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Ultra-sensitive conductometric detection of heavy metals based on inhibition of alkaline phosphatase activity from *Arthrospira platensis*

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ABSTRACT

This study is based on the conductometric measurement of alkaline phosphatase activity (APA) from the cyanobacterium, *Arthrospira platensis*, called Spirulina. Cyanobacterium cells were directly immobilized, by physical adsorption, on the ceramic part of gold interdigitated transducers. This activity was inhibited in the presence of heavy metals and a variation of the local conductivity was measured after addition of the substrate. The Michaelis–Menten constant (Km) was evaluated to be 0.75 mM through a calibration curve of the substrate, disodium 4-nitrophenylphosphate p-nitrophenyl phosphate (pNPP). Inhibition of APA was observed with cadmium and mercury with a detection limit of 10^{-20} M. The half maximal inhibitory concentration (IC50) was determined at 10^{-19} M for Cd^{2+} and 10^{-17} M for Hg^{2+}, and the binding affinity of heavy metal (Ki) was equal to the IC50. On the sensor surface, scanning electron microscopy (SEM) images revealed a remarkable evolution of the cyanobacterium's external surface that was attributable to the first defense mechanism against toxic heavy metals in trace. This effect was also confirmed through the important increase of response time $\tau_{90\%}$ recorded for APA response towards the substrate pNPP after cell exposure to metallic cations. Lifetime of the Spirulina-based biosensor was estimated to be more than 25 days.

1. Introduction

Existing as natural components of the Earth's crust, heavy metals refer to chemical components with low densities, yet highly toxic or poisonous when concentrated, for e.g. cadmium, lead, and mercury which pose the highest pollution risks. Soil and water systems are the most common entry points for heavy metals, whose tendency to bioaccumulate in animals and human organisms makes them dangerous from a public health standpoint. Hence, even in traces, the toxicity of heavy metals represents a serious threat to human health.

Substances such as cadmium or mercury have been classified as "priority hazardous substances" in Decision No. 2455/2001/EC [1] and Directive 2008/32/CE [2] for which industries should implement the necessary measures. This will avertably help to reduce human anthropic activity and, thus, preserve ecosystems. In this approach, quality control of aquatic ecosystems requires tools for continuous detection that are in situ of contamination sources or contaminated environments. Electrochemical platform detection [3] and biosensors are such devices that can be implemented.

Recently, the development of whole-cell biosensors has raised an increasing interest. Microalgae such as *Chlorella vulgaris* were used in various studies to develop whole-cell biosensors for the control of toxic pollutants in aquatic environments [4–7]. Conductometric biosensors based on detection of alkaline phosphatase activity (APA) for immobilized cells of Chlorella were studied for the detection of heavy metals. Alkaline phosphatase of *C. vulgaris*, like other enzymes, induces catalytic reactions generating ionic species leading to measurable variations in local conductivity [8]. Also, in a study by Berezhetskyy et al. [9], a detection of heavy metals has been recorded with low detection limits: 1 ppb for Cd^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+}, and 10 ppb for Zn^{2+}. Recently, Thengodkar and Sivakami [10] observed that monoesterase of Spirulina was able to hydrolyze under alkaline conditions for the organophosphorus pesticide. According to the literature [11], Spirulina contains APA. This filamentous cyanobacterium, has been used by Tekaya et al. [12], to characterize the global detection of heavy metals using an impedimetric device. In this work, we have developed a conductometric biosensor based on APA measurements. This activity can be inhibited with metal ions [4–7].

Arthrospira platensis is a Gram negative bacterium. It is also considered as a blue-green microalgae. Regarding the localization of APA, it was reported that APA is periplasmic in gram-negative bacteria, and that it also occurs at the cellular and extracellular surface [13].

