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Nanoporous impedemetric biosensor for detection of trace atrazine from water samples

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ABSTRACT

Trace contamination of ground water sources has been a problem ever since the introduction of high-soil-mobility pesticides, one such example is atrazine. In this paper we present a novel nanoporous portable bio-sensing device that can identify trace contamination of atrazine through a label-free assay. We have designed a pesticide sensor comprising of a nanoporous alumina membrane integrated with printed circuit board platform. Nanoporous alumina in the biosensor device generates a high density array of nanoscale confined spaces. By leveraging the size based immobilization of atrazine small molecules we have designed electrochemical impedance spectroscopy based biosensor to detect trace amounts of atrazine. We have calibrated the sensor using phosphate buffered saline and demonstrated trace detection from river and bottled drinking water samples. The limit of detection in all the three cases was in the femtogram/mL (fg/mL) (parts-per-trillion) regime with a dynamic range of detection spanning from 10 fg/mL to 1 ng/mL (0.01 ppt to 1 ppm). The selectivity of the device was tested using a competing pesticide; malathion and selectivity in detection was observed in the fg/mL regime in all the three cases.

1. Introduction

Pesticides belong to a class of analytes frequently probed by sensors owing to their increasing presence as contaminants in water or in agriculture products. During the past 50 years, pesticides have been used in increasing amounts throughout the world. Among pesticides, atrazine is the most extensively applied herbicide to control broad-leaf plants and grassy weeds in the world (Dagnac et al., 2005).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), because of its high relative mobility in the soil and other features, has become one of the most widely used herbicides (Pérez-Carrera et al., 2007). It is a putative endocrine disruptor (Hyer et al., 2001) and may cause serious health risks even at very low levels (parts-per-billion concentration) (Ackerman, 2007). Long-term exposure of humans and animals to atrazine at low concentrations may induce sub-acute injury and potential hazards to the body. Although studies on the toxicity of atrazine on humans have not been completely conclusive, atrazine exposure in rodent models has identified reproductive and developmental abnormalities (ETNR, 1996). The maximum level for atrazine contamination is 3.0 ppb in drinking water, as established by the US Environmental Protection Agency. Thus it is of great significance to accurately quantify the compound at low concentrations. Analytical techniques such as chromatography (Viglino et al., 2008), capillary electrophoresis (Tongbo Jiang et al., 1995), and flow injection immuno analysis (McArdle and Persaud, 1993; Javier et al., 2010; Jordi et al., 1997) have been developed for the trace atrazine determination. However, these methods have some drawbacks such as poor selectivity, high cost, slow response, poor stability, low sensitivity and efficacy (Zhimin et al., 2010a), which limit their application.

Enzyme-based biosensors for pesticide determination have caused public interest due to their reliability, fast response, high sensitivity and selectivity. Enzyme-linked immunosorbent assay (ELISA) for determination of atrazine herbicides has become one of the most active methodologies in recent years (Thomas, 1993). Several reports on the enzyme immobilization and inhibition of atrazine on the activity of enzyme have been published (Lima et al., 2011). But, the signal detected in an ELISA measurement, probes the reaction between the enzyme-tagged small molecule and its substrate; therefore it is not a direct measurement of antibody-pesticide binding. The combination of the recent advances obtained in electronics and biotechnology have resulted in sophistication and enhancement of the limits of detection of the

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diagnostic devices. In particular, electrochemical biosensors have demonstrated fast response with relatively simple manufacturing techniques (Reddy et al., 2008). The biomolecular recognition event on the device surface results in an electro-ionic transduction that produces changes to the measured electrical double layer capacitance that can be detected, by electrochemical impedance spectroscopy.

Electrochemical impedance spectroscopy (EIS) is a sensitive technique based on monitoring the electrical response of the device under test after the application of a periodic alternating current (AC) signal of small amplitude in a wide range of frequencies (Daniels and Pourmand, 2007), which provides significant information about the electric properties of the test sample. EIS has been used to monitor the immunological binding of large antibodies, MW 176.2 kDa, and large antigens, MW 76 kDa (Nagaraj et al., 2010). Due to advances in the field of immunology monoclonal and polyclonal antibodies can be raised against small antigens, <1000 Da. This advancement has led to the development of electrochemical impedance biosensors used for the detection of atrazine (Ionescua et al., 2010b).

The utilization of functional nanomaterial as the active component for achieving detection is a recent development in the field of label-free diagnostics devices. Nanomaterial such as carbon nanotubes, silicon nanowires, nanoporous alumina and a wide range of polymer, metal/metal oxide nanoparticles have been widely used toward designing ultra-sensitive and rapid biosensors (Wang, 2006). Nanomaterials offer increased surface area-to-volume and the use of the electrically active nanomaterials enhance the electron signal transduction which in turn amplifies the detected signal associate with a biomolecular recognition event.

A number of label free sensor techniques using a combination of EIS and nanomaterials have been developed for ultrasensitive detection of atrazine. A number of polymer based nanotextured active sensing surfaces have been developed for detecting atrazine using impedemetric methods such as impedance spectroscopy and cyclic voltammetry. Electro generated polyquinone films functionalized by a hydroxyatrazine moiety have been demonstrated for label-free electrochemical detection of atrazine with a limit of detection of 0.2 pg/mL in phosphate buffered saline (Tran et al., 2011). Similarly polypyrrole films using N-substituted by nitrilotriacetic acid (NTA) electro generated on a gold electrodes have been demonstrated for detecting atrazine with sensitivity of 10 pg/mL (Javier et al., 2008). While these sensors show ultra low sensitivity with relatively low cross reactivity, the sensor performance is dependent on the activity as well as the stability of the polymer films in addition to the stability of the antibodies when immobilized onto the polymer films, additionally most of the studies have been conducted in standardized buffers such as phosphate buffered saline. Impedemetric sensors on interdigitated metal electrodes with electro active polymer active sensing surfaces have been able to demonstrate sensitivity of 0.04 ng/mL from wine and phosphate buffer saline without the use of labels (Ionescu et al., 2010a).

