Whole cell environmental biosensor on diamond

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A whole-cell environmental biosensor was fabricated on a diamond electrode. Unicellular microalgae *Chlorella vulgaris* was entrapped in the bovine serum albumin (BSA) membrane and immobilized directly onto the surface of a diamond electrode for heavy metal detection. We found that the unique surface properties of diamond reduce the electrode fouling problem commonly encountered with metal electrodes. The cell-based diamond biosensor can attain a detection limit of 0.1 ppb for Zn^{2+} and Cd^{2+}, and exhibits higher detection sensitivity and stability compared to platinum electrodes.

Introduction

The past decade witnessed the rapid dwindling of fresh water sources in developing countries due to contaminations caused by industrial effluents and agricultural pesticides. The degradation of fresh water sources is also exacerbated by deforestation and climate change. Accompanying these changes, there are ongoing efforts to develop water monitoring systems that are portable and which can show rapid, sensitive response to a wide range of toxins. Biosensors have received much attention in water monitoring systems owing to their high sensitivity, low cost and easy adaptation for on-line monitoring.[1,2] A whole cell microorganism appears to be an attractive candidate, as it hosts a large number of enzymes and bioreceptors, which can be very sensitive to toxic compounds. The green alga *Chlorella vulgaris* has been used in constructing optical,[3,4] electrochemical[5] and conductometric[6] biosensors for environment monitoring. *C. vulgaris* is an ideal choice for a biosensing element because it is ubiquitous and grows year-round in all climatic conditions. Its wide availability in nature, simple cell structure and ability to acclimatize to low nutrient levels make it an excellent choice for biosensing applications. Phosphatase enzyme is one of the enzymes which can be found abundantly on the *C. vulgaris* cell wall. Under alkaline conditions, the activity of phosphatase enzyme, also known as alkaline phosphatase activity (APA), is inhibited by heavy metals ions. The APA activity is responsible for the dephosphorylation of *p*-nitrophenyl phosphate into *p*-nitrophenol and phosphate ions. Chouteau *et al.* developed a conductometric biosensor which monitors the inhibition of APA in the presence of heavy metal ions.[6] Marks *et al.* developed an electrochemical biosensor based on *C. vulgaris* immobilized on a platinum electrode and the APA was monitored by the oxidation current of enzymatically generated *p*-nitrophenol.[5] However, we found that rapid electrode fouling precluded the long term usage of this biosensor.

Diamond possesses outstanding electrochemical characteristics, such as a wide potential window,[7–9] low background current,[10,11] weak adsorption for organic molecules[12,13] and high stability of response.[14,15] Therefore, diamond electrodes are a suitable material for several purposes: synthesis of chemicals, electroanalysis,[9] energy conversion and destruction of organic/inorganic pollutants.[16] Moreover, the versatility of these materials has also been extended to develop sensors and biosensors.[9,17] In order to fabricate a biosensor which consists of alga cells immobilized on diamond, the growth conditions of alga cells on diamond, as well as the condition for stable and leach-free entrapment of these cells, have to be established. To address these issues, we have investigated the conditions for the immobilization of alga cells on diamond, and also fine tuned the parameters that allow sensitive detection of heavy metals. Comparative studies on platinum electrodes were also carried out to evaluate the sensitivity and resistance to electrode fouling.

Experimental

Chemicals

All the chemicals used in this experiment were purchased from Sigma Aldrich unless stated otherwise and were used as received.

Diamond electrode preparation

The boron-doped diamond electrode was grown on a 35 mm diameter niobium disk using conventional CH_4/H_2/diborane chemistry, with a 2.45 GHz commercial microwave chemical vapor deposition (CVD) system (Seki Technotron). Diamond samples were cleaned and chemically oxidized with hot 'Piranha' solution (30% H_2O_2 : 97% H_2SO_4 = 1 : 3) for 1 h, followed by rinsing with ultrapure water. The samples were then rinsed with tetrahydrofuran followed by hexane. After the wet chemical treatment, the diamond samples were treated by hydrogen plasma in a microwave plasma CVD system using 300 sccm hydrogen gas and 800 W plasma power.

