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CANTILEVER NANOBIOSENSOR BASED ON THE ENZYME UREASE FOR DETECTION OF HEAVY METALS

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Abstract - Cantilever nanobiosensors have emerged as an alternative to conventional methods of analysis to monitor heavy metals, which are being highlighted by detecting substances at the micro and nanoscale through the use of sensor layers. In the present study, a new cantilever nanobiosensor was developed functionalized with urease enzyme by self-assembled monolayers for the detection of heavy metals (lead, nickel, cadmium, zinc, cobalt, and aluminum) in water. From the calibration curves, using the statistical method of Principal Component Analysis, it was possible to observe that the nanobiosensor was highly efficient in heavy metal discrimination. The nanobiosensor presented high sensitivity, good stability, and a detection limit in the ppb range during 30 days of storage. The surface characterization by scanning electron microscopy confirmed the cantilever functionalization and the sensing layer deposition. The cantilever nanobiosensor designs based on tensile surface stress measured by changes in voltage suggest the proposed mechanism of the adsorption of heavy metal by the urease enzyme. Thus, the cantilever nanobiosensor with urease enzyme was able to detect heavy metals in water and could be a promising tool for heavy metal detection.
Keywords: Detection limit; Sensitivity; Storage; Functionalization.

INTRODUCTION

Heavy metals may occur in the aquatic environment in the ionic form, or in the form of organic or inorganic soluble complexes, remaining retained in the sediment and incorporating into the biota (Schulz et al., 2015). The ions are non-biodegradables pollutants, ubiquitously distributed, leading to a greater risk to human health and the environment. The main sources of heavy metal pollution are industrial effluents, mining and agricultural crops. Agriculture is the most affected field through the application of agrochemicals, where the heavy metals are released into the soil and leached to the rivers. This contamination persists in the aquatic environment, causing other environmental problems (Akpor et al., 2014; Vinodhini and Narayanan, 2008).

The problems involved in water body contamination by heavy metals clearly establish an urgent need for fast, sensitive and reliable methods for detection and treatment of these compounds (Kuang et al. 2016; Lin et al. 2017). According to the World Health Organization (WHO) heavy metal ions, based on their toxicity, are considered to be "Environmental health hazard" substances (Aragay et al., 2011; Ohanian, 1991). To avoid complications with conventional metal detection methods (flame atomic absorption spectrometry, inductively coupled plasma atomic emission spectrometry, among others), such as

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delayed responses, the need for large quantities of sample and highly trained professionals, there have appeared the cantilever nanobiosensors functionalized with biological material specific to detect the analyte of interest (Martinazzo et al., 2018; Muenchen et al., 2018).

Advances in nanobiosensors for heavy metal detection directly imply a better control of industrial processes, the earlier and accurate detection of contamination problems, the real-time monitoring of treatment procedures and remediation (Faria-Tischer and Tischer, 2012; Ejeian et al., 2018; Frutos-Puerto et al., 2019). The cantilever nanobiosensors are very versatile devices in terms of their detection and monitoring, require a small amount of sample, are easy-to-use, ultrasensitive, miniaturized and present fast-response (Muenchen et al., 2016). One of the benefits of using cantilever nanobiosensors is the ability to tailor the size and structure of the device (Steffens et al. 2012; Steffens et al. 2014a). Disadvantages are disturbance of fluid medium during the measuring and control of temperature (Lang and Gerber, 2008). They also present the facility of multi-agent detection, since they convert a biomolecular event into a specific measurable quantity. The concentration of the analyte can be determined from the deflection (measured by the pressure produced by the analyte on only one side of the cantilever surface) or by the frequency difference (measured by the mass change obtained by the oscillation of frequency) (Cherian et al., 2003; Steffens et al., 2014; Rotake and Darji, 2018).