Nanomaterials have been widely used in designing ultra sensitive impedance base atrazine sensors, most recently tyrosinase-immobilized vertically grown TiO_2 nanotubes (Tyr/TiO_2-NTs) have been used for detecting atrazine through impedance measurements from soil samples based on the inhibition of tyrosinase by atrazine, sensitivity of 0.2 ppt to 2 ppb was demonstrated. The sensitivity and reliability of the sensor is dependent upon the activity and stability of the enzyme (Zhimin et al., 2010b). Mass based sensors have also been developed using nanoparticles incorporated in to screen imprintable polymers, which have shown sensitivity of detection in the range of 0.05–7 mg/L (Yaqub et al., 2011). Finally, label-free optical techniques such as surface enhanced Raman scattering (SERS) have been used to demonstrate sensitivity of 0.1 μg/mL, this technique requires the use of complex equipment and the system is not suitable for portable applications (Jayawardhana et al., 2010). Table 1 in supplementary section provides an overview of all the label free techniques that have been designed for atrazine detection.

In this paper we have used nanoporous alumina membranes and interfaced them with printed circuit boards toward generating a high density array of nanoscale confined spaces. We have designed an assay similar to ELISA without the use of enzymes to achieve non-faradic detection, i.e. the transfer of charge between the biomolecule and the electrode is mediated without the use of a redox probe. Sensitivity in detection is achieved through nano confinement of the small molecules in size matched spaces resulting in amplification of the impedance signals measured due to the modulation of the electrical double layer. Unlike comparable impedemetric sensors for atrazine detection this sensor does not use electro active polymers/polymer composites to achieve amplification of the detected signal. Detection of atrazine from three types of water sources (i) phosphate buffered saline (ii) river and (iii) drinking water has been demonstrated with femtogram/mL level of sensitivity with cross-reactivity less than a third of the specific binding signal from malathion, with detection time in the order of minutes. We have leveraged some of the findings based on our previous work, where we have observed that localization of biomolecules in size matched nanoscale confined spaces enhances the sensitivity of the molecular detection (Kunduru et al., 2010).

2. Materials and methods

2.1. Biosensor platform

The biosensor platform developed for this application is based on a design that our group had previously developed on silicon substrates toward detecting protein biomolecules (Nagaraj et al., 2010). In our previous work we have established that interfacing alumina membranes with gold metal electrodes results in the generation of nanoscale confined spaces, the diameter and depth of the confined spaces play a key role in achieving biomolecule confinement. The conditions selected for this work is based on the optimization that has been previously performed (Nagaraj et al., 2010; Bothara et al., 2008; Vattipalli et al., 2010). We have also previously established that significant enhancement to the detection sensitivity as well as amplification during biomolecule detection can be achieved using this proposed configuration (Nagaraj et al., 2010). The key modifications that have been implemented for pesticide detection are changes to the base metallic electrode design to enhance small molecule (pesticide) binding and manufacturing the sensors on a printed circuit board platform to enhance the cost-effectiveness. The biosensor platform comprises of three parts (i) a printed circuit board platform, (ii) nanoporous alumina membrane and (iii) polydimethylsiloxane (PDMS) manifolds. The three components were heterogeneously integrated to assemble the biosensor chips. Printed circuit boards (PCB) of dimensions 19 mm × 30 mm were used in lieu of silicon due to reduced costs associated with manufacturing. The dimensions of the electrodes and the electrode design were identified to ensure maximum surface coverage with the nanoporous alumina membrane. The electrode design of the PCB chips comprised of concentric branched gold circular patterns as shown in Fig. 1. The outer diameter of the gold electrodes was 13 mm and the diameters of the inner branches were 10 mm and 5 mm. The gold electrode had two electrical leads connected to electrical outlet contacts of dimensions 2 mm in diameter with a 1 mm pore. There were two electrode leads one connecting the working electrode (WE) and the second connecting the counter electrode (CE). The gold electrodes

