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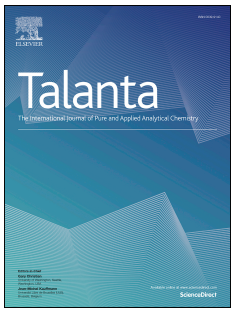

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A electrochemical biosensor for As(III) detection based on the catalytic activity of *Alcaligenes faecalis* immobilized on a gold nanoparticle–modified screen–printed carbon electrode

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A B S T R A C T

A electrochemical biosensor for As(III) determination has been developed by immobilization of the *Alcaligenis faecalis* bacteria on gold nanoparticle–modified screen–printed carbon (AuNPs–SPCE). The detection of As(III) is due to the catalytic activity of arsenite oxidase enzyme which oxidizes As(III) to As(V) producing an analytical signal. To enhance the performance of the biosensor, was optimized the amount of bacteria, amount of glutaraldehyde and incubation time applied in the preparation of the electrode, in addition to the effect of pH and applied potential. The analytical application was carried out applying 300mV (pH = 7) obtaining a LOD of 6.61µmol L^{-1} (R = 0.9975) and 700mV (pH = 12) obtaining a LOD of 1.84µmol L^{-1} (R = 0.9983). *AF*/AuNPs–SPCE was applied to the determination of total arsenic in Loa river water samples after reduction, with satisfactory results.

Keywords: *Alcaligenes faecalis*; As(III); Astotal; Water samples; Gold nanoparticle–modified screen–printed carbon electrodes.

1. Introduction

In the last decades, the field of biosensors has undergone enormous advances, with a great impact in the industrial area. The incorporation of enzyme cells, antibodies, DNA, microorganisms, among others, to analytical devices has allowed the specific and more sensitive determination of analytes of interest in the food industry, medical field, industrial sector, environmental sciences, etc. [1,2]. However, time consumed for

device development, stability, miniaturization, continuous and *in situ* monitoring in a complex matrix capacity and high fabrication costs prevent biosensors from entering the market massively [3]. These difficulties can be overcome looking for a simple development of immobilization methods and more robust biological materials. Along this line, the microbial life provides a wide variety of communities that have adapted to the most diverse and extreme conditions, preserving its biocatalyst functions. Among the microorganism extremophiles there are the metallophiles, which are microorganisms that grow in the presence of high metal concentrations, but despite this characteristic, the biotechnological application of enzymes is not always obvious [4,5]. Nevertheless, in view of the great potential of biocatalysts, this microbial community presents a niche rich for application in detection. Among this community, one of those studied in detail correspond to the microorganisms responsible for the respiratory oxidation of arsenite, As(III), to arsenate, As(V).

It is well known that the pollution of arsenic in the environment is a worldwide problem, and its toxicity is not only human but also microbial, the microorganisms involved in the redox transformations of arsenic are taxonomically very diverse and metabolically versatile gaining energy from oxidizing [6,7]. Arsenite functioning as an electron donor at the start of a membrane respiratory chain generating electron transfer, which provides a wide variety of applications [8–10]. The oxidative activity is the product of an enzyme called arsenite oxidase (Aro). The genes responsible for the encoding of this enzyme have been studied in species such as *Alcaligenes faecalis*, from which Ellis et al. [11] obtained the crystal structure. Aro corresponds to a 100 kDa protein, consisting of two subunits, a large one containing a molybdenum–pterin

center, and a small one of the Rieske type which contains a [2Fe–2S] center. It is located on the external surface of the internal membrane of this microorganism. Hoke et al. [12] studied arsenite oxidase from *A. faecalis*, showing that reduction or oxidation of the active site produces a two–electron signal associated with reversible reduction of the oxidized Mo(VI) from the molybdenum center to Mo(IV). Theses authors also showed that the catalytic current results from the continuous oxidation of the enzyme, following reduction by substrate. On the other hand, Male et al. [13] developed a biosensor for As(III) using Aro from chemolithoautotroph NT–26 by depositing enzyme galvanostatically onto a multiwalled carbon nanotube modified glassy carbon electrode reporting a LOD of 0.013µmol L^{-1} (E_{acc} = 300 mV, t_{acc} = 10 s). Prasad et al. [14] reported of a direct bacterial electron transfer reaction on a Screen–Printed Carbon Electrode (SPCE). These authors modified an SPCE with *Shewanella s.p.,* CC–GIMA–1, by means of a simple coating procedure, obtaining positive results when evaluating the capacity of bacteria for electrocatalytic responses with arsenite, hydrogen peroxide and nitrite, not reporting quantitative analytical data.