The choice of biological material is one of the major challenges for nanobiosensor construction, where the sensitive layer allows a high selectivity for particular analyte species. In addition, adapt and maintain biological activity is a challenge (Nugaeva et al., 2005). The use of a tool (principal component analysis) and techniques of surface modification give rise to highly selective heavy-metal ion device systems (Aragay et al., 2011; De Benedetto et al., 2019). The enzyme urease is one promising biological sensing layer, specific and able to recognize many heavy metals (mercury, cadmium, cobalt, lead, etc.) by their inhibition resulting from the reaction with sulfhydryl groups of the active site. Urease (EC 3.5.1.5) presents the advantages of low cost and high sensitivity (Berezhetskyy et al., 2008; Cherian et al., 2003; Pogorilyi et al., 2016).

In this context, the objective of this study was to develop a new cantilever nanobiosensor functionalized with the enzyme urease by the self-assembled monolayer (SAM) technique to detect heavy metals (lead - Pb, nickel - Ni, cadmium - Cd, zinc - Zn, cobalt - Co and aluminum - Al) in different concentrations (0.01 to 50 μg/L). The nanobiodevice was also applied in river water to investigate the detection of heavy metals. The nanobiosensor surface was characterized during the functionalization steps, and the sensitivity, detection limit, stability and reversibility obtained in the presence of heavy metals.

MATERIALS AND METHODS

Reagents

Isopropyl alcohol, 16-mercaptohexadecanoic acid (16-MHD), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sigma–Aldrich (USA), and were used without further purification. The enzyme urease was obtained commercially from Sigma Aldrich (USA) with 75.26 U/mg. The urease used in the immobilization was diluted in 0.2 M sodium phosphate buffer pH 7.0. Each heavy metal (Pb, Ni, Cd, Zn, Co and Al) working solution containing the background solution for the calibration curves was prepared by dilution of standard stock solutions. The standards were purchased from Alamar Tecno Científica (99% purity, Brazil). Ultrapure water (≥18 MΩ cm) was used throughout the experiments.

Cantilever nanobiosensor functionalization

The cantilever used for the development of the nanobiosensor was commercially purchased from BudgetSensors (ContGD-G, Switzerland), with a resonance frequency of 13.0 kHz and spring constant of 0.2 N/m. The cantilever material was made with silicon, coated with 70 nm of gold

The cantilever functionalization was performed on the upper side using the self-assembled monolayer (SAM) technique, by crosslinking agents (EDC/NHS) for covalent attachment with alkanethiol molecules, based on Velanki and Ji (2006) and Martinazzo et al. (2018). After surface cleaning with isopropyl alcohol, the cantilever was immersed in a 2 mM 16-MHD solution overnight, then, immersed in a solution containing 1 mM EDC/NHS (1:1, v/v) for 10 min. Next, urease was immobilized with the enzyme solution for 10 min, washed with ultrapure water (Millipore) and dried in a desiccator at 4°C.

Heavy metal detection

The performance of the cantilever nanobiosensor (calibration curves) with the heavy metals (Pb, Ni, Cd, Zn, Co and Al) in different concentrations (0.01; 0.1; 1; 10 and 50 μg/L) was studied based on current legislation for river water and drinking water (Brasil, 2011, 2005, 2004). The heavy metal solutions were prepared in ultrapure water, separately. The cantilever nanobiosensor response was evaluated by the change in the deflection of the coated and uncoated (reference) cantilever in an atomic force microscope - AFM (Nanosurf - C3000 Controller, Switzerland).

The deflections were measured in voltage, through monitoring of the laser beam position and obtaining an image in contact mode, resolution 1800 x 1800 points. All measurements were performed in triplicate.

For heavy metal analysis, 0.5 mL of each concentration (0.01; 0.1; 1; 10 and 50 µg/L) was injected with a Pasteur pipette on a stainless steel plate in the AFM, so that the cantilever nanobiosensor was fully immersed in this drop for reading the voltage. After the reading, the images were converted to a voltage (V) x contact time (min) graph using the Gwyddion software (Version 2.45). As response sensitivity and limit of detection were obtained. Additionally, the stability of the sensor response was determined during the storage time (30 days) in a refrigerator at 4°C.