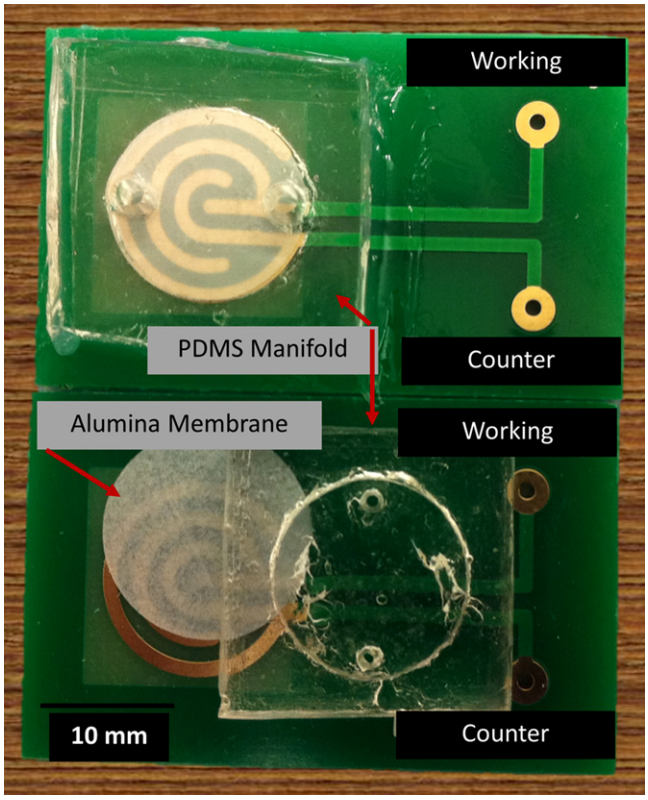

Fig. 1. Optical micrograph of the nanoporous biosensor platform with the concentric working and counter electrodes (WE and CE). Leads from the potentiostat (Gamry Reference 600) are connected to the WE and CE through soldered wires. The base platform was mounted by a PDMS manifold and the nanoporous alumina membrane was overlaid on top of the concentric gold electrodes. This biosensor platform was enclosed using a metallic calibration shield while acquiring the data to minimize stray electrical field effects.

were manufactured by depositing gold of thickness of 2–5 μin. over 125 μin. of nickel. This was plated on top of copper which was 1.4 milli-inches thick. The outer metal patterns were passivated with a polymer layer. The printed circuit board chip was connected to the impedance measurement unit through soldered contacts to the WE and CE. The metallic electrode patterns have been designed in such a manner to maximize the interaction area between the number of pores of the alumina membranes and the electrode. We have also investigated other electrode geometries such as the interdigitated pattern, but have found the concentric circle geometry to give the least electrical cross reactivity (Sierks et al., 2011).

The PCB chips were overlaid with nanoporous alumina membranes. The diameter of the nanoporous alumina membrane matched the dimensions of the circular metallic gold electrode. Commercially available nanoporous alumina membranes (Fisher Scientific, catalog number - 09-926-31), manufactured using electrochemical anodization methodology were used for this application. The nanopores were 20 nm in diameter and 250 nm in length. There were approximately 2 million nanopores (PRODA) interfaced with the metallic base electrode resulting in the formation of a high density array of nanoscale confined spaces of fixed volume. In order to achieve fluid confinement on the biosensor platform a PDMS manifold was bonded to the PCB chip using a biocompatible adhesion agent (3M adhesive tape – 300 LSE). The PDMS manifolds were manufactured by curing the elastomer on an aluminum master mold at 100 °C for 15 min. Before pouring the mixture of the resin and hardener on to the master mold, the mold was cured at room temperature for 30 min by spraying the water based release agent, "departure solution" (Zyvax corporation, Ellijay, GA). After curing, the PDMS manifolds were diced into individual components of 15 mm × 15 mm dimension. The PDMS manifold comprised of microscale wells with an inlet and an outlet port and a slot for immobilizing the nanoporous alumina membrane. The well had a fixed volume of 150 μL.

Prior to assembly, the manifold and the PCB chip were cleaned by ultra-sonication process for 10 min in isopropyl alcohol. They were then air dried for a few minutes. The nanoporous membrane was first overlaid onto the chip and the PDMS manifold was placed such that the membrane was immobilized with in the slot. The manifold was bound to the PCB substrate using biocompatible adhesives. The assembled chips were tested for fluid leaks through visual inspection.

2.2. Assay protocol

The sensor performance was first calibrated using 150 mM phosphate buffered saline (PBS). The sensor was then tested with river water and drinking water spiked with varying concentrations of the pesticide. After assembly, the chip was calibrated to obtain the baseline measurements. The chip was first incubated at room temperature with 150 μL of PBS. After a 15 min incubation impedance measurements were obtained using the potentiostat. After aspirating PBS, 150 μL of dimethyl sulfoxide (DMSO) was injected into the chip, after a 15 min incubation impedance measurements were obtained. The initial calibration with DMSO was performed as it was used as the solvent for the biochemical linker used for immobilization of the antibody for the small molecule atrazine. After aspirating DMSO, 150 μL of 10 mM of the biochemical linker, dithiobissuccinimidyl propionate (DSP) in DMSO was injected on the sensor, after 30 min incubation at room temperature, impedance values were measured. DSP underwent covalent conjugation with the gold surface through the thiol linkers and the amine group of DSP was used to immobilize the antibody of atrazine. Anti-atrazine (Abcam, catalog number: ab30533) was diluted in PBS to a 1:100 ratio of stock yielding a dose concentration of 25 μg/mL. This dose of antibody was experimentally identified to saturate the sensor chip. The optimal dose concentration of antibody was determined through an antibody saturation study, which has been previously been presented in detail (Bothara et al., 2008). Briefly, serial dilution of the antibody was prepared from stock. The serial doses were tested from the lowest dose on the sensor surface through a room temperature incubation process and the dose of the antibody that yielded a steady impedance value was identified to correspond to the saturation dose. The duration of incubation for individual steps was assay was determined through prior work (Nagaraj et al., 2010; Bothara et al., 2008; Vattipalli et al., 2010) where the time required for achieving steady state measurements correlating to diffusion mediated biomolecule immobilization in the nanoporous structures was determined by measuring the steady state impedance. 150 μL of the antibody solution was delivered in to the manifold after aspirating the DSP solution and aspirating the surface with DMSO. Antibodies conjugated to the amine end groups of the DSP linker molecules, after 15 min incubation at room temperature the impedance was measured, anti-atrazine was aspirated off the chip and was washed three times with PBS. Impedance was recorded. Superblock (Pierce, catalog number: 37515) was used as blocker to passivate the unconjugated surfaces of the sensor chip of. 150 μL of superblock was incubated on the substrate for 15 min and the impedance measurements were recorded and after a 3× aspiration with DI water impedance measurements were again recorded. Atrazine (Sigma-Aldrich, catalog number: 45330) was dissolved in 150 mM PBS solution to achieve a range of dilutions from 10 fg/mL to 1 μg/mL. Starting from the lowest dose 150 μL of atrazine was injected into the sensor chip, after 15 min incubation