Algae culture condition

The algae *C. vulgaris* strain was purchased from the company *Culture Collection of Algae and Protozoa* based in Cumbria, UK. The algae was cultured in an inorganic medium and sub-cultured every three weeks. In order to induce the maximum enzyme alkaline phosphatase activity (APA), the algae were

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centrifuged and suspended in the phosphate-free medium for 25 days before being immobilized on the diamond electrode surface.[18]

Diamond biosensor preparation

The algae were immobilized on the diamond electrode surface with bovine serum albumin (BSA) and glutaraldehdye as the crosslinker.[19] 100 μL of starved algae solution and 10% (w/v) BSA were mixed and deposited onto the diamond electrode surface. Finally, the electrode was placed in a saturated glutaraldehyde vapor for 20 min, followed by drying at room temperature for 30 min. The electrode surface area is 0.0962 cm^2.

Fluorescence observation

A fluorescence micrograph of the algae/BSA membrane after being immobilized on diamond electrode was observed with a fluorescence microscope (Olympus BX60) equipped with a rhodamine B filter (excitation: 540 nm; emission: 625 nm).

Electrochemical instrumentation

All electrochemical experiments in this work were performed using a potentiostat (Autolab/PGSTAT30, Eco Chemie B.V.). A platinum wire and a saturated Ag/AgCl electrode were used as the counter and reference electrodes, respectively.

Cyclic voltammetry and chronoamperometric

The electrochemical behavior of the algae–BSA membrane-coated diamond electrode was investigated by cyclic voltammetry (CV). The electrolyte used was 0.5 mM ferrocene carboxylic acid dissolved in 0.1 M tris-HCl containing 1 mM $MgCl_2$. For chronoamperometry, the algae-coated diamond electrode was immersed in 0.1 M tris-HCl (with 1 mM $MgCl_2$ as enzyme activator) solution. The electrode was biased at 1.2 V and *p*-nitrophenyl phosphate was added into the solution with constant stirring (100 rpm). The resulting current from the oxidation of enzymatically generated *p*-nitrophenol was recorded.

Heavy metals testing

Two heavy metals, namely cadmium and zinc ions, were used for testing in this work.

Results and discussion

Membrane permeability

The permeability of algae–BSA membrane on diamond electrode to charge transfer was investigated by cyclic voltammetry (CV) using ferrocene carboxylic acid as the redox indicator. Fig. 1 shows the CV recorded for the diamond electrode before and after coating with the algae–BSA membrane. The algae–BSA membrane coating imposed only a slight degree of steric hindrance to the heterogeneous electron transfer, as can be judged by the small decrease in the current of the reversible peaks, *i.e.* the anodic peak is decreased from 8.1 μA to 6.9 μA after the coating. The small current decrease demonstrates the good permeability of the BSA membrane. The stability of the membrane was confirmed by soaking the algae–BSA coated

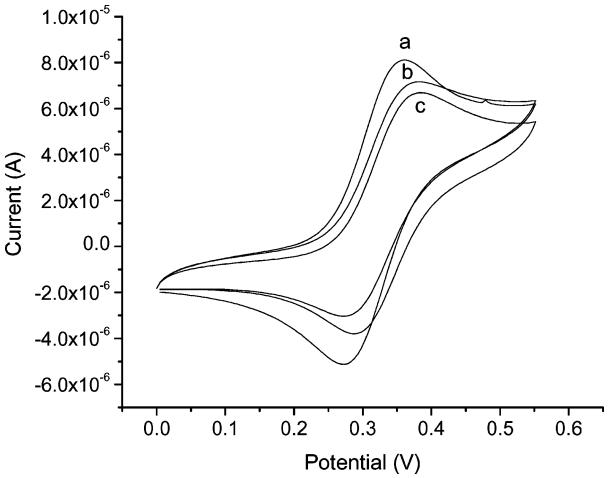


Fig. 1 Cyclic voltammograms of diamond in ferrocene carboxylic acid solution (a) before algae–BSA coating and (b) after algae–BSA coating; (c) after soaking; (d) after overnight in buffer solution. The CV shows only a slight current decrease, which attests to the good permeability and stability of the BSA coating.

diamond electrode in the buffer solution overnight, CV testing (Fig. 1c) revealed that the current response is consistent with that originally obtained.

