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Acoustic, electrochemical and microscopic characterization of interaction of *Arthrospira platensis* biofilm and heavy metal ions

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ABSTRACT

This study examines a biofilm of *Arthrospira platensis* and its interactions with cadmium and mercury, using electrochemical admittance spectroscopy technique combined with highly sensitive Love wave platform for the real-time detection in liquid medium. Spirulina cells were immobilized via multilayers of polyelectrolyte (PEM) on Si/SiO_2 surface of both transducers and characterized using atomic force microscopy (AFM). Scanning electron microscopy (SEM) cell images revealed a first defense mechanism against cadmium at 10^{-12} M and it immediately takes place after 4 s from injection. The cyanobacteria biofilm becomes more conductive, due to an increase of polyphosphate bodies. An increase of density induces a decrease of frequency. Response time $\tau_{90\%}$ of the biofilm toward Cd^{2+} was between 6 and 8 min, while it did not exceed a few seconds toward Hg^{2+} at 10^{-12} M. However, the initial rapid stage of mercury adsorption took 40 s to reach the saturated stage. Once external sorption reached the saturated stage, internal mercury uptake began; cations were transported across the cell membrane into the cytoplasm and a beta-HgS precipitation took place, inducing conductivity biofilm decrease, and generating an increase of density, and thus a frequency decrease. SEM images revealed the beginning cell damage at 10^{-06} M of cadmium and mercury.

Introduction

Environmental contamination with heavy metals has increased throughout the world due to the disposal of hazardous effluent into receiving waters [1,2]. Humans and animals are placed highly in the food chain and in particular the marine food chain. Non-biodegradable, heavy metals accumulate in photosynthetic organisms and transfer pollutants to consumers, including humans [3,4]. Indeed, substances such as cadmium or mercury have been classified as "priority hazardous substances" in Decision No. 2455/2001/EC [5] and Directive 2008/32/CE [6] for which industries should implement the necessary measures in order to reduce human anthropic activity. This is to preserve ecosystems. The U.S. Environmental Protection Agency's Roadmap for Mercury (July 5, 2006) promotes the reduction of mercury in processes and products. The overall goal of the Global Mercury Partnership of the United Nations Environment Program (Governing Council Decision 25/5, Nairobi, Kenya, February 16–20, 2009) is to reduce and eventually eliminate mercury use in products and processes and raising awareness of mercury-free alternatives. Quality control of aquatic ecosystems requires tools of in situ continuous detection of contaminated environments, such as electrochemical [7] and electromechanical [8] platform detection.

Biosensors that are emergent micro-technologies and characterized by their small size, rapid response would allow continuous in situ toxicity monitoring. Recently, the development of whole-cell biosensors has raised an increase 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 [9–12]. Acoustic biodetection platform based on the bacteria *Escherichia coli*, has been developed for heavy metals detection in liquid medium [8]. For the same purpose, a dried

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biomass of cyanobacteria, *Arthrospira platensis*, called Spirulina, was used, in the present study.

The choice of Spirulina was based on two principal reasons: the first is that its biomass is environmental friendly, easier and harmless when manipulated. The second is that Spirulina has never been used as a bioreceptor for biosensors destined to directly detect pollutants. Most previous work employed algal and bacteria biomass to extract heavy metals from effluent solutions and for bioremediation. In fact, some microalgae and cyanobacteria species (such as Spirulina) can bind a wide range of heavy metals in contaminated ecosystems [13–16]. Spirulina is a Gram negative bacterium, also considered as blue green microalgae. Components found in the cell wall of Spirulina, such as peptydoglycan, teichuronic acid, teichoic acid, polysaccharides and proteins [17] which display mainly carboxylic, hydroxyl and phosphate groups [18,19] may give algal wall binding properties. The cell wall of *A. platensis* has lots of negative carboxyl and phosphate groups, which are the dominant binding sites of toxic and metallic cations [20,21]. Furthermore, it has been found that microalgae possess a large surface area and high binding affinity [22], hence, they are a very effective biosorbents.