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Here, Luo et al. [13] predicted a much higher abundance of cytoplasmic activity phosphatase in marine bacteria. In a study on the cyanobacteria, *Anacystis nidulans*, Block and Grossman [14] reported that the majority of phosphatase activity is released into the medium in which *A. nidulans* is osmotically shocked, indicating that the enzyme is located either in the periplasmic space or weakly fixed to the cell wall. In a recent work [11], the authors reported that alkaline phosphatase was histochemically localized in the cytoplasm, the cell wall, and the sheath of Spirulina, using the enzyme-labeled fluorescent substrate (ELF) method. In fact, the ultrastructure cell of *A. platensis* observed by electron microscopy by Vonshak [15] is typical of that of prokaryotic organisms, without a morphologically limited nucleus and with an outer gram-negative type envelope on the cell wall [16–20].

In this study, kinetic proprieties of APA were directly determined using conductometric transduction which has been extensively used for probing enzymatic reactions for biomedical applications, such as urea [21] and glucose [22] detection or for environmental applications [23–25]. The geometry of the interdigitated transducers (IDTs) takes advantage of the changed conditions for the current flow which mainly occurs close to the surface and, thus, shows a much higher sensitivity towards surface change compared to the conventional flat design of electrodes.

Characterization of Spirulina cells was observed using scanning electron microscopy (SEM). This new technology allows the observation of microstructural changes of biological samples in their natural state under controlled conditions of temperature and pressure. Here, it was applied in this study for revealing heavy metal effects on Spirulina cells.

2. Experimental

2.1. Chemical and biological reagents

Disodium 4-nitrophenylphosphate p-nitrophenyl phosphate (pNPP) purchased from Sigma-Aldrich was used as the substrate for measuring alkaline phosphatase activity. It was dissolved in a buffer solution to prevent a change to the total conductivity of the reaction when adding the substrate. The buffer was HEPES ($C_8H_{18}N_2O_4S$) 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid and it was purchased from Sigma-Aldrich. HEPES was diluted in Milli-Q water to obtain a concentration of 5 mM and adjusted to pH 8.1 by adding NaOH solution. This pH was chosen for its good functionality with HEPES.

To inhibit APA, the stock solutions of cadmium (Cd^{2+}) and mercury (Hg^{2+}) were prepared from standard solutions purchased from Sigma-Aldrich. Stock solutions were stored at 4 °C and dilutions were prepared in the buffer solution (HEPES) 15 min before each series of analysis.

A. platensis (Compere 1968/3786 strain) or Spirulina was cultivated under sterile conditions in Zarrouk liquid medium containing: (g/L) $NaNO_3$, 2.50; K_2HPO_4, 0.50; $NaHCO_3$, 10.00; NaCl, 1.00; $MgSO_4$ $7H_2O$, 0.2; $CaCl_2$ $2H_2O$, 0.02; $FeSO_4$ $7H_2O$, 0.01. All salts were of analytical grade and were purchased from Acros Organics. The medium was adjusted to pH 9.0 using NaOH solution. Cultivation was conducted in 5 L Erlenmeyer flasks. Cultures were maintained at 26 ± 1 °C under air bubbling and continuously exposed to fluorescent lamps (100 μmol·photon/m^2 s). After, the biomass was recovered by filtration, washed with physiological water for the removal of nutrient salts, and then dried at 40 °C for 48 h. Then, it was lyophilized and the resulting powder was protected from moisture by storage in a closed vessel at 4 °C. The Spirulina was dissolved in HEPES and it was filtered (0.8 μm) before analysis. As Spirulina is made of transparent cells stacked end-to-end and entrapped with a sheath forming a spiral filament, the sheath was broken through filtration in order to separate individual cells. This step was very crucial for our studies.