Different analytical methods are used to determine trace levels of arsenic in a variety of samples. Hydride generation atomic absorption spectrometry (HG−AAS), hydride generation atomic fluorescence spectrometry (HG−AFS), electrothermal absorption spectrometry (ET−AAS); inductively coupled plasma (ICP) with atomic emission and mass spectrometry (ICP−AES; ICP−MS) as detectors. Electroanalytical techniques have important advantages that include a low detection limit, excellent portability for field analysis, relative simplicity, low equipment cost compared to other techniques. However, satisfactory results depend on the choice of a suitable working

electrode. For the specific case of arsenic, it has been reported that anodic stripping voltammetry (ASV) using gold electrodes have been established as the most important and widely used technique for the determination of trace amounts of arsenic. Table 1 summarizes the reported electroanalytical methods for arsenic determination in various water samples (River water, lake water, sea water, tap water, mineral water, etc) using modified and unmodified electrodes.

The aim of this work was to study the microbial ability to metabolize metals and its capacity to generate a redox response of *A. faecalis*, we decided to prepare electrochemical biosensors by a simple procedure of immobilization of this bacteria onto different screen–printed carbon electrodes and later use them in the quantitative detection of As(III). The electrodes modified with the bacteria were: screen–printed carbon (SPCE), gold nanoparticles modified screen–printed carbon (AuNPs–SPCE), graphene modified screen–printed carbon (GPH–SPCE), and graphene–gold nanoparticles modified screen–printed carbon electrode (AuNPs–GPH–SPCE). After having optimized the methodology, and choose the electrode whose analytical signal is bigger we proceeded to apply it in the determination of total arsenic in water samples from Loa river (North of Chile), which is a zone extremely arid and the only permanent superficial water is from this river containing arsenic concentrations that reach until 291µmol L^{-1} [40]**.** This source provides water for nearby cities (tap water), mining activity, and agricultural use. The quality of the water from the Loa River is poor due to high salinity and high dissolved arsenic and boron, because its main tributary, the Salado River, has its origin in the geothermal field of El Tatio [41]. Therefore, arsenic detection is critical to the health care of the population in areas like

this.

2. Material and methods

2.1. Apparatus

Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and amperometric measurements were carried out with a CHI 600E electrochemical analyzer (CHI Instrument, Austin, TX), and corresponding software (CHI–852D). The electrochemical experiments were performed with a gold–nanoparticle modified screen–printed carbon electrode DRP–110GNP purchased from Dropsens and modified with *A. faecalis*) bacteria. The GNP–Screen–printed carbon electrode is an integrated three–electrode strip consisting of an AuNPs–carbon working electrode, a carbon counter electrode, and a silver pseudo–reference electrode. Other electrodes studied were a screen–printed carbon electrode (DRP–110), a graphene modified screen–printed electrode (DRP–GPH), and a graphene–gold nanoparticle–modified screen–printed carbon electrode (DRP–110GPH–GNP). For pH measurements, an Orion model 430 pH–meter was used, and to study the bacterial growth curve a UV–VIS (Milton Roy 3000 diode array spectrometer) was used.