The results in this study help to provide an insight into the different interactions of dried biomass of *A. platensis* toward metallic cations using a combination of electrochemical, acoustic and microscopic tools. These three techniques have been chosen for specific reasons: first, admittance spectroscopy is a powerful tool for the study of dynamic electrical properties of dielectric materials [23]. Second, we applied acoustic wave platform in order to perform real time monitoring of the interaction of a heavy metal solution in contact with Spirulina biofilm. This will induce changes of its viscoelastic parameters. Due to their high sensitivity to surface perturbations and their transverse wave type (Shear horizontally polarized surface guided waves), sensors based on Love waves are ideally suited for (bio)chemical applications in gases and liquids [24]. The acoustic wave delay-line was inserted in an oscillation loop and associated with a Polydimethylsiloxane (PDMS) chip, resulting in a small platform convenient for fast detection. Therefore, admittance and acoustic characterization was carried after each injection of heavy metals. Third, microscopic tools (AFM and SEM) were used as complementary techniques in order to provide information of the observed Spirulina cells (shape, size, biovolume, etc.). AFM is a powerful imaging tool that mechanically probes a surface with a high resolution to give morphological (or topographical) details of sample surface. It can also provide information about the mechanical surface properties at the local scale such as viscoelasticity, chemical composition and morphology evolution [25–30]. The AFM can also be used to capture dynamic aspects of individual biological molecules [31–36] and their interactions with the environment [37,38] providing new insights into how macromolecules may work on the nanometer scale. AFM images were achieved to detect a change in Spirulina cells by measuring the elastic modulus via force curves at high concentrations of metallic cations. SEM was performed on Spirulina cells in order to characterize heavy metals effect at low concentrations (10^{-12} M). This technology allows the observation of microstructural changes of biological samples in their natural state, under controlled conditions of temperature and pressure.

Spirulina cells were immobilized on the electrode surface (Si/SiO_2) and on Love wave sensors with a silicon oxide surface, via a polyelectolyte multilayers (PEM) using a layer by layer (LBL) method. The LBL assembly technique consists in the alternate deposition of polyanions and polycations from aqueous solutions to build ultrathin multilayered films on flat substrates [39]. Currently these films are intensely studied because of their many potential applications [40,41] and recently, PEM were used to immobilize *E. coli* on Si/SiO_2 substrate [8].

Materials and methods

Chemical and biological

Two types of polyelectrolytes (PE) were used: polyallylamine hydrochloride (PAH), a cationic type, and polysodium 4-styrenesulfonate (PSS), an anionic type. PAH and PSS have the molecular weight of about 56,000 and 70,000 respectively, and they were purchased from Sigma–Aldrich. Solutions of PE (5 mg/mL) were prepared in TBS (Tris Buffered Saline) solution (pH = 7.2 at 0.15 M).

The stock solutions (1 g/L) of cadmium (Cd^{2+}) and mercury (Hg^{2+}) as heavy metals, were prepared from $Cd(NO_3)_2{\cdot}(H_2O)_4$ and $Hg(NO_3)_2{\cdot}(H_2O)$ in TBS, purchased from Sigma–Aldrich. Stock solutions were stored at 4 °C and dilutions were prepared before each series of measurements.

A. platensis (Compere 1968/3786 strain) or Spirulina, was cultivated under sterile conditions in Zarrouik liquid medium containing: (g/L) $NaNO_3$, 2.50; K_2HPO_4, 0.50; $NaHCO_3$, 10.00; NaCl, 1.00; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.02; and $FeSO_4{\cdot}7H_2O$, 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.

Love wave delay lines

The Love wave sensor, resulting from previous studies [42], is an electromechanical sensor based on a SAW (surface acoustic wave) delay line with metalized interdigital transducers (IDT) to generate and receive an acoustic wave on a piezoelectric substrate (Fig. 1a). It consisted of a dual delay line deposited on AT cut quartz substrate (Euler angles: 0°, 121.5°, and 90°) used as the piezoelectric material. IDTs were made by sputtering 70 nm of gold on top of a 30 nm titanium adhesion layer to achieve a good surface adhesion (Fig. 1a). Each IDT was composed of 44 split pairs of electrodes with a 40 μm periodicity which defined the wavelength λ. Each electrode was 5 μm wide with an aperture w of 40λ, while the center to center path length between electroacoustic transducers (L_{cc}) was equal to 209λ. A 4 μm plasma enhanced chemical vapor deposited (PECVD) SiO_2 layer was used as the guiding layer. It generated a guided shear horizontal surface acoustic wave (guided SH-SAW), also called Love wave. This confinement of the wave energy near the surface maximized the sensor sensitivity. It also ensured mechanical isolation of electrodes from biological samples. These characteristics led to a 117 MHz synchronous frequency f_0.

The obtained Love wave delay lines were inserted into an oscillation loop. Physicochemical perturbation onto the sensor surface will alter the wave velocity, which can be measured with high accuracy through the frequency shifts of the oscillation loop of a radio frequency amplifier. This leads to the achievement of an oscillator with ultra-high stability, which had a considerable impact on the detection threshold of the Love wave sensor. This oscillator reached a short-term stability lower than 1 Hz/s at working frequencies higher than 100 MHz. The resonant frequency