Algae viability

Fluorescence observation of the algae–BSA membrane was performed using 540 nm light for excitation. This excitation wavelength lies close to the Soret band maximum absorption of chlorophyll b in algae.[20] The emission wavelength was monitored at 625 nm, which corresponds to the Photosystem II (PS II) fluorescence emission peak in algae.[21] From Fig. 2, red fluorescence can be seen from the algae–BSA membrane, indicating that most of the green algae remain in the viable state.

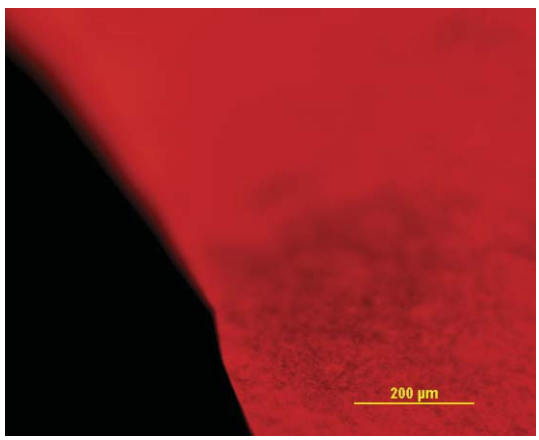


Fig. 2 Fluorescence image of algae–BSA membrane. Photosystem II (PS II) fluorescence emission indicates that the alga remains viable after BSA entrapment.

Alkaline phosphatase activity detection

The principle of the diamond biosensor is based on the dephosphorylation properties of the enzyme alkaline phosphatase

which can be found abundantly on the algae membrane, as depicted in Fig. 3. The added *p*-nitrophenyl phosphate, which is electro-inactive, will act as the substrate for the enzymatic action to generate the product *p*-nitrophenol, which is electro-active. The chronoamperometry experiments monitor the oxidation current of *p*-nitrophenol.

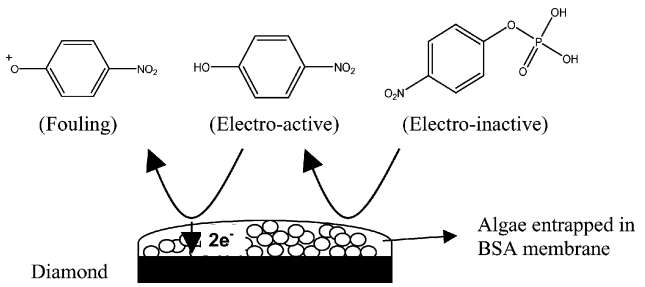


Fig. 3 Detection principle of the diamond biosensor. The electro-inactive substrate *p*-nitrophenyl phosphate will be dephosphorylated by enzyme alkaline phosphatase at the algae membrane to produce electro-active *p*-nitrophenol, which is subsequently oxidized at the diamond electrode. The oxidation of *p*-nitrophenol causes fouling of metal electrodes.

The density of the immobilized cell has to be controlled to optimize the amount of enzyme loading on the electrode. On the other hand, overloading of the diamond platform with cells will passivate the electrical response of the diamond and block the diffusion of analytes to the diamond surface. The current response for diamond sensors loaded with different concentrations of algae is shown in Fig. 4a. It is clear that the optimum cell density can be obtained at 5×10^7 cells mL^{-1}, where the highest oxidation current is obtained. Fig. 4b shows that the optimum pH for the enzyme alkaline phosphatase activity is at pH 9, where the highest current response in the presence of excess substrate could be detected.

