuit board, fixed and wirebonded with Al wires (25 μm Ø), which were protected with a silicone gel (Elastosil E41, Wacker) also to produce an open chamber ($\sim 1 \text{ mm}$ Ø) for further sensing experiments. Electrical measurements were made using a 4155 C Agilent semiconductor parameter analyzer, which was linked to a closed test fixture (Agilent 16442A) where the devices were positioned; in the test fixture, the drain, gate, and source of each FET were connected to respective terminals to provide electrical circuit for sensing measurements.

2.4. Immunosensor surface functionalization

Immunosensor surface functionalization was performed in two steps. Firstly, the FET surface was washed with acetone, 1-propanol, distilled water and dried under N_2 and then, 2 μl of

SWCNTs dispersion was deposited on the clean FET surface for 15 minutes at room temperature, in order to obtain the CNTFETs. Anti-ATZ, which is the recognition molecule, was immobilized over the surface of the CNTFETs by dropping 2 μL of an anti-ATZ solution in PBS (10 mg L^{-1}) onto the surface of CNTFETs. After adsorption of the anti-ATZ, the CNTFETs were dried with N_2 and electrically characterized the I_D vs. V_D , between 0 and +2 V.

2.5. Detection of atrazine

In order to detect the atrazine, 2 μL of ATZ solution at different concentrations from 0.001 to 10 ng mL^{-1} (5 decimal concentrations) were dropped into five individual CNTFETs per concentration with two replicates (total of 10 CNTFETs), and incubated 15 minutes at room temperature. CNTFETs were washed with distilled water, dried with N_2 , and their electrical characterization was immediately processed. The reproducibility was evaluated performing all measurements in five individual FET devices. The analytical response (ΔI_{ATZ}) was calculated by the difference between the I_D obtained after ATZ measurement (I_{ATZ}) and the I_D obtained after the anti-ATZ measurement ($I_{\text{antibodies}}$).

3. Results and discussion

3.1. Characterization of SWCNTs

The UV-Visible spectroscopy was used for the characterization of the dispersions of SWCNTs as evaluation of their stability, which will further constitute the conducting channel of CNTFETs. Fig. 2 shows the UV-Visible spectrum obtained from the SWCNTs dispersed in SC and used in this work.

< Fig. 2 >

From the Fig. 2, the characteristic electronic transitions of SWCNTs are shown: 400-600 nm assigned to the metallic SWCNTs (main features: 455, 510, and 570 nm) and 600-800 nm assigned to the semiconducting SWCNTs (main features: 655 and 735 nm) [30,31].

3.2. Performance of CNTFETs for the detection of atrazine

After the deposition of specific antibodies for ATZ in CNTFET surface, a decrease of I_D values from 193 ± 3.4 nA (for CNTFETs only with SWCNTs) to 123.4 ± 6.8 nA was observed at

$V_D = +1$ V. Such 36% decrease can be associated with the direct charge transfer which occurred when charged analytes are immobilized at the nanotubes surface, affecting their conductance, as reported by Grüner [26]. The changes in I_D values in each CNTFET after the exposure of any solution to the CNT surface can also be explained by the variation of the flow of electrons through the nanotubes between the source and the drain of each NTFET, as reported by Kamahori et al. [32].

The performance of the proposed immunosensor was evaluated by determining ATZ standard solutions at different concentrations (0.001, 0.01, 0.1, 1.0, 10 ng mL⁻¹) at $V_G = +1$ V and $V_D = +1$ V, for five individual CNTFETs for each concentrations (and two replicates). The variation of the analytical response (ΔI_{ATZ}) and the calibration curve obtained with CNTFETs for ATZ are shown in Fig. 3. Each point represents an average value based on ten measurements, corresponding to two replicates in five individual CNTFETs for each concentration and the error bars represent the variation calculated from the standard deviation.

< Fig. 3 >

As shown in Fig. 3, the current change of the immunosensor decreases with increasing ATZ concentrations from 0.001 to 10 ng mL⁻¹. This can be attributed to the formation of an immunocomplex resulting from the incubation of the immunosensor with ATZ solutions. Such analytical response, the lower variation of current change in the CNTFETs with the addition of increasing concentrations of ATZ, could also be explained by the charge tunnelling with the receptor analyte (anti-ATZ), which reduces the mobility of holes (major carriers) [33], thus leading to a decrease of the current in the devices.

The coefficients of variation calculated from the standard deviation (for the 5 CNTFET per concentration) are 1.90, 1.48, 2.01, 1.75, 2.16% between 10⁻³ and 10¹ ng mL⁻¹, with an average CV of $1.86 \pm 0.26\%$, which shows a low variability of the analytical response (Fig. 3). The ANOVA applied to the experimental data showed that there is no statistically significant difference ($p=0.184$) between various CNTFETs for the same concentration of ATZ. However, there is a statistically significant difference ($p<0.001$) between the different levels of concentrations, which means that CNTFETs respond differently to each ATZ concentration.

Based on the analytical response of CNTFETs to atrazine as shown in the inset of Fig. 3, the linear relationship of atrazine from the log-log calibration curve can be expressed by the following regression equation ($p<0.001$): $\log y = 1.007(\pm 0.0133) - 0.233(\pm 0.00768)\log x$, with a

R of 0.975 (an adjusted R^2 of 0.949 and a fit standard error of 0.077), where y is the current change and x the concentration of ATZ.