The experiments were performed at a constant temperature (25 ± 0.2°C), which was maintained through a cooling system in the AFM chamber, assembled on a Peltier pellet.

Characterization of the cantilever nanobiosensor surface

Scanning electron microscopy (SEM) (JEOL, model - JSM6510, Japan) was used to characterize the surface of the nanobiosensor, being performed at each stage of cantilever functionalization.

Principal Component Analysis (PCA)

The response of the cantilever nanobiosensor to the heavy metals was evaluated by the statistical method of Principal Component Analysis (PCA) using OriginPro 5.0 (© Origin Lab Corporation) software. The multivariate analysis was performed to evaluate the discrimination between the different heavy metal responses in voltage. PCA is a projection method that allows one to observe all the information present in a database, assisting in the recognition of differences in samples and the variables. The score plots of PCA represent in a bi-dimensional or tri-dimensional plane the linear combinations of the original nanobiosensor voltage signal and results closer together indicate more similar samples in relation to the heavy metal and concentration (Santonico et al. 2008). The discriminative detection was achieved in terms of the correlation between the heavy metal concentration and voltage signal.

RESULTS AND DISCUSSION

Characterization of the cantilever nanobiosensor surface

Fig. 1 presents the micrographic images obtained at high resolution by SEM of the cantilever surface during the coating steps. Fig. 1A corresponds to the cantilever surface without coating, where a uniform surface can be observed. With the deposition of