Having identified the optimum conditions for the experiment (5×10^7 cells mL^{-1}; pH 9), the analytical performance of the diamond biosensor was investigated. Fig. 5 shows the calibration curves for substrate *p*-nitrophenyl phosphate when diamond and platinum were used as electrode material. The enzymatic activities on both electrodes show typical Michaelis–Menten behavior. The diamond and platinum electrode showed sensitivities of 8.03 mA M^{-1} cm^{-2} and 4.03 mA M^{-1} cm^{-2}, respectively. It can be noted that for the diamond biosensor, the oxidation current reaches saturation at a lower substrate concentration (0.125 mM) compared to the platinum biosensor (0.2 mM).

The biofouling resistance of the diamond biosensor was further evaluated by monitoring the current response after repetitive usage in the presence of excess substrate (0.5 mM). Fig. 6 shows that the oxidation current remains stable on the diamond biosensor compared to platinum biosensor after 20 scans. The resistance to fouling on both electrodes after 20 CV scans is summarized in Fig. 7. It can be observed that the current only fluctuates within 10% of the initial oxidation current on diamond, compared to a decrease of more than 40% on the Pt electrode. A previous report by Rodrigo shows that continuous oxidation of *p*-nitrophenol will generate a *p*-nitrophenoxy radical and this radical will be oxidized subsequently to a nitrophenoxy cation, as shown in eqn (1).[22] The nitrophenoxy cation is very reactive, its tendency to undergo polymerization on the surfaces of electrodes causes electrode fouling. The diamond surface is chemically inert and shows good fouling resistance, due possibly to the hydrophobic, hydrogen-terminated surface,[23] compared to the more hydrophilic metal

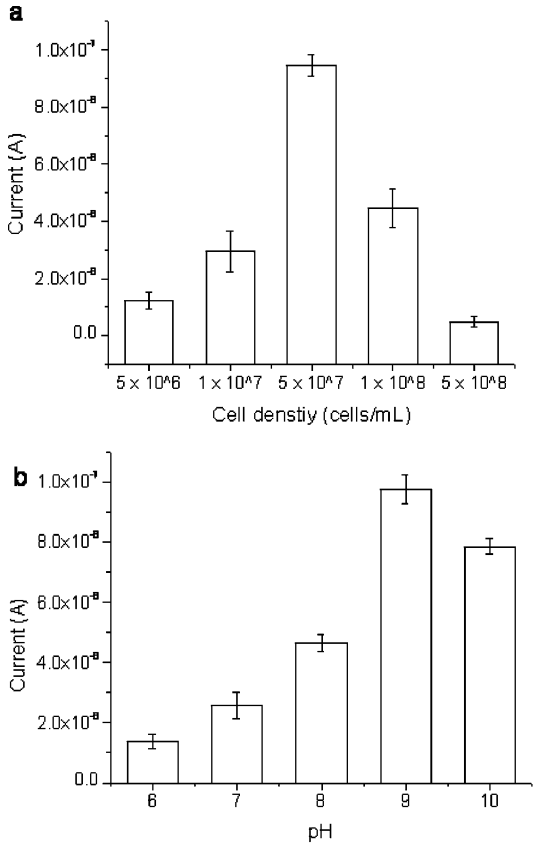


Fig. 4 Current response of diamond sensors immobilized with (a) different concentration of algae and in (b) different pH solution with excess substrate (0.5 mM). The optimal operating conditions for the diamond biosensor can be obtained with 5×10^7 cells mL^{-1} and at pH 9.

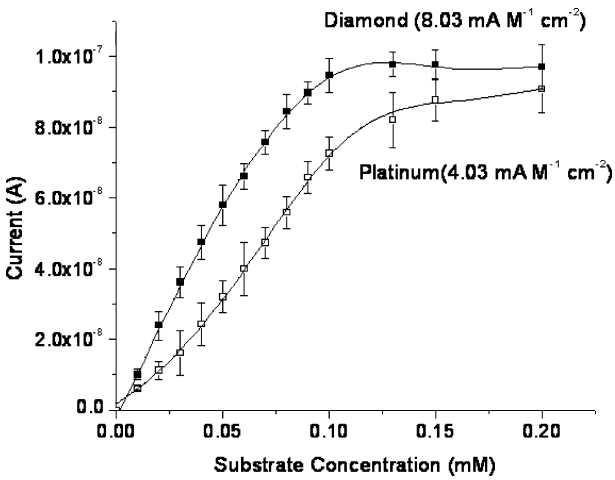


Fig. 5 Substrate calibration curve. Both samples exhibit typical Michaels–Menten behavior but the algae–diamond sensor shows higher sensitivity compared to the algae–platinum sensor.