at room temperature the impedance measurements were recorded. After a 3× wash with DI water, impedance measurements were again recorded and the next highest dose of atrazine was then injected on to the sensor. Hence the dose response for atrazine was obtained. Similar protocol was adopted for obtaining atrazine dose response in river water. Water was collected from Arkansas River (collection site: Sim Memorial Park – East side of the river at the Central, McClean and Meridian road intersections.) and was filtered using a 200 nm pore filter (Whatman, Fisher Scientific, PA)) before spiking with specific doses of atrazine. Drinking water (Kroger Purified Drinking Water, Processed by the Kroger, Co. in Cincinnati, OH, using Advanced Filtration, Ozone, and Reverse Osmosis Technologies) was spiked with the specific dose range of atrazine. The testing was conducted in a similar manner as in the case of PBS.

2.3. Electrochemical impedance spectroscopy (EIS)

The modulation to the electrical double layer, which extends approximately 50 nm from the solid/liquid interface within the nanoscale confined spaces, is the detection mechanism that has been adopted for measuring atrazine binding to the sensor surface. Perturbation to the charge in the electrical double layer occurred due to the following bio-recognition events (linker-antibody-small molecule). The charge perturbation was measured using the EIS module of the Gamry Reference 600 potentiostat (www.gamry.com). For the sensor configuration that has been presented in this paper, the immobilization of biomolecules as described in Section 2.2, modulated the charge of the EDL, this modulation resulted in a change to the measured capacitance associated with the EDL, when there was specific binding between the small molecule and its receptor there was a significant increase in capacitance which translated into a measured decrease to the impedance. EIS measurements have been represented for a peak-to-peak voltage of 10 mV and an applied frequency of 100 Hz. Immobilization of the small molecules through the adopted biochemical technique and the associated capacitance change has been schematically represented in Fig. 2. The assay as represented in Fig. 2 is expected to replicate in the array of nanoscale confined spaces. The electrical equivalent circuit representation of the individual nanoscale confined spaces is also shown in Fig. 2. It comprises of electrical double-layer capacitances (C_{EDL}) that are in conjunction with the solution resistance. Other components that play a role in designing this equivalent circuit are the electron-transfer resistance (R_{et}) and the Warburg impedance (Z_W). At low frequencies, the dominant component of the impedance measured in the fluid is the double layer capacitance. C_{EDL} from the individual nanoscale confined spaces are summated (Bothara et al., 2008). As the array of the nanoscale confined spaces are connected to a single base measurement site, signal enhancement was achieved through summation which in turn enhanced the sensitivity of the sensor.

3. Results and discussion

The data presented in this section comprises of three categories. The sensor performance has been evaluated in (i) phosphate buffered saline (PBS) (ii) river water and (iii) drinking water. Determination of sensor performance in PBS was used to calibrate sensor performance under controlled conditions. The sensor performance in "real-world" solvents was then evaluated with river and drinking water. A set of 15 independent measurements were performed for each dose of the small molecule for each type of sample. All the data represented in Section 3 were collected at 100 Hz and 10 mV peak-to-peak voltage. The optimal frequency for data representation was identified through a calibration study (Supplementary Fig. 1). The data was averaged and the error was expressed as the ratio of the standard deviation to the mean. The sample volume throughout the assay was maintained at 150 μL and the association time of the pesticide with its antibody was approximately 15 min.

3.1. Sensor performance with phosphate buffered saline (PBS) samples

The sensor was prepared following the assay protocol detailed in Section 2.2. Antibody concentration study with atrazine was performed on the antibody immobilized sensor surface starting at the lowest dose (10 fg/mL) following the protocol detailed in Section 2.2. Fig. 3 demonstrates the dose response of atrazine in PBS and the associated non-specific binding of malathion over the same dose range. Interaction of PBS with antibody saturated sensor surface produces a decrease in the impedance from a dry chip measurement as the ions in PBS will interact with the EDL thus producing changes to the capacitance of the EDL which translates into an impedance change (impedance associated with dry chip = 0.6–0.8 GΩ; impedance of chip after PBS wash = 520 Ω). The PBS measurements were conducted with 150 mM strength PBS at neutral pH ~7.4, higher ionic strength of PBS produced charge screening effects which reduced the sensitivity of the sensor device. The limit of detection with atrazine in PBS was determined to be 10 fg/mL and the associated measured impedance value was 154.51 Ω which was a decrease of 83.20 Ω from the antibody baseline measurement of 237.72 Ω. This impedance change was expressed as a percentage change from the antibody baseline which translated to 35% change; this is shown in the inset of Fig. 3. The percentage change in impedance increased from 35% to 95% over the dose range of 10 fg/mL (0.01 ppt) to 1 ng/mL (1000 ppt). A linear trend with a regression value (R^2) was observed to be 0.97 over the linear dynamic range of the dose response (10 fg/mL to 1 ng/mL). To verify the cross reactivity of the sensor, malathion, (diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate) was chosen, as it had similar binding profile as atrazine and it was aliquoted using the same protocol as that adopted for atrazine. The percent change in the impedance from the antibody saturation base line ranged from 5% to 30%, with respect to the antibody saturation baseline. The percentage changes in impedance over the concentration range of 10 fg/mL to 1 ng/mL were calculated using the same approach as was discussed for atrazine. Reduced binding of malathion to the atrazine antibody can be attributed to the selectivity of the atrazine antibody. A linear trend was observed with the non-specific small molecule; malathion with a regression value of $R^2 = 0.97$. The calculated error for both atrazine dose response and malathion dose response in PBS was <1% of the measured signal. Detection of atrazine from PBS buffer containing equal dose concentrations of atrazine and malathion has also been demonstrated. The dose response shows the atrazine binding signal after subtracting the non-specific signal from malathion. This was represented in Fig. 3 with the violet bars. The R^2 value for the linear regression fit was 0.97. The specific binding of atrazine small molecules with the antibody results in an increase to the double layer capacitance, which in turn translates into a decrease in the measured impedance. The double layer capacitance changes can be most accurately monitored at low frequencies (Nagaraj et al., 2010), hence the impedance measurements were taken at 100 Hz. As the antibody used for the assay was purchased from a commercial vendor, there is some non-specific binding, which has been obtained from the assay. The amount of non-specific binding has been minimized by controlling the antibody orientation through controlled attachment to the linker molecules (Nagaraj et al., 2010). The amount of non-specific binding is less than one third of specific binding signal for any given dose over the dose response regime.