2.2. Scanning electronic microscopy

The SEM images were captured using a Quanta™ 250 microscope (FEI). A pretreatment of cells was required before imaging. Cells were fixed with covalent links using glutaraldehyde solution (3%, v/v) purchased from Sigma-Aldrich, specially purified for use as an electron

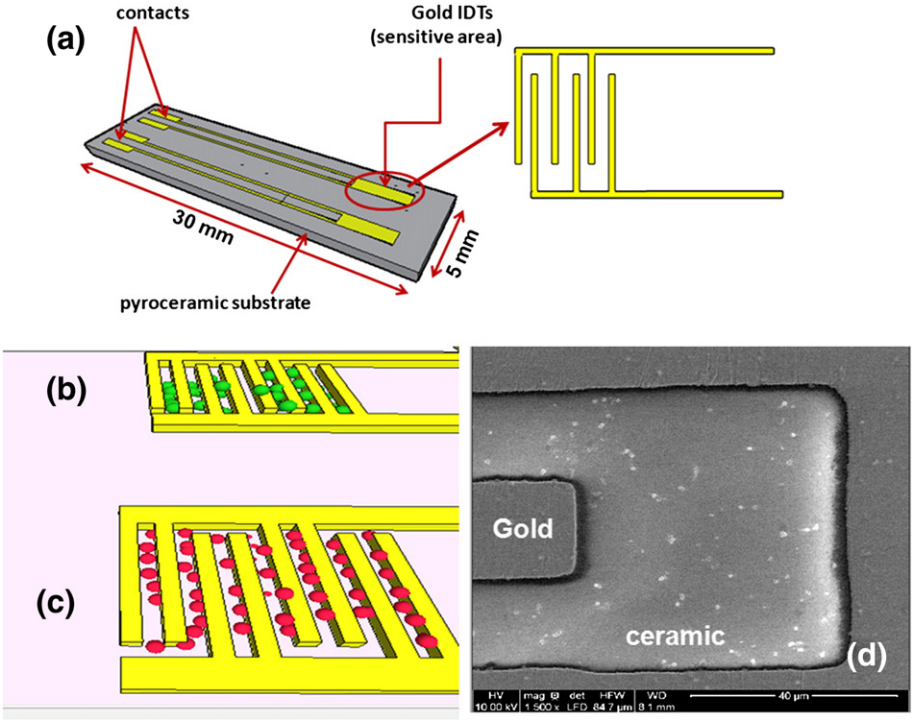

Fig. 1. (a) Scheme of microelectrodes with gold interdigitated transducers, (b) Spirulina cells immobilized on the working electrode, (c) Spirulina cells, with inhibited APA immobilized on the reference electrode, and (d) SEM image of Spirulina cells population performed between gold fingers of interdigitated transducers.

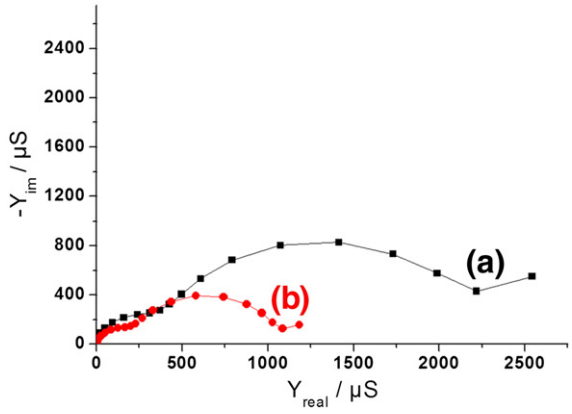

Fig. 2. Complex admittance spectra of (a) bare electrode and (b) modified electrode, where: frequency range = 100 mHz–100 kHz, amplitude potential = 10 mV, with measurements made in HEPES (5 mmol/L at pH 8.1).

microscopy fixative. Spirulina-based biosensors were immerged in this solution for 45 min. After, a series of dehydration in successive absolute ethanol solutions were applied for 10 min using increased concentrations (20%, 40%, 60%, 80%, and 100% ethanol).