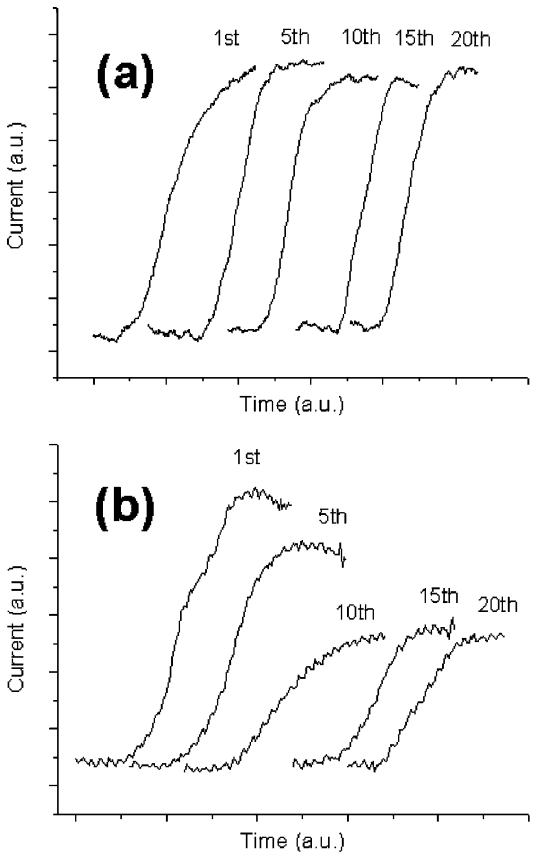


Fig. 6 Chronoamperometry current response for (a) algae–diamond biosensor and (b) algae–platinum biosensor after multiple CV cycles.

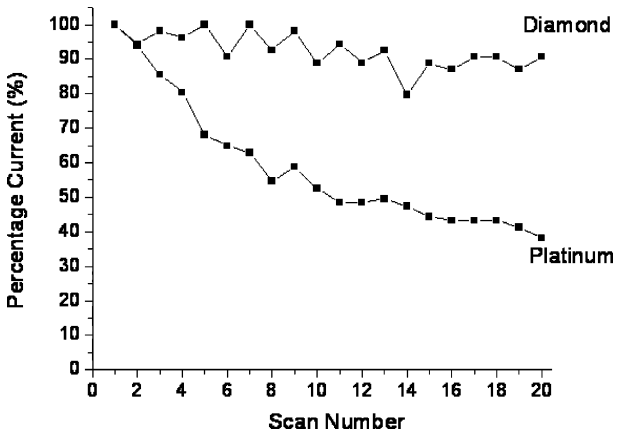


Fig. 7 Biofouling resistance of algae–diamond and algae–platinum sensors after repetitive usage.

electrode surfaces. In order to evaluate the long term stability of these sensors, the diamond and Pt biosensors were stored in the phosphate-free medium and tested over a period of 14 days in excess substrate (0.5 mM). As shown in Fig. 8, the diamond biosensor exhibits excellent long term stability with minimal (±10%) current fluctuation compared to the platinum biosensor, the current response of the latter drops to 60% of the initial oxidation current at the end of 14 days.

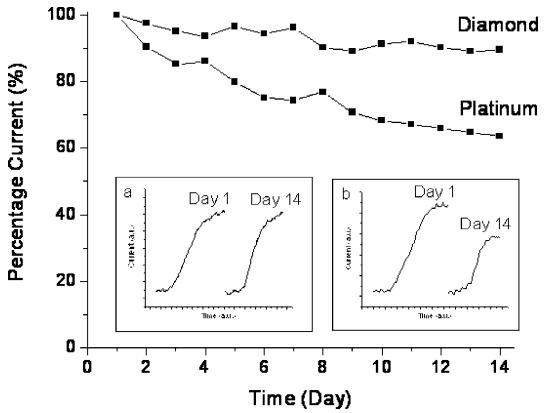
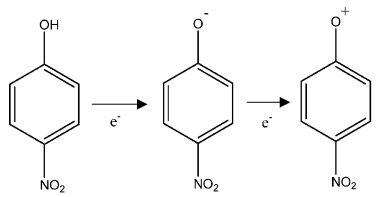