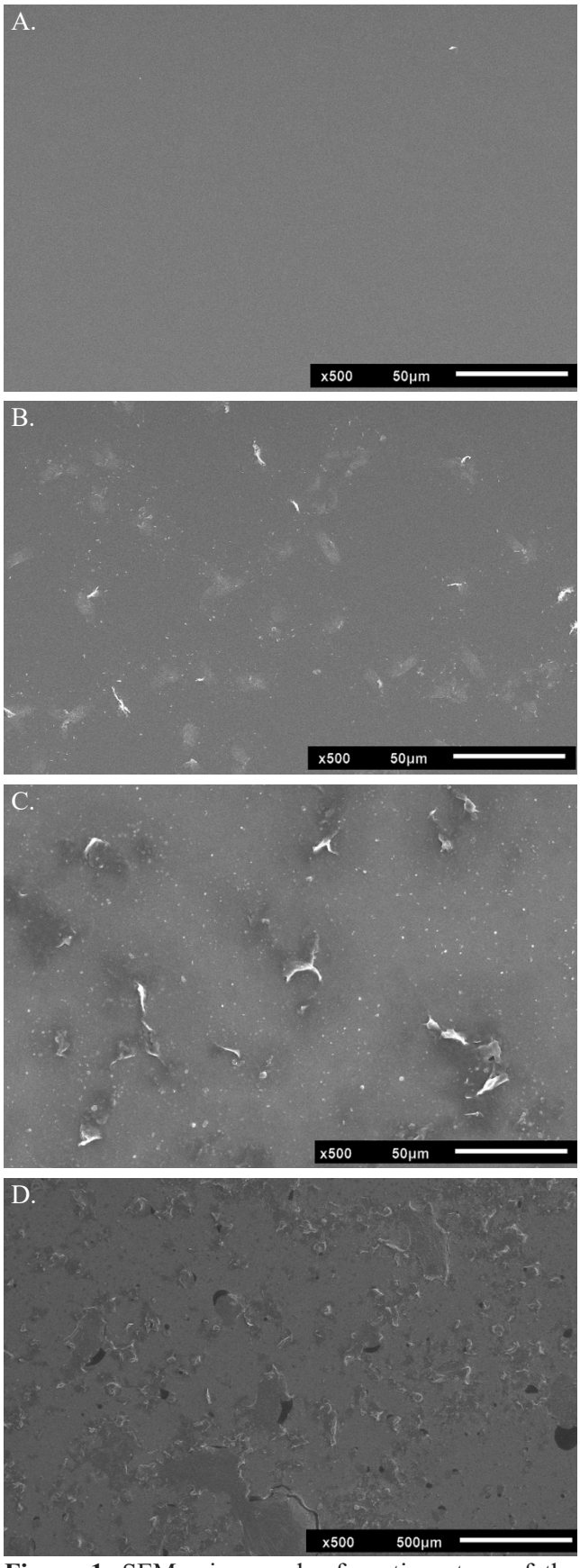

Figure 1. SEM micrograph of coating steps of the cantilever surface (A) gold (B) 16-MHD (C) EDC/NHS and (D) urease enzyme.

16-MHD (Fig. 1B) and EDC/NHS (Fig. 1C) on the surface, a formation of scattered clusters was observed. This change in the surface can be due to groups of the 16-MHD conjugate with the gold layer (over the cantilever surface) by covalent bonding. The amine ($–NH_3^+$) groups change the surface with a well distributed surface layer, and with the enzyme urease (Fig. 1D) a more uniform surface deposition is observed. In this way, the SAM technique resulted in a homogeneous layer on only one side of the sensor, which is very important, due to the control of adsorption of molecules on the surface.

Detection of heavy metals by the cantilever nanobiosensor

Fig. 2 shows the detection response of the cantilever nanobiosensor functionalized with urease enzyme to the heavy metals as a function of time. Each data point represents the average voltage signal of the cantilever nanobiosensor. The response was different between the concentrations of heavy metals and ultrapure water, revealing that it can detect the presence of heavy metals in the medium. The difference in the voltage response between the concentrations studied can be due to the absorption of molecules on the