2.3. Sensor design

The conductometric transducers (Fig. 1.a) were fabricated at the V.Ye. Lashkaryov Institute of Semiconductor Physics (Kyiv, Ukraine). Each of them consisted of two identical pairs of interdigitated thin film electrodes (150 nm thick), fabricated by gold vapor deposition onto a non-conducting pyroceramic substrate (5 × 30 mm). The technique of sensor manufacturing has been reported previously [26,27]. A 50 nm thick intermediate Cr layer was used to improve the adhesion of Au to the substrate. Both the digit width and interdigital distance were 10 µm, and their length was ~1.5 mm. As a result, the sensitive area of each pair of electrodes was ~2.9 mm^2 (Fig. 1.a).

2.4. Conductometric device

A biosensor analyzer consisted of a sensor block and an electronic measuring block (portable four-channel biosensor analyzer) was developed in collaboration with Institute of Electrodynamics of National Academy of Sciences of Ukraine and it was reported by Dzyadevych et al. [27]. The sensor block consisted of a stand with a fixed block of holders; each holder was connected to the fingers of an appropriate conductometric biosensor. An electronic measuring block consisted of the following modules: a secondary transducer and a basic measurement-control module. A personal computer with a specific software support was an integral part of the measuring block. Using a generator, a sinusoidal excitation signal with a frequency of 30 kHz and amplitude of 10 mV was applied to the two exciting electrodes. These conditions avoided faradic processes, double-layer charging, and polarization of the microelectrodes. The signals measured were processed using the lock-in amplifier method, i.e. they were filtered using a low bandwidth centered on the excitation frequency and then transmitted to the synchronous detection which provided the difference of conductivity between the working electrode and the reference electrode. In fact, this system transformed the local variations in conductivity to a change of the active conductivity which can be recovered as information at the output of the system, such as variations in the output signal versus time.

Measurements were performed at room temperature in a glass cell of 4 mL. The biosensor was immersed gently into the buffer (HEPES, 5 mM). A magnetic stirrer ensured solution homogeneity throughout experimentations. After the signal stabilization, different quantities of the substrate were added in the measurement cell. A rinsing step was performed between two successive measurements.

2.5. Electrochemical impedance spectroscopy (EIS) measurements

The measurements were performed with an electrochemical impedance analyzer "Voltalab PGZ 402" (Radiometer Analytical, France) in a two electrode configuration. Data acquisition and processing via "Voltamaster" software was provided by the same company. All EIS measurements were taken at a frequency range of 100 mHz to 100 kHz at room temperature. The same glass cell (4 mL) was filled with HEPES (5 mM) under vigorous magnetic stirring.

3. Results and discussion

3.1. Immobilization of Spirulina cells

IDT electrodes were cleaned by sonication for 10 minutes, followed by chemical treatment in Piranha solution (H_2SO_4/H_2O_2, 3:1 v/v) for 5 minutes. The electrodes were then rinsed with absolute ethanol and finally dried under a flow of nitrogen.

Finally, cyanobacteria solution (0.7 µL) was deposited directly onto the sensitive area of the first pair of IDTs representing the work electrode (Fig. 1.b). The cells were adsorbed preferentially to the ceramic substrate as observed through SEM (Fig. 1.d). Throughout the study, the cyanobacteria concentration was 0.3 g/L and it was chosen after optimization. The variation of Spirulina concentration in the initial solution did not affect the shape of the curve, while at the saturation concentration of the substrate the curve varied slightly (data not shown).