2.2. *Reagents and materials*

Alcaligenes faecalis bacteria (NCIB8687/LMG3368) were obtained from BCCM/LMG Laboratorium Voor Microbiologie, Universiteit Gent (UGent). Glutaraldehyde 25% in water (CAS 111–30–8) was used for the enzyme immobilization and was purchased from Sigma–Aldrich. Standard solutions of arsenic were prepared by diluting a stock solution of 1000mg L^{-1} of arsenic standard for AAS

(in nitric acid) (TraceCert, Fluka Analytical, CAS 39436). All the chemicals (nitric acid, hydrochloric acid, 30% sodium hydroxide solution, etc.) were suprapur grade purchased from Merck, Darmastadt. Phosphate buffer was prepared with ptassium dihydrogen phosphate (KH_2PO_4) (CAS 7778–77–0) and dipotassium hydrogen phosphate (K_2HPO_4) (CAS 7758–11–4). The pH was adjusted with 4mol L^{-1} sodium hydroxide solution. Interference studies were carried out diluting standard solutions of Cd, Ni, Li, Al, Co, Mg, Be, Se, Fe, Sb, Mo, Ca, Zn, Tl, Mn, K, Bi, and Pb (1000mg L^{-1} CertiPUR. Merck). The method was applied to the determination of total arsenic in river water from the arsenic rich zone in the north of Chile (city of Calama). Ascorbic acid (CAS 50–81–7) was used as a reducing agent and was purchased from Sigma–Aldrich. All solutions were prepared with deionized water (LiChrosolv, Merck CAS: 7732–18–5).

2.3. Microbial growth conditions

Alcaligenes faecalis strains were grown on media composed of Difco nutrient broth (10gL^{-1}), K_2HPO_4 (25mmol L^{-1}), KH_2PO_4 (40mmol L^{-1}) and supplemented with $NaAsO_2$ 0.1% (w/v) at pH = 7. The culture was incubated at 30ºC under aerobic conditions, stirring at 150rpm. The turbidity of the culture broth was measured at 660 nm and cells were collected by centrifugation when absorbance was 1 (≈ 48 hours). They were then washed twice with sterile phosphate buffer solution 0.1mol L^{-1} pH = 7, and suspended in the same buffer by adjusting cell density at 1mgmL^{-1} wet weight [42].

2.4. *Preparation of electrochemical biosensors*

The bacterial suspension was homogenized by stirring and coated 5µL over the working electrodes surface, and dried at room temperature for 20min. After the incubation period, biosensors were cross–linked with 5µL (13µmol) of glutaraldehyde 25% in water and incubated for 15 min at room temperature. Then the SPCE was immersed in a phosphate buffer solution (PBS) (0.1mol L^{-1} pH = 7) during 30 min with constant stirring. They were finally stored at 4ºC until use.

2.5. Electrochemical measurements

Cyclic voltammetry (CV) was performed from –800 to 800mV with a sweep speed of 50mV s^{-1}. Impedance measurements were performed applying 100mV at an equilibrating time of 2s. Amperometric detection of As(III) was carried out at two different potentials: 300 and 700mV; in both cases an electrochemical cell (10mL) containing 0.1mol L^{-1} phosphate buffer at pH = 7 and 12, respectively, was used. The calibration curves, linear ranges, detection limit, and sensitivity of the method were obtained using the optimal parameters employed in the preparation of the electrochemical biosensor (*AF*/AuNPs–SPCE) and their application in As(III) detection. An optimized method was applied for the determination of total arsenic in water samples from the Loa River, Chile, (after reduction of As(V)), using the standard addition method in order to eliminate matrix effects and comparing with the classic calibration curve method.

2.6. Limits of detection and quantitation (LOD, LOQ)

The LOD was calculated using the approximation of Miller and Miller [43] for calibration curves with $X_{LOD} = 3\ \sigma_{XY}/b$, where σ_{XY} is the standard deviation of the calibration curve and *b* is the slope. The LOQ was calculated from $X_{LOQ} = 10\ \sigma_{XY}/b$.

