Simultaneous determination of Paraquat and Atrazine in water samples with a White Light Reflectance Spectroscopy biosensor

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Abstract An optical immunosensor based on White Light Reflectance Spectroscopy for the simultaneous determination of the herbicides atrazine and paraquat in drinking water samples is demonstrated. The biosensor allows for the label-free real-time monitoring of biomolecular interaction taking place onto a SiO_2/Si chip by monitoring the shift in the reflected interference spectrum during the reaction. Dual-analyte determination is accomplished by functionalizing spatially distinct areas of the chip with protein conjugates of the two herbicides and scanning the surface with an optical reflection probe. A competitive immunoassay format was adopted, followed by reaction with secondary antibodies for signal enhancement. The sensor was highly sensitive with detection limits of 50 and 100 pg/ml for paraquat and atrazine, respectively. The assay duration was 12 min. Recovery values ranging from 88 to 112% were determined for the two pesticides in spiked drinking water samples. In addition, the sensor could be regenerated and re-used at least 12 times without significant effect on the assay characteristics. Its excellent analytical performance and short analysis time combined with the small sensor size should be helpful for fast on-site determinations of these analytes.

Keywords: paraquat, atrazine, white light reflectance spectrometry, label-free biosensor, drinking water

1. Introduction

Nowadays, the necessity for point-of-need/care (PON/POC) devices which combine excellent analytical performance and portability for in-situ applications is becoming more and more evident (Petryayeva and Algar, 2015). To this direction the development of several types of biosensors has been exploited as an alternative to the conventional analytical techniques (Saidur *et al*., 2017; Gaudin, 2017). Biosensors applications extend to a wide range, with the determination of pesticides in water, food or feed to present an immensely expanding field (Liu *et al*., 2013; Verma and Bhardwaj, 2015). Broad spectrum synthetic pesticides have been extensively used worldwide in agricultural practice due to their beneficial effects in crop protection and agricultural economy. Nevertheless, the irreversible effects in human health and the ecosystems due to overexposure and bioaccumulation of pesticides is a reality that humanity has to manage (Kim *et al*., 2017). In order to harmonize the necessity for pesticide usage and the obligation to protect human health and the environment, the European Union has set the frame for the pesticides management (Directive 2009/128/EC), including the maximum allowable limits of various pesticides in unprocessed and processed foods (EC regulation 396/2005) and a list of substances for which manufacturing, use and distribution is banned within the European Union borders (Directive 2000/60/EC; Directive 2006/118/EC).

Paraquat and atrazine (Figure 1a) are two examples of herbicides which have been banned within EU since 2007 and 2004, respectively (Judgment of the Court of First Instance Case T-229/04, 2007; Commission decision of 10 March 2004, Official Journal of the European Union, 2004). Paraquat is highly toxic for humans and animals (Li *et al*., 2004) and recent studies have associated exposure to this substance with Parkinson's disease (Moretto and Colosio, 2011). Several methods have been reported in the literature for the determination of paraquat in water, food and environmental samples or even biological fluids such as serum. These methods include chromatographic techniques coupled to mass spectrometry or ultraviolent spectroscopy (Sun and Chen, 2015; Zou *et al*., 2015), electrochemical sensors based on square wave voltammetry or anodic stripping voltammetry (Valera *et al*., 2014; de Figueiredo-Filho *et al*., 2017), as well as immunoassays (Garcia-Febrero *et al*., 2014). Atrazine, though not as toxic as paraquat, it has been banned within European Union due to its ability to remain for long periods in soil and groundwater (Jablonowski *et al*., 2009). Despite the fact that studies suggest atrazine to be a possible endocrine disruptor to animals and humans (Cragin *et al*., 2011), it remains one the most commonly used herbicides in many countries outside EU. A large

number of methods has been reported in literature regarding the detection of atrazine, including classical chromatographic gas or liquid separation technics combined with a variety of detectors (Zarejousheghani *et al*., 2014; Nascimento Andrade *et al*., 2016), immunoassays (Sai *et al*., 2016; Barchanska *et al*., 2012), and sensors (Tortolini *et al*., 2016; Belkhamssa *et al*., 2016). The latter present the only viable solution towards the development of point-of-need devices due to increased potential for miniaturization as compared to other instrumental methods of analysis. Among the various types of sensors, optical label-free biosensors offer unique advantages over those employing labels due to the absence of labelling which can alter the binding affinity, increase the analysis cost, and complicate the assay procedure moving away the potential for point-of-need determinations.

a b

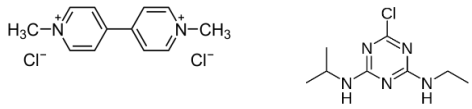

Figure 1: Chemical structures of paraquat (a) and atrazine (b).