Fig. 8 Time-dependent stability test carried out on the algae–diamond and algae–platinum biosensors over a period of 14 days. Insets show the chronoamperometric response at day 1 and day 14 for (a) algae–diamond biosensor and (b) algae–platinum biosensor.



Equation 1. Stepwise oxidation of *p*-nitrophenol to nitrophenoxy radical and nitrophenoxy cation.

(1)

Heavy metals detection

The diamond biosensor was used for the detection of two heavy metals, namely cadmium and zinc. It is known that the enzymatic activity of alkaline phosphatase will be inhibited in the presence of heavy metals. This will result in reduced *p*-nitrophenol production and a lower oxidation current will be detected. The diamond biosensor was exposed to the heavy metal solution for 5 min before excess substrate (0.3 mM) was added into the solution. The results were summarized in Fig. 9. For both heavy metals, it can be noted that the oxidation current decreased linearly with increasing concentration of heavy metals. The activity of the enzyme alkaline phosphatase is inhibited at high concentrations

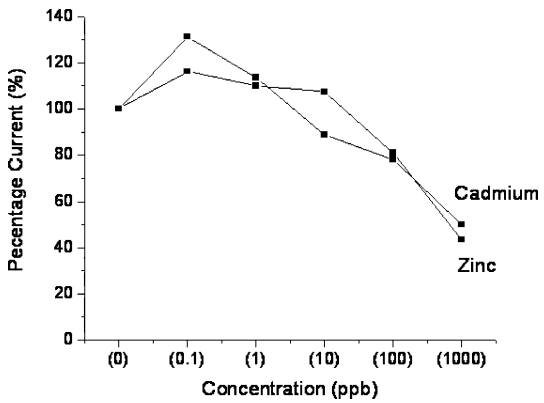


Fig. 9 Heavy metals detection by the algae–diamond biosensor. The oxidation current decreases linearly with increasing concentration of heavy metals, with the detection limit of 0.1 ppb.

of cadmium ions (10 mM) and zinc ions (100 mM). Slight increase in oxidation current can be observed for low concentration of heavy metals (for cadmium < 10 mM; zinc < 100 mM). The presence of low concentrations of heavy metals produces stress promoters in the cells which increase the enzyme activity.[24] Detection limits for the diamond biosensor was determined to be 0.1 ppb for both cadmium and zinc ions, which is well above the contamination level for heavy metals set by the US for drinking water (5 ppb for cadmium and 5 ppm for zinc). Although the antibody-based biosensor reported by Blake *et al.*[25] shows a better detection limit for cadmium ions (30 ppt), the diamond biosensor we fabricated here exhibited better detection limit compared to other types of *Chlorella vulgaris*-entrapped biosensors, such as the *Chlorella vulgaris* optical biosensor[26] (10 ppb detection limit for cadmium) and *Chlorella vulgaris* conductometric biosensor[27] (10 ppb detection limit for cadmium). One drawback of all enzymatic-based sensors is that re-activation of the enzyme is not possible after exposure to heavy metals, this means that although the algae–diamond sensor may have a longer shelf-life compared to metal-based electrodes with regard to the problem of biofouling, ultimately, the shelf-life of the sensor depends on the saturation threshold of the enzyme for heavy metals.

Conclusions

This paper demonstrates the application of diamond as a signal transduction platform for algae cells owing to its excellent property in resisting electrode fouling by *p*-nitrophenol oxidation. The conditions for optimizing algae cell density on the BSA membrane-diamond platform have been identified. Comparative studies carried out on the Pt electrode showed that diamond was more resistant to electrode fouling, and also showed better long term stability. The detection limit for cadmium and zinc ions is 0.1 ppb.

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