3. Results and discussion

3.1. Amperometric responses and characterization of electrochemical biosensors

A biosensor was designed by entrapment of *A. faecalis* into glutaraldehyde, with the detection of As(III) due to the catalytic activity of enzyme arsenite oxidase (Aro) which generates the oxidation of As(III) to As(V) producing a two electron signal. Initially, the direct electrochemistry transfer of *A. faecalis* was studied by amperometric detection of As(III) in four different electrochemical platforms modified with 5µL of the bacterial suspension and unmodified. The signal was monitored by holding the biosensor at 300mV in PBS 0.1mol L^{-1} at pH = 7. The four electrodes studied were: screen–printed carbon (SPCE), gold nanoparticle modified screen–printed carbon (AuNPs–SPCE), graphene modified screen–printed carbon (GPH–SPCE), and graphene–gold nanoparticle modified screen–printed carbon electrodes (AuNPs–GPH–SPCE) (Fig.1a). The results proved that when SPCE was used in the absence and presence of *A. faecalis*, no oxidation signal of As(III) was seen. Likewise, when the unmodified GPH–SPCE was used, no oxidation signal of As(III) was seen either, however a very small signal was observed (0.2µA), when *A. faecalis* was immobilized on the electrode (*AF/*GPH–SPCE). Better results were obtained when the graphene electrode was modified with gold nanoparticles (AuNPs–GPH–SPCE), obtaining values of 0.5µA without bacteria and 1.2µA in the presence of *A. faecalis*

(*AF*/AuNPs–GPH–SPCE). This already shows us a positive effect of the presence of the bacteria and an increase in the presence of gold nanoparticles. Both effects are enhanced. However, when measurements are made only in the presence of gold nanoparticles, with and without bacteria, the effect on sensitivity is much more significant if graphene is not present. Comparing AuNPs–GPH–SPCE with AuNPs–SPCE electrodes the currents obtained were 0.25 and 0.5μA, while when *AF*/AuNPs–GPH–SPCE and *AF*/AuNPs–SPCE electrodes were used the currents obtained were 1 and 2.25μA, respectively. Comparing all the electrodes, the signals from highest to lowest were: (*AF*/AuNPs–SPCE) > (*AF*/GPH–AuNPs–SPCE) > (AuNPs–SPCE) > (GPH–AuNPs–SPCE) > (*AF*/GPH–SPCE) > (GPH–SPCE) = (SPCE) = (*AF*/SPCE) (Fig. 1a). The results prove that the *A. faecalis* has an electroactive nature, generating a signal corresponding to oxidation of As(III), giving the best sensitivity when AuNPs–SPCE was used, so it was selected for further measurements, showing that AuNPs generated a more biocompatible medium with the bacteria. It has been reported that gold nanoparticles deposited on different electrode materials have been widely used for the detection of metals and metalloids as arsenic, the effective electrode surface area not only promoting electron transfer between analyte and electrode, but also enhancing the sensitivity and catalysis of the redox reaction. On the other hand, the gold nanoparticles do not inhibit the behavior of the bacteria, enhancing both effects. In the presence of arsenic the formation of stable inter–metallic of Au–As compounds has been reported, allowing its detection [44–48]**.**

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS)

measures were used to characterize the biosensors. The analyses were carried out in 5mmol L^{-1} $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ containing 0.1mol L^{-1} KCl. Fig. 1b shows the CVs of SPCE, AuNPs–SPCE, and *AF*/AuNPs–SPCE. Although the currents are similar, the signal with AuNPs–SPCE is slightly higher. On the other hand, the potential values E_{pa}/E_{pc} were 114/5, 239/26, and 242/6 mV/mV for *AF*/AuNPs–SPCE, AuNPs–SPCE, and SPCE, respectively. As can be seen in these CVs, when immobilizing the *A. faecalis* in a gold nanoparticles modified screen–printed carbon electrode (*AF*/AuNPs–SPCE) the reversibility of the model system $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ increased markedly and the signal was shifted to less positive potentials. Fig. 1c shows the Nyquist diagrams obtained for the same electrodes. As can be seen, the magnitude of the electron–transfer resistance is greater in SPCE, decreasing when the electrode is modified with AuNPs. In the presence of bacteria (*AF*/AuNPs–SPCE) there is a marked decrease in the diameter of the semicircular zone, evidencing that the bacteria do not block the electronic transfer.