In this work, a label-free sensors based on White Light Reflectance Spectroscopy (WLRS) is employed for the fast simultaneous determination of the two herbicides, paraquat and atrazine, in water samples. This sensing approach has been already used for the immunochemical determination of high molecular weight analytes (Koukouvinos *et al*., 2015; Koukouvinos *et al*., 2016) as well as for pesticides in wine samples (Koukouvinos *et al*., 2017). The particular sensor allows for the label-free and real-time monitoring of biomolecular interaction by following the shift in the interference spectrum reflected from SiO_2/Si chip on the top surface of which the reaction takes place (Figure 2a and b). Dual-analyte determination is accomplished by functionalizing spatially distinct areas of the chip with protein conjugates of the two herbicides and scanning the surface with an optical reflection probe. A competitive immunoassay format was followed, according to which mixtures of calibrators or samples with antibodies specific against the two herbicides were run over the surface, followed by reaction with secondary antibodies for signal enhancement.

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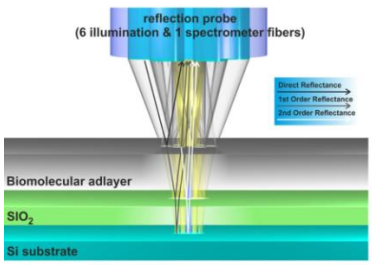

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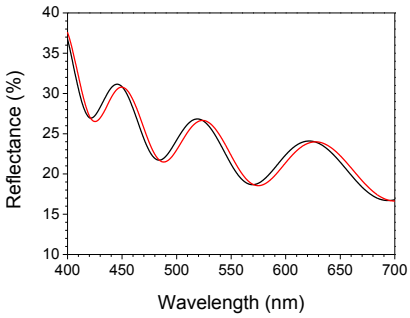

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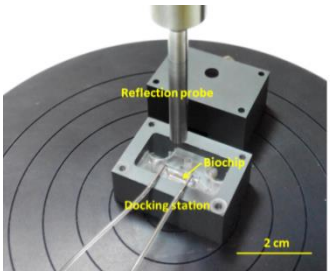

Figure 2: (a) Optical transduction principle of the WLRS biosensor. (b) Interference spectrum shift due to the biomolecular reaction. The black line corresponds to initial spectrum and the red to that obtained after the reaction. (c) Photograph of the biochip assembled with the docking station and placed under the reflection probe.

2. Materials and Methods

a. Reagents

Four-inch Si wafers (<100>) were purchased from Si-Mat Germany (Kaufering, Germany). After cleaning, a 1000-nm thick silicon dioxide layer was grown on the wafers by thermal oxidation at 1100°C in the clean room facility of the Nanotechnology & MEMS Laboratory of the Institute of Nanoscience and Nanotechnology of NCSR "Demokritos". Bovine serum albumin (BSA), (3-aminopropy)-triethoxysilane (APTES), Paraquat dichloride hydrate (PQ) pestanal® and Atrazine (ATR) pestanal® were purchased from Sigma-Aldrich (St. Louis, MO). Anti-atrazine rabbit polyclonal antibody and atrazine-BSA conjugate were purchased from United Immunoassay (San Bruno, California), protein G purified sheep polyclonal anti-paraquat antibody was purchased from Abcam (Cambridge, UK) and paraquat-BSA conjugate was prepared in-house following a published method (Garcia-Febrero *et al*., 2014). Rabbit anti-sheep and goat anti-rabbit antibodies were purchased from Thermo-Fisher Scientific Inc. (Paisley, UK) and Merck-Millipore (Billerica Massachusetts, USA), respectively. The water used throughout was doubly distilled.