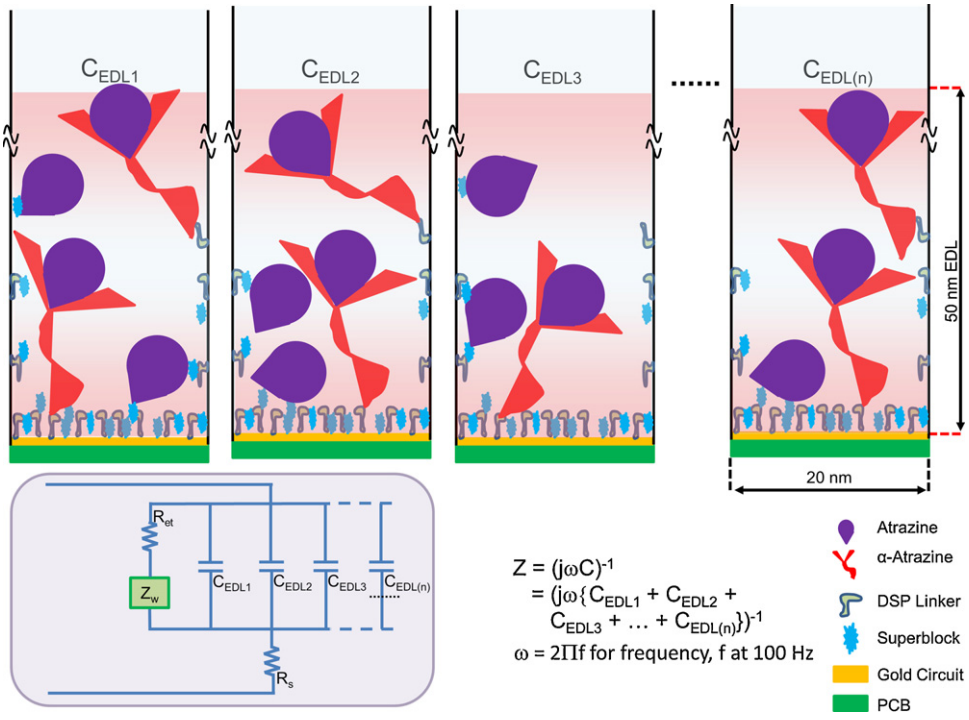

Fig. 2. The assay chemistry with each of the nanopores is illustrated in the schematic. The assay comprises of immobilizing linker molecules on the gold substrate of the nanopore and covalently conjugating the pesticide antibody to the linker. Binding of the pesticide small molecules to the antibodies can be measured by changes to the impedance associated with the perturbation to the capacitance of the electrical double layer, EDL (nearly 50 nm from the gold substrate). Each of these nanopores can be electrically represented as a combination of the resistors and capacitors connected across the same two points (in parallel). C_{EDL1} represents the electrical-double layer capacitance of the first nanopore, C_{EDL2} of the second nanopore and so on, $C_{EDL(n)}$, adding up to the final capacitance, C. R_{et} is the electron transfer resistance, Z_w is the Warburg resistance and R_s is the solution resistance. The impedance, Z, can be calculated from the capacitance based on the equation in the Figure.