3.2. Optimization of the preparation conditions of the electrochemical biosensor. AF/AuNPs–SPCE

The optimum conditions of biosensor preparation were investigated through the oxidation current response of the 200µmol L^{-1} As(III) solution applying 300mV, PBS 0.1mol L^{-1} at pH = 7. The variables studied were amount of cell suspension, amount of glutaraldehyde and incubation time.

3.2.1 Optimization of amount of cell suspension over AuNPs–SPCE

The influence of the amount of bacteria on the amperometric response of As(III) was evaluated by varying the volume of bacterial suspension deposited on the electrode. This parameter was studied in the 1 to 7μL range. The current increased by adding from 1 to 5μL and then slightly decreased by adding 7μL, probably due to the fact that a greater number of microorganisms overlap each other, hindering electronic transfer (Fig. S1). On that basis, a volume of 5μL of bacterial suspension was used for further studies.

3.2.2 Optimization of the amount glutaraldehyde and incubation time over AF/AuNPs–SPCE

A. faecalis was immobilized on electrode surface by cross–linking with glutaraldehyde due this agent can react with several functional groups (such as amine, thiol, imidazole, amino acids) [49]. The cross–linking with bacteria is possible to occur with functional group present in periplasmic proteins like arsenite oxidase [9].

The volume of glutaraldehyde 25% (GA) was evaluated in the 1 to 10 μL range (2.6 to 26 μmol), and the incubation time was chosen from 0 to 30min. The highest current was obtained when coating the electrode surface with 13μmol of GA and incubating for 15min. Small GA volumes are not sufficient to immobilize completely the bacteria dispersed in the wash step, while larger volumes block electronic transfer. On the other hand, incubation times of less than 15min are not enough to complete the fixation and longer times showed no effect on the arsenic signal current (Fig. S2a and Fig. S2b).

3.3. Optimization of AF/AuNPs–SPCE experimental conditions

3.3.1. Influence of the applied potential

The influence of the applied potential on the amperometric response was studied in 100 to 800 mV range using 200µmol L^{-1} As(III) solution. Fig. 2 shows two maximums signal at 300 and 700mV. Initially, an increase in the current was seen at 300mV, following a decrease until 500mV; subsequently, the current showed a rapid increase that attained the maximum signal at 700mV, decreasing again to highest potentials. Male et al. [13] made experiments with arsenite oxidase under similar conditions in terms of potential, pH, and buffer type, but this effect was not reported. On the other hand, Silver and Phung [9] have described an arsenic gene island associated with the arsenic resistance in *A. faecalis* (NCBI AY297781), so it is not possible to attribute with certainty that this effect is due only to the enzymatic activity of arsenite oxidase without considering an inorganic arsenic metabolism in *A. fecalis*. However, considering only that arsenic oxidation by Aro, applying a potential of 300mV, an amperometric response of oxidation for these conditions was described by Male et al. [13] (Fig. 3a). Regarding to the amperometric response obtained at 700mV, it is possible that the applied potential would change the electronic transfer pathway generating a response corresponding to the reduction of Mo(VI) to Mo(IV) as described in Fig. 3b.

3.3.2. Effect of pH

The effect of pH was studied in the 5 to 12 range using phosphoric acid 0.1µmol L^{-1} and adjusting the pH with 4µmol L^{-1} of sodium hydroxide solution. The study was

carried out at both potentials: 300 and 700mV. As can be seen in Fig. 4, the current increased until pH = 7 in both cases. When 300mV were applied, the amperometric response decreased to higher pH values, while when 700mV was applied, the response showed a maximum pH = 12. Ellis et al. [11] reported two types of conformation for Aro, P1 and P2, whose formation depends on the pH. At pH = 8.5 P2 is formed and at pH = 6.4 structure P1 is formed. These conformations differ in the dimensions of the unit cells that compose it, increasing the distance at higher pH. Considering this, it is possible that by increasing the distances between the enzyme subunits there is a probability that the electrons corresponding to the reduction of Mo(VI) to Mo(IV) are transferred directly to the surface of the electrode, generating a reduction response. However, this interruption of the electron transfer pathway has a negative effect on the respiratory chain of *A. faecalis*, causing instability in Aro until loss enzymatic activity and the subsequently death of the microorganisms.