On the second pair of IDTs representing the reference electrode (Fig. 1.c), Spirulina cells where the APA had already been inhibited

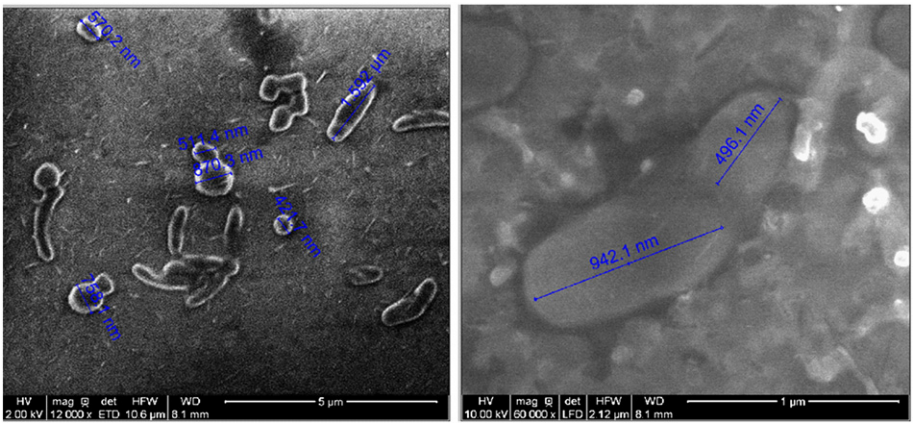

Fig. 3. SEM images of Spirulina cells.

by mercury solution (10^{-6} M) were immobilized using the same volume (0.7 μL). These cyanobacteria cells were inhibited in mercury solution; this was made 24 h before immobilization. This configuration allowed differential mode measurements.

EIS is a valuable tool to characterize phenomena at the interface. For this, electrochemical admittance spectra were recorded for a bare microelectrode and after immobilization of Spirulina cells. An optimization of the functionalization time was performed (data not shown) and revealed that immobilization was not detectable after 6 h of deposition. After 24 h from the deposition of the Spirulina cells on sensors, Fig. 2 shows an evolution of complex admittance spectra validating the immobilization process.

SEM images revealed the coexistence of several populations (Fig. 3): single cells with average sizes between 0.4 and 0.9 μm, while other cells were clustered in groups of two or three (Fig. 3).

3.2. Characteristics of kinetics for alkaline phosphatase

When the substrate was added to the solution it penetrates the biofilm based on Spirulina cells and the enzymatic reaction induced local variations of the ion concentration. The response time $\tau_{90\%}$ was deduced at 12 s (Fig. 4.a). Therefore, the variation of the electrical conductance was proportional to the concentration of the injected substrate.

Fig. 5 shows the calibration curve of the APA of Spirulina cells with pNPP concentration. The corresponding equation is:

$$G/\mu S = (0.0056)[pNPP]/\mu M + 0.0615, \quad (1)$$

where the regression coefficient is 0.0056.

The sensor response increased linearly versus the concentration of substrate (pNPP) to reach a level corresponding to enzyme saturation at concentrations greater or equal to 1500 μM pNPP. The obtained curve presents a Michaelian shape, as seen in previous works performed with other microalgae [7,8]. Mean values and error bars were calculated from three measurements using different biosensors (RSD<10%). The Michealis–Menten constant (Km) was calculated at 0.75 mM. This value is close to Km (0.69 mM) determined by Yang et al. [28] and Km (0.83 mM) [29] where APA was extracted from *Bos taurus*. It was also close to Km (0.7 mM) [30], where APA was extracted from *Gallus gallus*.