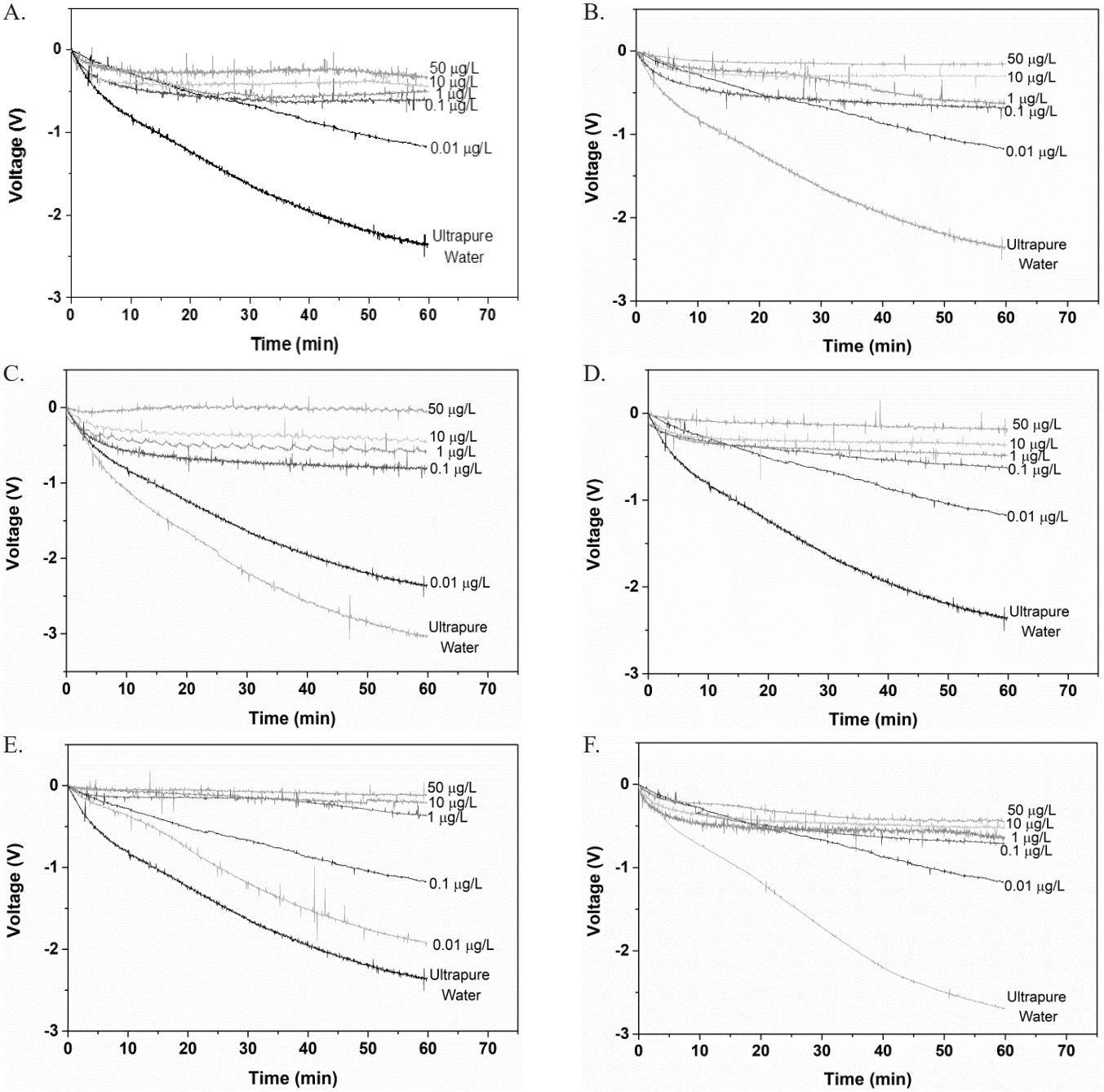

Figure 2. Response of the nanobiosensor functionalized with the enzyme urease to heavy metals (A) Pb, (B) Ni, (C) Cd, (D) Zn, (E) Co and (F) Al.

surface, resulting in a cantilever deflection. As the cantilever surface was functionalized in the upper side with gold, 16-MDH, EDC/NHS and enzyme, the reaction of the heavy metals with the enzyme resulted in a downward bending due to the surface stress in the cantilever. According to Bhushan (2010) the cantilever functionalized with the SAM technique on the upper side will cause a downward bending in a presence of an analyte, and called this process compressive surface stress. Lang and Gerber (2008) reported that the absorption of molecules on the surface (functionalized on only one side) will result in upward or downward deflection due to tension between the functionalized and non-functionalized sides. The heavy metals recognition by the immobilized enzyme (specific binding to each heavy metal) on one side of the cantilever causes a change in surface stress that induces cantilever deflection.

Urease is a common biocatalytic receptor, which can recognize heavy metals through the mechanism of inhibition of the enzyme, oxidative stress, or impaired antioxidant metabolism based on the interactions between heavy metal and chemical groups present in the active center of the enzyme. Also, the metal ions have a toxic effect on enzymes due to their binding to thiol groups next to the active site/center, resulting in an irreversible inhibition (Ogończyk et al., 2005; Bagal-Kestwal et al., 2008). In this way, Gabrovska and Godjevargova (2009) stated that the reduction of urease activity in the presence of different concentrations of heavy metals can be used for quantitative analysis of these metals in solution. Also, Gabrovska and Godjevargova (2009) utilized a reduction of the urease activity to determine the concentrations of heavy metals, and obtained the following sequence of activity inhibition: Cu> Cd> Zn> Ni> Pb.