2.2. Calibrator and water samples preparation

A stock solution with concentration of 1 mg/mL was prepared for each pesticide (PQ, ATR) in methanol and stored at -20 ^{o}C. From this solution, calibrators with concentrations ranging from 0.05 to 5.0 ng/mL were prepared for each pesticide in 0.05 M phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.2% (w/v) BSA (assay buffer). Water samples fortified with the two

pesticides were prepared in commercially available bottled natural table water.

2.3. Preparation of the biochip

The immobilization of biomolecules onto silicon dioxide chips requires activation of the surface with a silane layer. Prior to activation, the chips were cleaned/hydrophilized by O_2 plasma treatment for 30 s at 10 mTorr. Then, they were immersed in a 2% (v/v) aqueous APTES solution for 20 min, washed with distilled water, dried under a nitrogen stream, cured at 120 ^oC for 20 min and kept at room temperature in a desiccator for at least 24 h before usage. The pesticide-BSA conjugates were then deposited as two spatially distinct bands on the APTES-modified chips by means of spotting from solutions prepared in 100 mM carbonate buffer, pH 9.2, using the Bio-Odyssey Calligrapher microarray spotter (Bio-Rad Laboratories, Hercules, CA). After spotting, the chips were incubated in a controlled humidity chamber (75% humidity) overnight at 4 ^oC. Then, they were washed with 10 mM phosphate buffer, pH 7.4, containing 9 g/L NaCl (washing solution), and immersed in blocking solution (10 mg/mL BSA in 0.1 M $NaHCO_3$, pH 8.5) for 1 h at room temperature, rinsed again with washing solution and distilled water, dried under a nitrogen stream and used for the assay.

2.4. Dual-analyte assay

The detection system employed in the present study consists of: a) a visible/near infra-red light source and a miniaturized spectrophotometer, b) a reflection probe, placed over the chip, which delivers the incident light to the surface and collects the reflected light, and c) a reaction cell assembled by an open compartment laser-cut on double-sided adhesive and a top cover with embedded fluid inlet and outlet tubing (Figure 2c). In order to perform the assay the biofunctionalized chips (biochips) were assembled with the fluidic compartment and loaded in the docking station. The docking station was mounted on a commercial computer controlled stage that was operated through the same software performing the spectra acquisition and adlayer thickness calculation. A precision micro syringe pump (Cole-Palmer, Vernon Hills, IL) provided the continuous delivery of solutions in the fluidic compartment, while the biochip surface was scanned with a step of 0.5 mm, with respect to the reflection probe, giving well discriminated responses from each pesticide conjugate band. Firstly, assay buffer was run over the biochip to acquire a baseline and then mixtures of calibrators or spiked water samples with a solution containing the two analyte-specific antibodies (2 μg/ml of rabbit polyclonal anti-atrazine antibody and 1/3000 diluted sheep anti-paraquat antibody in assay buffer) at a 1:1 volume ratio were passed over the biochip for 7 min with a flow rate of 50 μL/min (total sample volume 350 μL). After that, a solution containing 5 μg/mL of anti-sheep IgG antibody and 5 μg/mL of anti-rabbit IgG antibody in assay buffer were run for 5 min at the same flow rate. Finally, the biochip was regenerated by passing a 0.5% (w/v) SDS solution, pH 1.9, for 2 min, followed by equilibration with assay buffer. The calibration curves were created by plotting the effective biomolecular layer thickness (signal) obtained for the different calibrators (S_x) as percentage of the zero calibrator signal (maximum signal; S_0) against the respective analyte concentration in the calibrator solutions in linear/log scale.

3. Results and discussion

a. Assay optimization

The optimization of the assay for the two pesticides was performed separately using biochips with one immunoreactive band. The two most important parameters for a competitive immunoassay are the concentration of analyte-protein conjugate used for coating and the concentration of analyte-specific antibody; since they define the absolute signal but also the assay sensitivity. For paraquat, maximum signal values were obtained when a conjugate concentrations equal to 50 μg/mL was used for coating in combination with a 1500-times dilution of anti-paraquat antibody. Nevertheless, based on the assay sensitivity (% inhibition caused by pesticide calibrators), a 1/3000 anti-paraquat antibody dilution was finally selected. Similar optimization experiments for atrazine showed that satisfactory zero calibrator signal and assay sensitivity was obtained using a 10 μg/mL BSA-atrazine solution for coating in combination with a 2 μg/mL anti-atrazine antibody solution.