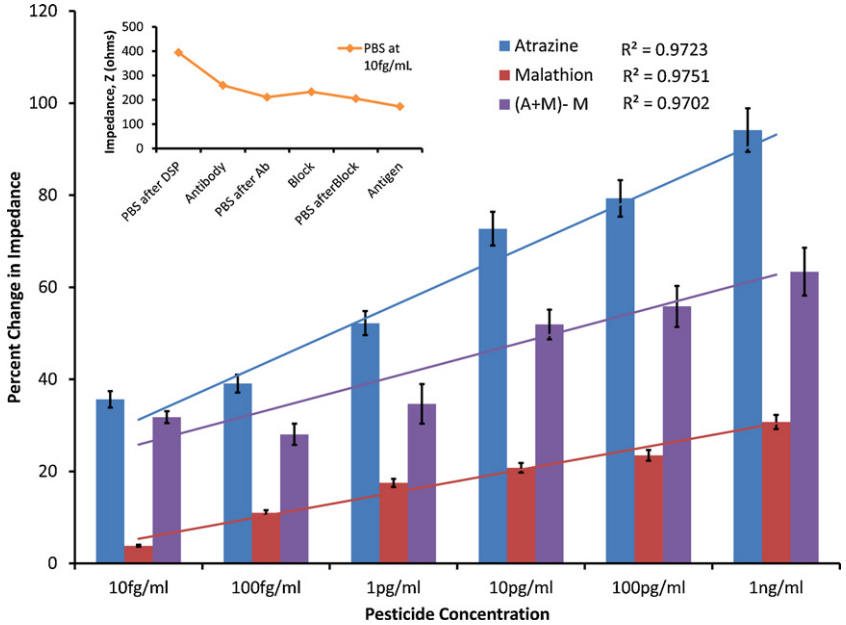

Fig. 3. Dose response of atrazine (blue bars) in phosphate buffered saline buffer indicating the lowest detected concentration as 10 fg/mL (0.01 parts-per-trillion). The linear changes to the impedance ranges from 35% to 95% of the baseline impedance associated with the anti-atrazine saturation of the substrate over the dose range from 10 fg/ml to 1 ng/ml. Non specific binding with malathion (red bars) varies from 5% to 30% over the same concentration range. A 1:1 (vol/vol) concentration ratio of atrazine (A) and malathion (M) concentrations were combined and tested on the atrazine antibody saturated sensor surface. The (A + M) – M (violet bars) indicate the percentage change in impedance over the entire dose range after subtracting out the non-specific binding contribution at each dose due to malathion. Regression values on all the trend lines are 0.97 implying the linear trend in the dose response for all the tests performed on the sensor. Inset indicates the step-wise change in the measured impedance spectra for one representative concentration dose in the atrazine dose response (10 fg/mL) as highlighted with a dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

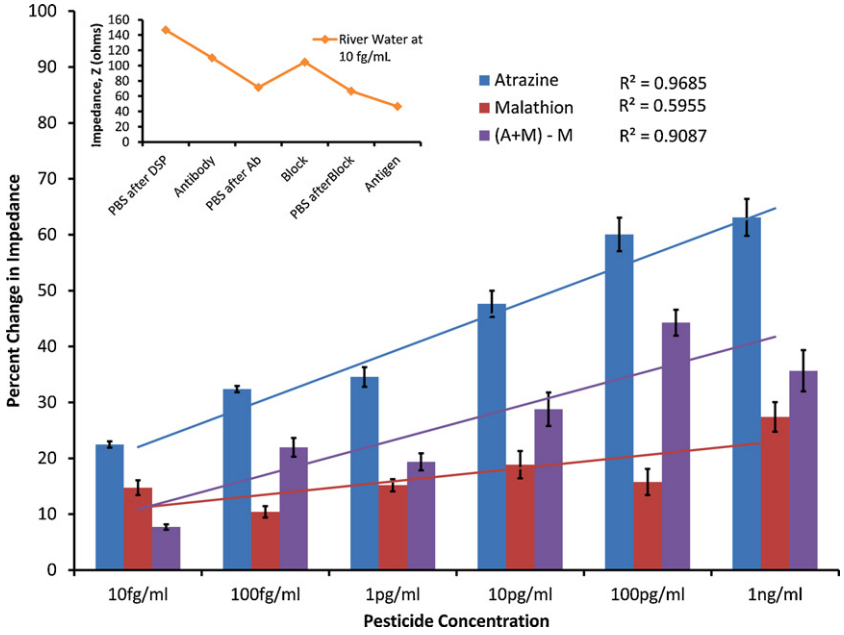

Fig. 4. Dose response of the atrazine (blue bars) dissolved in river water indicating the lowest detected concentration as 10 fg/mL (0.01 parts-per-trillion). The linear changes to the impedance ranges from 26% to 62% over the dose range 10 fg/mL to 1 ng/mL (0.01 parts-per-trillion to 1 parts-per-billion). The percentage change in impedance is calculated from the baseline impedance associated with anti-atrazine saturation of the substrate. Non-specific binding with malathion (red bars) varies from 14% to 25% over the same concentration range. Equal volume mixtures of the concentrations of atrazine and malathion were tested against the atrazine antibody and the impedance changes due to atrazine (violet bars) was determined by subtracting the impedance values obtained from malathion non-specific binding for a particular dose from the impedance values measured due to the combined interaction of atrazine and malathion. Regression values with atrazine were much higher than with the non-selective pesticide, malathion. Inset indicates the step-wise change in the impedance for a concentration (10 fg/mL) of atrazine detection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Hence, it is possible to clearly distinguish between specific and non-specific binding in PBS at the 10 fg/mL regime.

3.2. Sensor performance with river water samples

River water from the Arkansas River in Wichita KS was utilized for testing the sensor performance. Arkansas River has atrazine contamination ranging from 0.02 μg/L to 0.1 μg/L (kdheks); hence the test aliquots prepared with river water were prepared such that the atrazine concentration ranged from 10 fg/mL to 1 ng/mL. River water was filtered twice using a 0.2 μm syringe filter. The quality of water and the associated trace presence of atrazine were ascertained using Kansas DEQ water quality analytical methodologies. The river water was then treated such that the concentration of atrazine in river water was 1 mg/mL. Serial dilutions were then performed in a manner similar to that adopted for PBS.

