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Graphene immunosensors for okadaic acid detection in seawater

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ABSTRACT

In this work, we report the fabrication of immunosensors based on field effect transistors with graphene (Gr-FET) for the detection of okadaic acid (OA) in real seawater samples. OA is a marine toxin, and specifically a diarrheic shellfish poison produced by the dinoflagellate species *Dinophysis* and *Prorocentrum*. The analytical results obtained with fabricated Gr-FET were compared with those obtained with a traditional methodology (enzyme-linked immunosorbent assay, ELISA) in order to validate the immunosensors for OA detection. Standard solutions of OA with concentrations between 0.05 and 300 ng mL^{-1} were tested to construct the calibration curve and four spiked real seawater samples were used to validate the immunosensors. With the obtained good reproducibility (0.54–2.19%) and repeatability (0.05–2.06%) as well as low limit of detection (0.05 ng mL^{-1}) and adequate recovery range (98.2–100.7%), the proposed immunosensors are useful for the detection of OA in real seawater samples and could be advantageously extended for detection of other toxins or agents of marine environmental pollution.

1. Introduction

Seawater contamination by small molecule pollutants is nowadays a major issue worldwide. Particularly, the marine harmful algal blooms have been released toxins, which can accumulate in shellfish, echinoderms, tunicates, and gastropods and their ingestion can lead to toxic symptoms in humans and mortality of marine organisms [1,2].

OA is a low-molecular-weight marine toxin produced by dinoflagellates, commonly microalgae species such as *Dinophysis* and *Prorocentrum* and it is responsible for the diarrheic shellfish poisoning [3]. OA is ingested through filter feeding by various species of shellfish such as bivalves (e.g., mussels, scallops, oysters, and clams) and the consumption of contaminated shellfish by humans induces diarrheic shellfish poisoning (DSP). This syndrome is responsible for gastrointestinal disturbances, such as diarrhea, vomiting, and abdominal pain.

The mechanism of action of OA is based on the reversible inhibition of protein phosphatase of type 1 and 2A, which are enzymes that play an important role in the protein dephosphorylation in cells. This toxin binds to the receptorial site of the enzyme, blocking its activity [3,4]. The diarrheic symptom is due to the hyperphosphorilation of intestinal epithelia, with the loss of intestinal structure, producing an important loss of water [5]. OA can also alter cell morphology, induce apoptosis and cell death, as well as modify cell physiology [5,6].

The detection of OA remains a challenging and important economic issue for shellfish industries worldwide. The European Commission on the basis of toxicity to humans set up a provisional guideline limit of 160 μg kg^{-1} of shellfish for OA [7]. Food and Drug Administration (FDA) has established action levels for natural toxins and for OA, this level is about 0.16 ppm [8]. It should be highlighted that no legislation is currently available for the testing of marine toxins in seawater samples, and the regulation is only available for shellfish [9].

Preliminary toxicity screening for OA has been traditionally performed using the mouse bioassay, the method of reference recommended in European Union since 1990, but currently it is prohibited due to ethical problems by the use of laboratory animals [7]. In addition, they have poor selectivity (no differentiation between the various components of DSP toxins) and accuracy, as well as it is time consuming and expensive [7]. Alternative analytical methods for detection of OA usually involve liquid chromatography coupled to mass spectrometry (LC-MS) and planar waveguide microarray [10,11]. Although these techniques are well-proven and widely accepted, but there is still need of sensitive and consistent methods to perform rapid monitoring of real samples. Immunoassays, based on the affinity interaction between antibodies and antigens, have also been developed for OA detection; the most commonly format is the colorimetric enzyme-linked immunosorbent assay (ELISA), where enzymes are used as labels to detect the interaction between polyclonal or monoclonal antibodies and the toxins [12].

As alternative, the continuous monitoring of environmental contaminants can be performed by marine sensors and biosensors due to their

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capability of miniaturization, small scale networks, and wireless communication [13]. In recent years, immunosensors play an important role in the detection of toxins for environment monitoring and food safety, mainly due to the highly sensitive and selective nature of the recognition between antigen and antibody [14–16]. In the latest years, a large number of sensors and biosensors based on graphene and graphene related materials (graphene oxide and reduced graphene oxide) have been reported in various areas such as clinical, environmental, and food sciences research [17]. In addition, the use of graphene as transduction element, mainly in electrochemical sensors and biosensors, is due to its fast electron mobility, high current density, high mechanical strength, and large surface area [17–19].

Most of the biosensors for detection of OA are label-dependent immunosensors, in which labelled secondary antibodies are required to convert the interaction into a detectable electrochemical signal, and they were applied to the detection of OA in shellfish samples [20–24]. Label-free immunosensors, in which the affinity interaction between antigen and antibody is directly monitored without labelling of any partner, have received much attention for their sensitivity, low cost, and simplicity of operation [25–27]. Few works have reported the detection of toxins such as OA in seawater through biosensors [9], and to the best of our knowledge, the determination of toxins such as OA in seawater through sensing systems based on disposable biosensors and employing graphene has not been reported up to date. In this work, electrochemical immunosensors with graphene were applied as disposable and label-free analytical devices to detect and quantify OA in real seawater samples with improved analytical performance such as low limit of detection.