Based on the Fig. 3, it is shown that CNTFETs are able to detect concentrations of atrazine at the nanomolar range well below the legal limits, since ATZ limit for drinking water and food samples is 0.1 ng mL^{-1} [18]. The limit of detection found for this sensor is 0.001 ng mL^{-1} , which is lower than that of other methods for the ATZ detection, as shown in Table 1.

< Table 1 >

The CNTFETs were applied to the detection of ATZ in 5 surface seawater samples from Costa Nova (Aveiro, Portugal) and in 5 surface riverine water samples from Rio Novo do Príncipe (Aveiro, Portugal). For all the samples, the I_D obtained after atrazine measurement (I_{ATZ}) was lower than the I_D obtained after the anti-atrazine measurement ($I_{antibodies}$), which means that the analytical signal (ΔI_{ATZ}) is negative. Thus, the NTFETs are not able to detect atrazine in real water samples. Therefore, CNTFETs were also used to the determination of ATZ in spiked water samples (deionised water), spiked seawater samples, and spiked riverine water samples by standard addition methodology with ATZ standard solutions at 0.01 and 1.0 ng mL^{-1} (with 5 individual CNTFETs for each concentrations).

Based on the analytical response of CNTFETs to atrazine (Fig. 3), the concentration of ATZ found in spiked samples of deionised water and real water was calculated, as shown in Table 2.

< Table 2 >

The average recovery was in the range of 87.3-108.0%, which corresponds to a good accuracy of the CNTFETs based sensor. In addition, the recovery showed for the ATZ concentrations of 0.01 ng mL^{-1} is better for all the samples than that obtained for the concentration of 1.0 ng mL^{-1} , which demonstrates the ability of the CNTFETs to detect ATZ at low levels. Thus, CNTFETs fabricated in this work can be used to detect ATZ in water samples as a simple, label-free, and disposable methodology.

The cross-reactivity of the ATZ to two atrazine degradation products was also assessed by evaluating the CNTFETs results for water spiked with 3 ng mL^{-1} hydroxyatrazine or 3 ng mL^{-1} desethylatrazine, and with no ATZ. Atrazine was detected at an average concentration, for 3

individual CNTFETs, of 0.0010 ng mL⁻¹ in the water sample containing only hydroxyatrazine and at an average concentration of 0.0013 ng mL⁻¹ in the water sample containing only desethylatrazine, which suggest a higher cross-reactivity for this compound.

4. Conclusion

A fast disposable immunosensor for the quantification of atrazine has been developed and demonstrated to work under field conditions. This immunosensor is able to detect ATZ in a range between 0.001 and 10 ng mL⁻¹ with a limit of detection of 0.001 ng mL⁻¹, which is lower than some other detection methods for ATZ reported so far in the literature. In addition, the short analysis time of CNTFETs (15 minutes) and the low variability of the analytical response (CV of 1.86 ± 0.26%, when tested 5 CNTFETs with two replicates for each concentration of atrazine measured) are advantages of immunosensors developed in this work. It was also shown that CNTFETs are able to detect concentrations of atrazine below and above the legal limits (0.1 ng mL⁻¹). Thus, CNTFETs can be used to detect atrazine in water samples as a simple, label-free, and disposable methodology.

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Figure Captions

Fig. 1. Schematic a) cross view and b) top view with final architecture of fabricated FET (at blue: Au layer; at red: Ti layer; green: Cr/Au layer - gate electrode; at grey: SiO₂ layer; at black: Si wafer).

Fig. 2. UV-Visible spectrum of SWCNTs dispersion in sodium cholate.

Fig. 3. Variation of the analytical response obtained at different concentrations of ATZ from 0.001 to 10 ng mL⁻¹ at V_G = +1 V and V_D = +1 V, and from five individual CNTFETs; the error bars represent the standard deviation. The inset graph shows the log-log regression analysis for the mean response of CNTFETs to ATZ.

Table 1. Comparison with other reported methods for the detection of atrazine.

Method	Working range (ng mL ⁻¹)	Limit of detection (ng mL ⁻¹)	Reference
ELISA	0.0005-0.1	0.03	[12]
Optical immunosensor	0.005-100	0.155	[22]
SPR biosensor	0.001-1000	0.07	[21]
Electrochemical sensor	0.001-1000	0.04	[16]
Electrochemical immunosensor	0.05-0.5	0.016	[34]
Electrochemical immunosensor	0.001-10	0.001	This work

Table 2. Recovery of the CNTFETs in deionised water and real water samples.

Sample	Background concentration (ng mL ⁻¹)	Spiked concentration (ng mL ⁻¹)	Found concentration (ng/mL, mean and standard deviation for n=5)	Mean recovery (%)
Deionised water	ND	0.01	0.0102 ± 2.55x10⁻³	102.3
	ND	1.0	0.873 ± 0.189	87.3
Riverine water	ND	0.01	0.0108 ± 1.42x10⁻³	108.0
	ND	1.0	0.948 ± 0.174	94.8
Seawater	ND	0.01	0.00976 ± 1.31x10⁻³	97.6
	ND	1.0	0.910 ± 0.186	91.0

ND – Not Detected

Figure 1

Figure 2

Figure 3