Fig. 4 demonstrates the dose response of atrazine in river water and the associated non-specific binding of malathion over the same dose range. The limit of detection for atrazine detection in river water was 10 fg/mL. At lower doses the measured impedance change was comparable to the baseline signal associated with antibody saturation. A dose dependent decrease to the impedance was observed, which translated to increasing percentage changes to the impedance when compared to the antibody baseline. The dynamic range of detection varied from 10 fg/mL to 1 ng/mL. The measured values of impedance were in the range of hundreds of ohms. The impedance changes ranged from 26% to 62% over the dose range, and a linear trend was observed with the R^2 value of 0.96. Percentage changes in impedance were calculated based on the method as described in Section 3.1. The changes to the impedance for malathion in river water over the same concentration range varied from 14% to 25% with respect to the antibody base line The R^2 value of the malathion dose response was at 0.59. The calculated error for both atrazine dose response and malathion dose response in river water was <1% of the measured signal. Atrazine detection from sample of river water containing equal concentrations of atrazine and malathion has also been represented. The impedance changes shown (violet bars) in Fig. 4 were obtained after subtracting the non-specific binding due to malathion. There is a linear trend in the dose response with the R^2 value to be at 0.90. It is possible clearly distinguish atrazine binding from the non-specific matrix from 100 fg/mL to 1 ng/mL. There is more background signal which may be attributed to increased non-specific binding from river water due to the presence of non-malathion and organic and inorganic contaminants which may also show some affinity to anti-atrazine. The step-wise change in impedance due to the specific binding of atrazine has been shown for one representative concentration of 10 fg/mL. The trend is similar to the one seen in PBS samples.

3.3. Sensor performance with drinking water samples

Drinking water from Kroger Company was utilized for the dose response analysis. Drinking water had 36–99 dissolved units per liter and was primarily dissolved salts such as sodium, magnesium, potassium and calcium (niagarawater). The analytical quality control tests indicated the absence of any dissolved pesticides. Drinking water was treated with 1 mg/mL of atrazine and the subsequent lower doses up to 10 fg/mL were aliquoted through serial dilution. The same methodology was adopted for generating the dose range for malathion in drinking water. Fig. 5 demonstrates the dose response of atrazine in drinking water and the cross reactivity of malathion in drinking water over the same concentration range as atrazine. The lowest dose of atrazine that produced a measurable change in the impedance from the antibody baseline was 10 fg/mL. The range of detection for atrazine was from 10 fg/mL to 1 ng/mL, and the corresponding percentage change to the impedance as

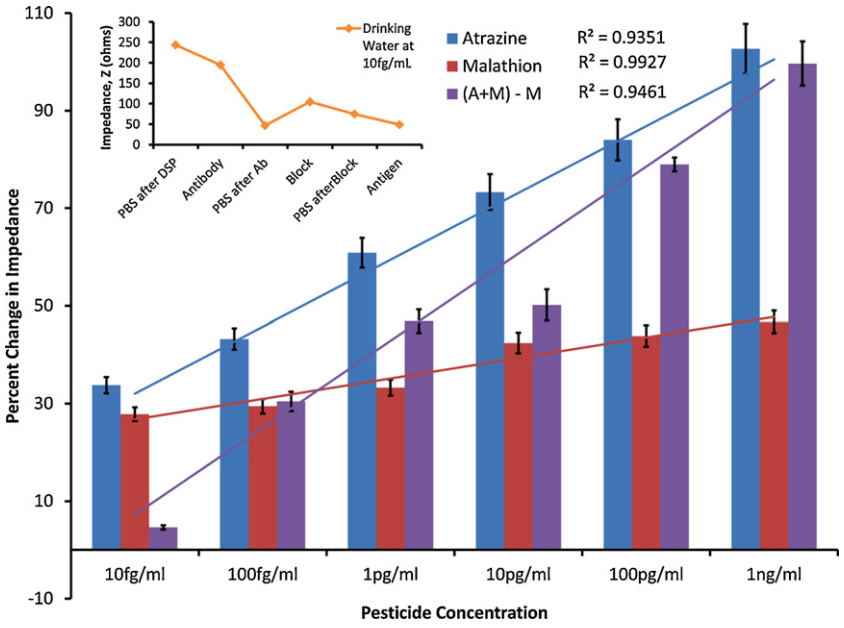

Fig. 5. Dose response of atrazine (blue bars) spiked in drinking water indicating the lowest detected concentration as 10 fg/mL (0.01 parts per trillion). The linear changes to the impedance ranges from 37% to 102% over the dose range from 10 fg/mL to 1 ng/mL (0.01 parts-per-trillion to 1 parts-per-billion). The percentage change in impedance was calculated with respect to the baseline impedance associated with the anti-atrazine saturation of the substrate. Non-specific binding with malathion (red bars) varies from 36% to 55% over the same concentration range, indicating that the lowest concentration dose of atrazine distinguishable in drinking water is 100 fg/mL. Equal volumes concentrations of atrazine and malathion over the concentration range in drinking water were tested against the atrazine antibody. Equal volume mixtures of the concentrations of atrazine and malathion were tested against the atrazine antibody and the impedance changes due to atrazine (violet bars) was determined by subtracting the impedance values obtained from malathion non-specific binding for a particular dose from the impedance values measured due to the combined interaction of atrazine and malathion. Regression values in all the cases were high (0.99, 0.94, and 0.93 respectively) indicating the linearity of all the three assays. Inset indicates the step wise change in the atrazine binding assay at the 10 fg/mL concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

compared to the antibody base line varied from 37% to 96% with a linear increase in the percentage change in impedance with an R^2 value of 0.93. The percentage changes in impedance were estimated in the similar approach as described in Section 3.1. Additionally, the step wise change in impedance for one representative dose of the atrazine was shown as an inset for 10 fg/mL. The non-specific binding changes due to interaction of malathion resulted in a percentage change in impedance from antibody baseline ranging from 28% to 38% for the concentration ranges of 10 fg/mL to 1 ng/mL with a linear trend and the regression, R^2 value was 0.99. The calculated error for both atrazine dose response and malathion dose response in drinking water was <1% of the measured signal. The dose response for detecting atrazine from drinking water containing equal volume concentration of atrazine and malathion after subtracting the contribution of non-specific binding due to malathion has been shown. There is a linear trend in the dose response with a R^2 value of 0.94 (violet bars). It is clearly possible to clearly distinguish atrazine binding from the specific matrix over the concentration range from 1 pg/mL to 1 ng/mL.