2. Experimental

2.1. Materials

Graphene nanoplatelets (#799084), sodium dodecyl sulphate (SDS), domoic acid (DA), acetone, 1 propanol, Dulbecco's phosphate buffered saline solution (PBS, pH 7.4), and 3 in. silicon wafers were purchased from Sigma-Aldrich. Antibodies specific to OA (anti-OA, #ABIN615668) and kainic acid (KA) was purchased from antibodies-online. Anti-OA was dissolved with PBS (dilution of 1:1000) and stored at 4 °C. The standard solutions of OA (0.05, 0.5, 5, 50, 100, and 300 ng mL^{-1}) were prepared in 5 mL volumetric flasks through a stock solution of OA of 100 μg mL^{-1} from Biovision (#1543) in Milli-Q water.

The ELISA was performed with a commercially available ELISA kit from Abraxis® (#PN520021), according to the protocol provided by the manufacturer (http://www.abraxiskits.com/wp-content/uploads/2014/07/AOkadaic-Acid-DSP-PL-Users-Guide-R4.pdf). The kit was stored at 4 °C but allowed to reach room temperature before use.

2.2. Microfabrication of FET and electrical characterization

The FET were microfabricated in 3 in. silicon wafer and in the final configuration, they were based on ten interdigitated electrodes of 1.5 μm of width and 1000 μm of length. For that, various steps were followed using standard microfabrication procedures:

1) passivation of wafer with SiO_2 (400 nm-thick) through plasma-enhanced chemical vapor deposition;
2) deposition of Ti (10 nm-thick) and Au (100 nm-thick) on Si/SiO_2 substrate through physical vapor deposition by sputtering;
3) definition of source and drain metal electrodes through optical lithography, using a 1.5 μm-thick photoresist layer coated in a standard spin coating system;
4) ion milling of Ti/Au films to remove undesired zones;
5) removal of 500 nm of SiO_2 by reactive ion etching after the coating of a photoresist film (1.5 μm) on the wafer and definition of a second mask (non-inverted) by optical lithography in order to define the back gate electrode with definition of an opening in the resist;
6) deposition of Cr (50 nm-thick) and Au (100 nm-thick) films by ion beam deposition immediately after ion beam etching (without vacuum break); and,
7) removal of the photoresist and the Cr/Au films by liftoff, immersing the wafer in a microstrip solution at 65 °C.

After such processing steps, the wafers were cleaned with isopropanol and distilled water, dried with N_2 and the silicon wafer was diced to promote individualized FET (~3 × 2 mm^2). Each FET was then mounted into a printed circuit board (PCB), fixed and wirebonded with Al wires (25 μm Ø), which were protected with a silicone gel also to produce an open chamber (~1 mm Ø) for further sensing experiments, as shown in Fig. 1a and b. The FET surface was washed with acetone and 1-propanol, rinsed twice with distilled water, and dried under a N_2 flow before use.

Electrical measurements were made using a semiconductor parameter analyzer (Agilent 4155C, Japan), which was linked to a closed test fixture (Agilent 16442A, Japan) 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. All electrical measurements were performed in a room under controlled temperature of 25 °C. For the acquisition of data [output characteristics, that is, drain current (I_D) as a function of applied drain voltage (V_D)], the software Desktop EasyExpert was used through an USB/GPIB interface. The electrical signal (I_D) was measured at a fixed drain voltage ($V_D = +1$ V) and against a back-gate voltage (V_G) of +1 V, after graphene deposition, antibody immobilization, and introduction of each solution (standard solution of OA or seawater sample).

2.3. Preparation of Gr-FET

In order to prepare graphene samples (1 mg mL^{-1}), 20 mg of graphene nanoplatelets were weight in a round-bottom flask and 20 mL of aqueous solution of SDS, previously prepared in Milli-Q water (1 mg mL^{-1}) were added. The graphene samples were maintained in a water bath ultrasonicator (Branson 2510) for 60 min at room temperature, and 9 mL of the round-bottom flask was transferred to each of two tubes for centrifugation (Pro-Research K2015 Ambient Centrifuge) during 10 min at 2000 rpm. The resulting supernatant of each tube was collected for subsequent optical characterization. Then, for the preparation of Gr-FET, a droplet of graphene dispersion (2 μL) was deposited on the clean FET surface for 15 min at room temperature, and then it was blown off with N_2. The schematic view of the obtained Gr-FET is shown in Fig. 1c, where the three electrodes (drain, source, and gate) are identified as well as the active area of the FET with graphene.

The graphene dispersions were analyzed by ultraviolet-visible spectroscopy (UV–Vis) through a Shimadzu UV-2101PC spectrophotometer and UV–Vis optical absorbance spectra (200–600 nm) were recorded using a quartz optical cell (10 mm). In addition, transmission electron microscopy (TEM) was carried out to examine the morphology of graphene layers. A FEG-TEM Hitachi H 9000 microscope operating at 300 kV and a Bruker EDX analyzer were used. The samples were prepared by placing a graphene dispersion drop on a copper grid coated with an amorphous carbon film. Concerning Raman spectroscopy, a Horiba micro-Raman spectrometer (model HR800) was used to obtain Raman spectra (1200–3000 cm^{-1}) for the graphene samples dispersed in SDS. The spectrometer has a He-Cd laser of 442 nm, and the spectra were obtained using a 600 lines mm^{-1} grating and a 100× objective (numeric aperture = 0.9).

2.4. Detection of OA by Gr-FET and ELISA

For the detection of OA with Gr-FET, 2 μL of anti-OA solution was dropped on the Gr-FET surface and it was incubated at 4 °C for more than 12 h. The output characteristics of Gr-FET with anti-OA were monitored after drying their surface with N_2 and then 2 μL of each standard