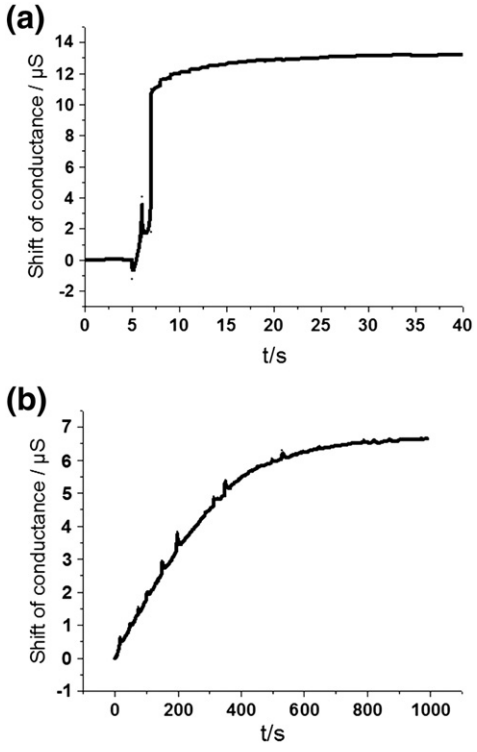

Fig. 4. Typical real time response of the conductometric transducer based on Spirulina to the substrate pNPP: (a) before incubation in mercury (10^{-16} mol/L) and (b) after 24 h of incubation.

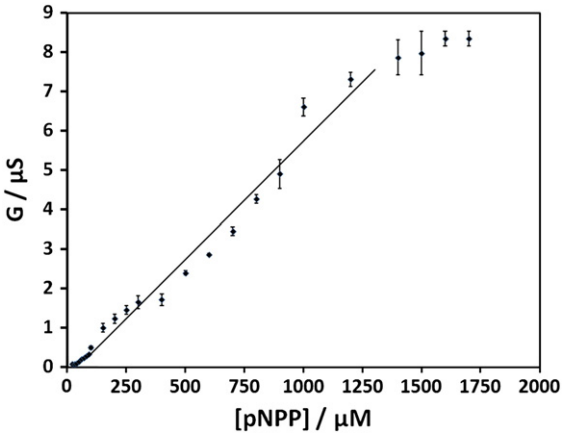

Fig. 5. Calibration curve of the alkaline phosphatase activity with pNPP concentration. The mean values and error bars have been calculated from 3 experimentations with different biosensors, under the same experimental conditions (HEPES, 5 mmol/L at pH 8.1).

Fig. 6 presents the evolution of the conductance monitored during a period of 25 days for three biosensors stored in dry conditions at 4 °C. Mean values and error bars have been calculated from three experimentations with three different biosensors. Measurements showed a continuous decrease of 40% of the initial conductivity after 25 days. Therefore, the lifetime of the biosensor can be estimated to be more than 25 days.

3.3. Inhibition measurements

To evaluate the effects of heavy metals on the enzymatic activity of the biosensors, measurements were performed before (dS_{before}) and after (dS_{after}) incubation in four concentrations of cadmium and mercury: 10^{-20} M, 10^{-18} M, 10^{-16} M, and 10^{-14} M. Incubation time has been optimized (data not shown) and a time of 24 h was adopted in this study.

The typical real time response acquired by our system after inhibition by mercury is shown in Fig. 4.b. Response time $\tau_{90\%}$ before and after incubation appears to be quite different. In fact, conductance

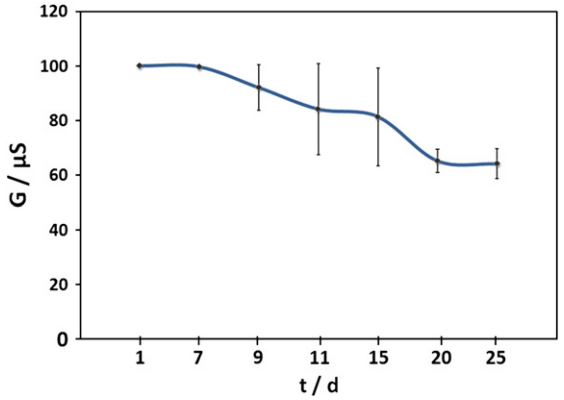

Fig. 6. Evolution of the signal of conductometric biosensors stored at 4 °C in dry conditions. The mean values and error bars have been calculated from 3 experimentation with different biosensors, under the same experimental conditions (pNPP, 1500 μmol/L and HEPES, 5 mmol/L at pH 8.1).