The maximum amount of non-specific binding has been observed in drinking water, which may be attributed to the presence of a number of dissolved salts (sodium, potassium and calcium), which have high surface charges and contribute to changes to the double layer capacitance at neutral pH. In contrast the organic and inorganic impurities in river water do not have as high surface charges and may not contribute to measurable changes to the double layer capacitance. Hence, we do observe reduced amount of non-specific binding from river water as compared to drinking water. As the aim of the paper is to demonstrate the ability of the sensor to distinguish atrazine binding without pretreatment of the water samples or the substrate, we have presented the data as obtained which gives a true measure of the sensor performance.

The enhanced sensitivity in detection as observed in all the three cases of detection may be attributed to the novelty in the sensor design. In the design of the pesticide sensor, the nanoporous alumina is overlaid on to the base metallic electrodes. The nanoporous structure serves in the spatial confinement of the biomolecules. To support this there is a compelling argument in the realm of biophysical understanding of bio macromolecules such as proteins/antibodies and their structures. There exists a crowded environment within every biological cell, the cytoplasm which is typically different from the dilute solutions that are general used for in vitro detection studies of these biomolecules. There exists a school of thought that states that this may affect the behavior of these biomolecules significantly, such as their stability and efficacy (Zimmerman and Minton, 1993; Zimmerman and Trach, 1991). The ability of the biomolecules to undergo association is adversely affected when the biomolecule efficacy is impacted. Hence, in order to enhance the sensitivity of a sensor, it is essential to ensure biomolecule efficacy for low dose detection.

To attain the crowding effect usually experienced in the biological environments, such as the interiors of the cell, the biomolecules need to be confined to regions or compartments which are of dimensions that are comparable to the size of the biomolecules (Chebotareva et al., 2004). This increases the packing density of the molecules. This principle is called “macromolecular confinement” and it suggests that confining biomolecules within the pores of the alumina membrane helps the biomolecules sustain their conformation (Cheung et al., 2005). This in turn maximizes the biomolecule activity and hence increases the overall probability of association between the antibodies and the small molecules, resulting in increased sensitivity of detection. To our knowledge this work is the first experimental demonstration of applying the macromolecular crowding principle that is prevalent in nature for maintaining the efficacy and stability of protein

macromolecules to an in vitro system for small molecule detection.

The effect of enhancement to the sensitivity in detection of atrazine molecules due to nanoscale confinement within the nanoporous alumina membranes by mimicking the principle of "macromolecular crowding" has been clearly established by comparing the sensor performance for three specific cases (i) planar sensor (ii) sensor with 200 nm alumina pores and (iii) sensor with 20 nm alumina pores (Supplementary data Fig. 2). The sensor performance clearly demonstrates enhancement in the sensitivity with the smaller pore size, where the size of the detected molecule is comparable to the pore size.

4. Conclusion

In this paper we have demonstrated the ability to leverage size based nano confinement in designing biosensors toward trace detection of pesticides from water samples. We have tested the technology using a standard herbicide; atrazine. In our previous work we have identified the role of size based confinement in localizing protein biomolecules and enhancing the sensitivity of protein biosensors designed using nanoporous arrays of confined spaces (Bothara et al., 2008; Vattipalli et al., 2010). We have adapted some of our findings toward achieving ultra-low sensitivity coupled with relatively low cross-reactivity in designing a label-free electrochemical impedance based biosensor for pesticide detection. We have demonstrated that the limit of detection is in the fg/mL both in the calibration buffer as well as in more complex solutions such as river and drinking water, we attribute the sensitivity to the immobilization and localization of the atrazine molecules in size matched nanoscale confined spaces. The nanoporosity of alumina offers enhanced surface area to volume, in addition to signal amplification associated with the electro-ionic transduction associated with the binding events. The perturbation to the electrical double layer at the solid/liquid interface at the base of the array of nanoscale confined space produces a cumulative enhancement to the double layer capacitance. This double layer capacitance is modulated due to the binding of the target small molecules with the antibody. The specificity in detection is primarily dependent upon the antibody efficacy. Hence we do observe some amount of cross reactivity with malathion molecules. Despite some non-specific binding there is a significant difference between the specific and non-specific binding signals, with specific binding yielded a signal 1.5*X* or greater than the non-specific binding signal.

To apply the research described in this paper to environmental monitoring, the sensor will be optimized for multiplexed detection of several well-established trace pesticides found in ground water on a single chip using fluids such as river and drinking water in a simultaneous manner. Several key engineering- and biochemistry- related challenges will have to be addressed to reach this goal. To overcome specificity issues with certain antibodies, the identification and use of other ligands such as aptamers and peptides with greater specificity and avidity toward the pesticide small molecules is necessary. The sensor platform needs to be developed for the simultaneous detection of multiple species of trace analytes ranging from metabolites, micro organisms, to chemicals from the same sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.11.055.

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