Based on the heavy metal detection results, a schematic mechanism was constructed using the cantilever nanobiosensor obtained by the SAM technique (Fig. 3) to explain the mechanism of detection. The cantilever was functionalized with chemical modification on the surface (70 nm of gold, 16-MHD) using self-assembled monolayers (SAM); the thiol group (-SH) located at one end of the 16-MHD adsorbs to the gold surface, while the carboxyl group present at the other end is available. To covalently immobilize the urease enzyme on this cantilever surface, the carboxyl groups of 16-MHD were coupled to the primary amine groups present in the structure of the enzyme with the EDC and NHS crosslinking agents. Then NHS is added to increase the efficiency, bound with the primary amine present in the enzyme by the carboxylic acid (Janegitz et al., 2011). The heavy metals present in the water solution bind to the active site groups of the urease enzyme by reacting

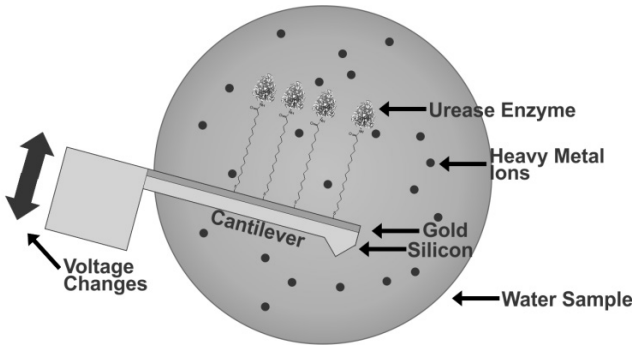

Figure 3. Schematic representation of the heavy metal detection by the cantilever nanobiosensor.

with the sulfhydryl groups, causing a stress tension on the cantilever surface, resulting in a deflection that was measured by the voltage change of the cantilever nanobiosensor. Also, the mechanism of heavy metal ions can be related to enzyme inhibition, oxidative stress or impaired antioxidant metabolism (Bagal-Kestwal et al., 2008). The mechanism that governs the surface stress generation with functionalization of biological molecules on cantilevers is attributed to an expansion (compressive surface stresses) influenced by electrostatic repulsion of surface groups, and conformational changes in the enzyme result in formation of ordered structures, bending the cantilever upward to the tensile direction (Buchapudi et al., 2012; Cherian et al., 2003; Karnati et al., 2007).

The responses of the cantilever nanobiosensor functionalized with the urease enzyme were analyzed by the PCA statistical technique, which is a pattern recognition technique that reduces the number of variables with redundant information and uses only the most important data (Rañola et al., 2016). The PCA plot in Fig. 4 shows the response of the cantilever nanobiosensor obtained in the detection of heavy metals. The analysis of the discrimination of different

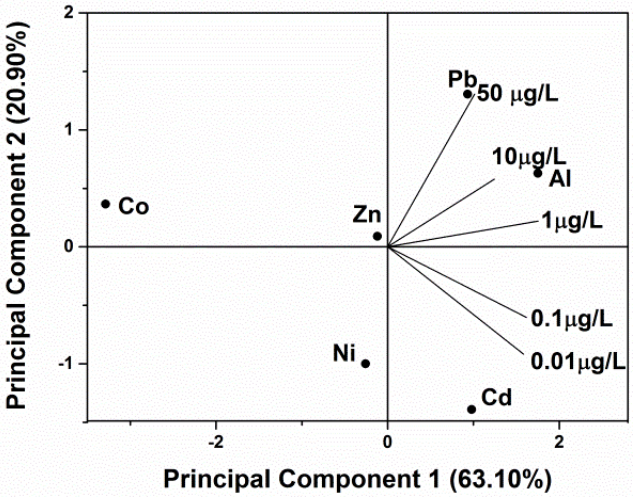

Figure 4. PCA plot obtained from the cantilever nanobiossensor response to the heavy metals.

concentrations of heavy metals was evaluated on the *Loading plot*.