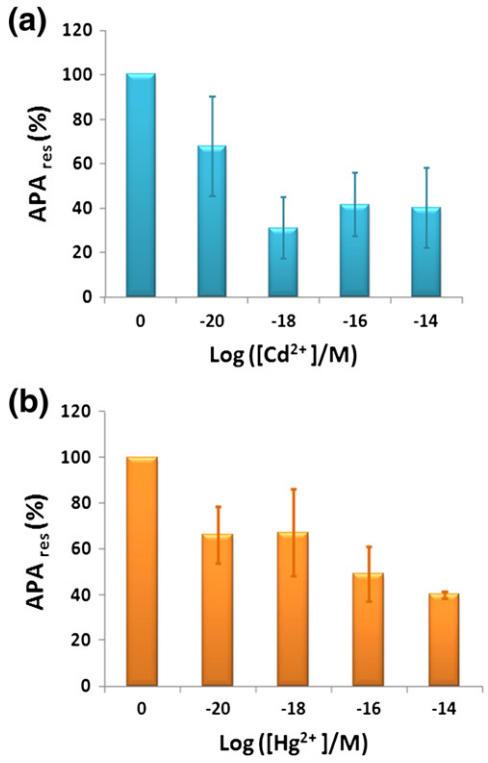

Fig. 7. APA_{res} (residual) for 24 hour exposure to (a) $Cd^{2+}$and (b) Hg^{2+}. The mean values and error bars have been calculated from 3 experimentations with different biosensors under the same experimental conditions (pNPP, 1500 μmol/L and HEPES, 5 mmol/L at pH 8.1).

variation towards the substrate (pNPP) injection was rapid before incubation in mercury (10^{-16} M). However, the response time $\tau_{90\%}$ reaches 8 min after 24 h of incubation. This was due to the phosphatase activity inhibition effect caused by mercury cations (Fig. 4). In fact, after APA inhibition, the presence of mercury cations prevents the substrate from reaching the phosphatase sites of Spirulina cells easily. This was due to the changes on the cells morphology in the presence of heavy metals.

Results were expressed as a percentage of residual activity (A_{res}) given by the following formula:

$$A_{res}(\%) = \frac{dS_{after}}{dS_{before}} \times 100 \quad (2)$$

A control measure has been carried out in the same conditions, to ensure that enzyme inhibition and not a phenomenon of electrode blocking by electrostatic interactions was occurring. This measurement was performed with a non heavy metal that was calcium. We have not recorded an inhibitory effect in low concentrations of calcium. Residual activity calculated from measurements recorded with calcium ions was more than 90% after incubation in the same concentrations 10^{-20} M, 10^{-18} M, 10^{-16} M and 10^{-14} M of calcium.

Residual phosphatase activity rates are presented in Fig. 7. These histograms show that the phosphatase activity can be inhibited at 10^{-20} M, 10^{-18} M, 10^{-16} M, and 10^{-14} M of cadmium and mercury. Here, the detection limits were lower in comparison with previous studies performed with conductometric biosensors based on *Chlorella*, where the detection limit was 5×10^{-9} M [8], 10^{-9} M [9], and 10^{-8} M [7] for cadmium. The detection limit in our study is less than 10^{-20} M. The half maximal inhibitory concentration (IC50) was evaluated at 10^{-19} M with cadmium as the inhibitor and 10^{-17} M with mercury as the inhibitor. The binding affinity of heavy metals (Ki) was equal to the IC50, and this suggests of non-competitive inhibition.

SEM images were performed on cyanobacteria exposed to heavy metals after conductometric measurements. The images reveal modifications on Spirulina cell walls exposed to 10^{-16} M of cadmium (Fig. 8.a) and mercury (Fig. 8.b). The external surface changes seem to be the first defense mechanism against toxic heavy metals, and this is in agreement with Swift and Forciniti's observations with cyanobacteria studies [31].