Another parameter that was optimized was the duration of the two immunoreaction steps i.e., the reaction of the pesticide-specific antibody with the immobilized conjugate and the reaction of the immunoadsorbed onto the biochip primary antibodies with the secondary antibody. The total assay time finally adopted included 7-min duration for the primary immunoreaction and 5-min for the secondary since more than 80% of the maximum plateau signal values were obtained in this period for both analytes.

Another important parameter that was optimized was the composition of the regeneration solution. The solutions tested were: a commercially available IgG elution buffer for immunoaffinity columns, a 0.1 M glycine-HCl buffer, pH 2.5, a 50 mM NaOH, a 100 mM HCl, and a 0.5% (w/v) SDS, pH 1.9. To select the optimum one, the amount of analyte-specific antibody remaining on the surface after regeneration was determined through incubation with the respective secondary antibody. Thus, 0.5% (w/v) SDS, pH 1.9, was selected as regeneration solution since it was found to be the one that removed more efficiently the bound antibodies (less than 5% of bound antibody molecules remain). Then, the stability of the biochip towards regeneration was assessed through repetitive assay/regeneration cycles. It was found that at least 12 assay/regeneration cycles could be performed without affecting the immobilized biomolecules (all 12 values were within the mean value ± SD limits). The ability to regenerate the biochip is a significant asset since it could reduce considerably the analysis cost.

3.2. Analytical characteristics of the dual-analyte assays

Typical calibration curves obtained with the dual-analyte sensor for paraquat and atrazine are provided in Figure 3.

The assays limit of detection (LOD) was calculated as the concentration corresponding to mean value -3 standard deviations (SD) of 8 replicate measurements of zero calibrator and was 50 and 100 pg/mL, for paraquat and atrazine, respectively. The limit of quantification (LOQ) was determined as the concentration corresponding to mean value of zero calibrator -6SDs (n=4), and was 100 and 200 pg/mL for paraquat and atrazine, while the linear response dynamic range reached up to 2.5 and 5.0 ng/mL, respectively.

The assay repeatability was determined by analyzing three control samples prepared in bottled water and spiked with the pesticides at three different concentration levels. The intra-assay coefficient of variation (CV) was determined by running 3 replicates of each control in the same day while the inter-assay CV was determined by duplicate measurements performed in 10 consecutive days and their values for the three controls were less than 10% and 12%, respectively, for both assays.

The assays accuracy was also evaluated through recovery experiments. For this purpose, three bottled water samples were spiked with 3 different concentrations of each one of the pesticides (0.2, 0.4 and 1.2 ng/mL). All samples were analyzed in triplicate both prior to and after the addition of pesticides and the recovery values were calculated (% recovery = [amount determined/amount added] × 100). The recovery values determined were ranged between 88.0 and 112% for both analytes, indicating the good accuracy of the immunosensor developed.

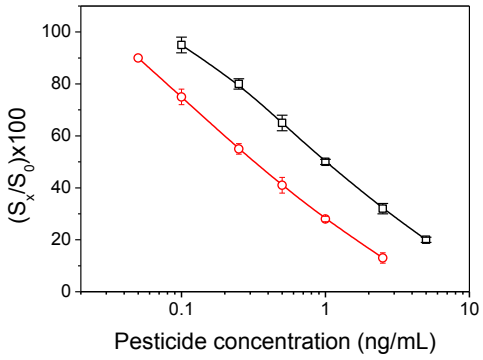

Figure 3: Typical calibration curves for paraquat (red curve) and atrazine (black curve) obtained with the dual-analyte WLRS sensor. Each point is the mean value of tree replicates ± SD.

4. Conclusions

A sensor for the simultaneous determination of paraquat and atrazine in drinking water samples was developed. The sensor was based on WLRS which allowed for the label-free and real-time monitoring of immunochemical interactions. The assays developed were highly sensitive, rapid and accurate. Thus, taking into account the excellent analytical performance and short analysis time along with the small sensor size it seems that it can be evolved in a portable device for determinations of pesticides at the point-of-need.

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