The PCA plot (Fig. 4) shows the distribution of the heavy metals in separate quadrants of the plot and describes the greatest amount of sample variability. Furthermore, the variances of the first two main components (PC1 and PC2) account for 84 % of the total information collected. This projection (PC1 versus PC2) plot translates similarity of the total variation of the samples. PC1 presented a great amount of information (63.10%), and therefore the analysis of heavy metals should be based on this axis; although small, the contribution of PC2 should be considered for analysis. In this case, samples close to each other on the principal component plot resemble each other, indicating in this way the difference between the heavy metals. The amount of information in PC1 and PC2 is relevant, and therefore both contribute to the analysis in the Cartesian plane, in which case it is possible to discriminate the heavy metals using the cantilever nanobiosensor.

The limit of detection and sensitivity values for the heavy metals of the cantilever nanobiosensor functionalized with the urease enzyme were evaluated at 1 and 30 days of storage (Fig. 5 A and B). In general, an increase of the limit of detection value, and a decrease in the sensitivity were observed, from the first to 30 days of storage.

The values of the limit of detection ranged from 0.286 ppb (0.286 µg/L) for Pb and 0.751 ppb (0.751 µg/L) for Co. Taking in account the maximum limits (Brasil, 2011, 2005, 2004) allowed for these heavy metals in drinking water, which are in the range of 1 to 5000 ppb, it is observed that the cantilever nanobiosensor can detect these heavy metals.

The maximum admissible limit set by the United States Environmental Protection Agency (U.S.E.P.A., 2008) to drinking water contaminants for heavy metals (ppb or µg/L) are 15 Pb, 100 Ni, 5 Cd, 5 Zn, 100 Co, and not mentioned for Al. The World Health Organization (World Health Organization, 2011) stipulated limits of 10 for Pb, 20 for Ni, 3 for Cd, 3 for Zn, 5 for Co and 100 µg/L for Al in drinking water. According to the limits of these agencies, the cantilever nanobiosensor can detect the maximum allowed contaminant in drinking water.

The sensitivity of the cantilever nanobiosensor obtained by the angular coefficient of concentration versus voltage (calibration curve), showed that the device presented high values for Pb. It is worth noting that the higher the sensitivity values, the more sensitive are the sensors and the better their responses. In this sense, the nanobiosensor developed in this study shows great potential for the detection of heavy metals in water.

The useful life of a sensor is extremely important since, depending on the situation, the ideal is that