According to Rangsayatorn et al. [32] transmission electron micrographs of Spirulina sections revealed the effects induced by cadmium: disintegration and disorganization of thylakoid membranes, presence of large intrathylakoidal space, reduction of gas vacuoles and an increase of polyphosphate bodies (PPBs). This increase of polyphosphate bodies can be the most important factor in the phosphatase activity inhibition mechanism during our study. It can explain the increase of the response time $\tau_{90\%}$ recorded for APA response towards the substrate (Fig. 4.b).

The increase in the number of PPBs was also reported in studies of heavy metal toxicity in cyanobacteria. PPBs were suggested as being the site for metal sorption in cyanobacteria [33]. The strongly negative surface charge of the polyphosphate in the PPBs and the lipids and proteins also found in the PPBs all act to adsorb the metal. After treatment with zinc, the number of PPBs in cells of *Anabaena flos-aquas* and *Anabaena variabilis* increased [34]. Similarly, their studies on *Spirulina platensis* cells showed an increase in the number of PPBs when the exposure time was increased. In addition, Swift and Forciniti [31] investigated different lead uptake mechanisms in various subcellular regions of cyanobacteria, *Anabaena cylindrica* and found that phosphate was precipitated both on the cell wall and inside the cell. Consequently, the inhibition mechanism in our study was induced by the presence of metallic cations on the external surface cell and also by the increase

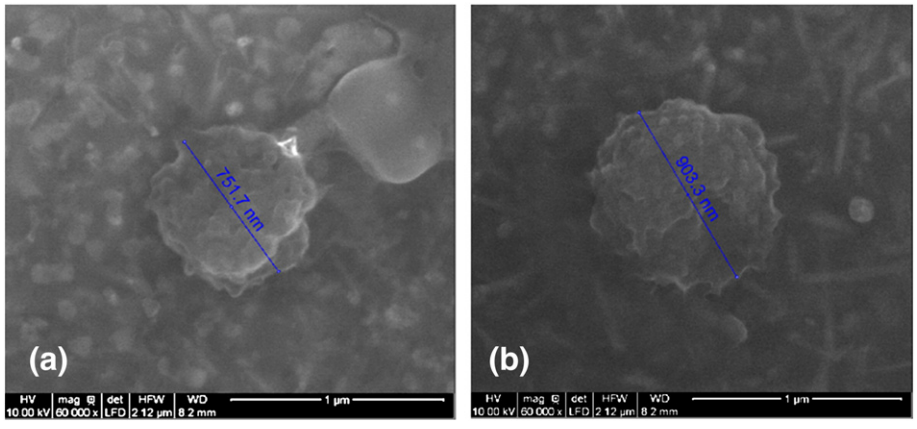

Fig. 8. SEM images of: (a) cell exposed to Cd^{2+} (10^{-16} mol/L) and (b) cell exposed to Hg^{2+} (10^{-16} mol/L).

in the number of PPBs. This can explain why a very low concentration of metal induces a high inhibition effect on APA activity.

4. Conclusion

The originality of this work consisted of the study for APA activity using conductometric transduction with a direct immobilization of Spirulina cells on the transducer surface. The cells were preferentially adsorbed to the ceramic substrate. The value of the Km can be evaluated to be 0.75 mM. The APA of Spirulina cells can be inhibited with heavy metals for concentrations higher than 10^{-20} M of Cd^{2+} and Hg^{2+}. The value of the IC50 was determined to be 10^{-19} M and 10^{-17} M with cadmium and mercury respectively. The effect of heavy metals on Spirulina cells morphology has been deduced from an increase in the response time $\tau_{90\%}$ recorded by APA response towards the substrate pNPP and SEM images. This was especially observed on the external surface as this is the first defense mechanism against toxic heavy metals in trace. Such results are very promising and provide a first proof of concept for an ultrasensitive biosensor based on Spirulina cells for the detection of heavy metals in a liquid medium. This biosensor was not specific for a single metal, but it will provide a global response to the presence of heavy metals in trace for natural waters and those in effluents.

Acknowledgments

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