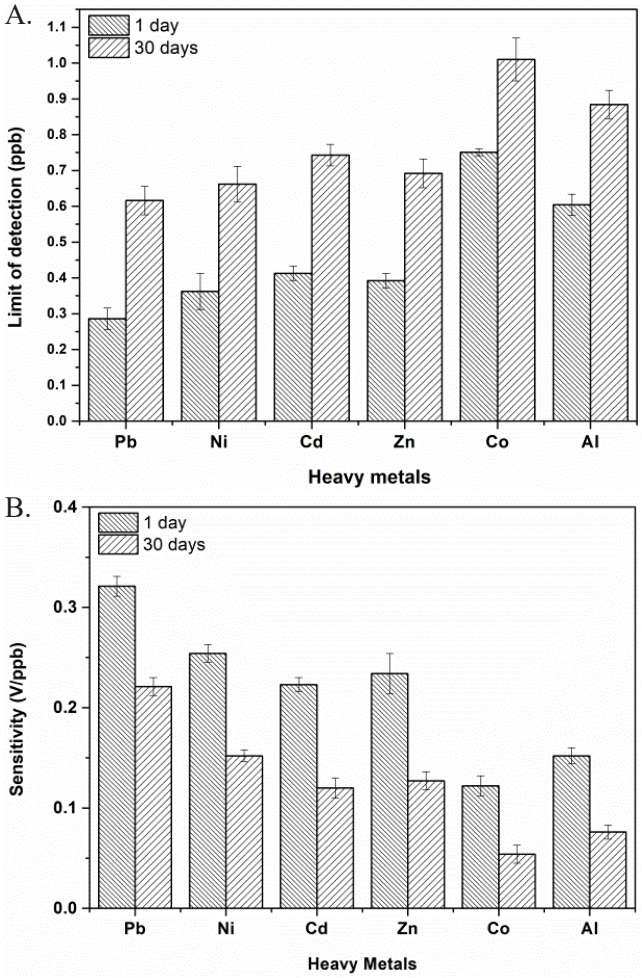

Figure 5. Detection limit (A) and sensitivity (B) of the cantilever nanobiosensor to the heavy metals during the storage.

the sensor can be used longer and maintains the characteristics in relation to the sensitivity and limit of detection. In Fig. 5 it is verified that after 30 days of storage an increase in the limit of detection occurs, and a decrease in the sensitivity, but with very low values. Decreases in the stability of nanobiosensors after storage have also been reported in the literature. Upadhyay and Verma (2015), using a condutimetric biosensor functionalized with alkaline phosphatase, observed a decrease of 12% in the response after 30 days of storage at 4 °C in a buffer (Tris HCl, pH 9.0). Rodriguez et al. (2004), using urease and glutamic dehydrogenase enzymes in an amperometric biosensor, obtained detectable limits of Cu(II), Cd(II) and Zn(II) of 8.5 ppm, 0.3 ppm and 0.2 ppm, respectively. A sol-gel-immobilized-urease conductometric biosensor was applied to detect Cd, Cu and Pb, and observed a storage time of 6 to 7 days (Ilangovan et al., 2006).

For the cantilever nanobiosensor also evaluated was the reversibility to the exposure to the heavy metal and the return of the signal to its original condition after the end of the interaction with the

heavy metal and exposure to ultrapure water. For this purpose, the lower concentration of 0.01 µg/L was evaluated for each heavy metal. For all metals evaluated, the nanobiosensor presented reversibility above 98%, indicating that there is no loss of signal during the various measurements. A typical response is demonstrated for Pb in Fig. 6, since all the heavy metals showed the sample behavior of reversibility.

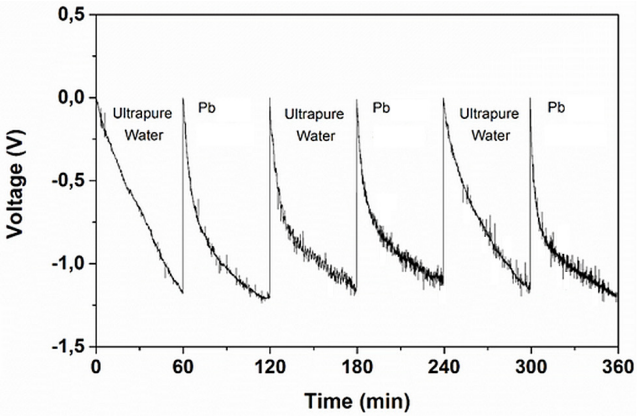

Figure 6. Reversibility of the cantilever nanobiosensor functionalized with urease enzyme to 3 cycles of exposure to Pb solution at a concentration of 0.01 µg/L and ultrapure water.

CONCLUSION

The performance of a nanobiosensor based on the immobilized urease enzyme proved to be an efficient tool for the determination of heavy metals. It is possible to verify, through the PCA analysis, a good discrimination between Pb, Ni, Cd, Zn, Co and Al in different concentrations. In addition, it showed limits of detection in the ppb range with high sensitivity. In relation to the characterization of the nanobiosensor surface by SEM analysis, it was possible to observe a homogeneous morphology with larger and more defined contours when functionalized with the enzyme. This framework highlights the significance of further functionalization of urease enzyme on cantilever nanobionsensors, and shows a bright prospect in practical applications for heavy metal detection and discrimination in water. Thus, we can say that the methodology developed is feasible and promising for the determination of heavy metal ions in water samples.

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