3.4. Analytical performance of the AF/AuNPs–SPCE *biosensor*

The analytical performance was realized applying the optimal conditions of preparation of *AF*/AuNPs–SPCE the electrochemical biosensor. The linear range, the calibration curves of As(III), and the method's sensitivity were studied using the two conditions described: PBS of pH = 7 applying 300mV (methodology 1, M1), and PBS of pH = 12 applying 700mV (M2).

When 300mV (pH = 7) were applied, the results showed that the current is proportional to the As(III) concentration over the range 6.67 to 200µmol L^{-1} (Fig. 5a). However three zones with different slopes and linear relationships are seen: the first

concentration range was evaluated from 6.67 to 53µmol L^{-1}, obtaining a correlation coefficient of 0.8652; the second concentration interval was evaluated from 53 to 140µmol L^{-1}, obtaining a sensitivity of 1.845 x 10^{-8} ± 2.782 x 10^{-10}µA/µmol L^{-1}, intercept was –7.668 x 10^{-7} ± 2.870 x 10^{-8}µA/µmol L^{-1}, LOD and LOQ of 6.61 and 22µmol L^{-1}, respectively (R = 0.9975). For the higher concentration, the range was evaluated from 140 to 200µmol L^{-1}, the sensitivity was 4.023 x 10^{-9} ± 7.132 x 10^{-10}µA/µmol L^{-1}, intercept was 1.389 x 10^{-6} ± 1,316 x 10^{-7}µA/µmol L^{-1}, LOD and LOQ were 32.25 and 107.48µmol L^{-1}, respectively (R = 0.9983).

When 700mV (pH = 12) (M2) were applied, the results showed that the current is proportional to the As(III) concentration over the 6.67 to 66.74µmol L^{-1} range (Fig. 5b). However, two zones are seen with different slopes and linear relationships: the first As(III) concentration range was from 6.67 to 26.69µmol L^{-1}, the sensitivity was 0.017 ± 7.025 x 10^{-4}µA/µmol L^{-1}, intercept was 0.071 ± 0,013µA/µmol L^{-1}, LOD and LOQ were 1.84 and 6.22µmol L^{-1}, respectively (R = 0.9983). The second range As(III) concentration was from 26.69 to 66.74µmol L^{-1}, the sensitivity was 0.055 ± 0.002µA/µmol L^{-1}, intercept was –0.101 ± 0.104µA/µmol L^{-1} LOD and LOQ were 3.06 and 10.17µmol L^{-1}, respectively (R = 0.9974). However, these LOD are higher than most the previous results summarized in Table 1. We could have better sensitivity by working with the enzyme directly. In these reported methods (Table 1) are remarkable the lower LOD (7.1pmol L^{-1}) obtained by Ma et al. [15] using a AuE modified with ion imprinted polymer and AuNP; Sanghavi et al. [26] (8pmol L^{-1}) using a GPE modified with a thiacrown ether and AuNPs; Gao et al. [28] (10pmol L^{-1}) using a SPCE modified with ionic liquid–Fe_3O_4 and Zhao et al. [17] (130pmol L^{-1})

using a GCE modified with rGO supported with Fe_3O_4 nanoparticles and Ag/Au hollow nanoshells.

3.5. Selectivity and stability of the (AF/AuNPs–SPCE) *electrochemical biosensor*

Interference caused by other metals or metalloids always present in water samples may occur, decreasing specificity of the *AF*/AuNPs–SPCE biosensor. With this purpose, the effect of the presence of 10mg L^{-1} of Cu, Pb, Cd, Mn, Mg, Fe, Bi, Zn, Se, Al, Sb and As(V) in the presence of 5mg L^{-1} of As (III) were studied under both conditions (M1 and M2). The results show that under M1 conditions As(V), Cd(II), and Bi(III) do not generate signals (Fig. S3a and Fig. S3b). The other metals show a signal at the level of 66.74µmol L^{-1}, but the current is lower than that of As(III). In the study carried out at M2 conditions, none of the metal ions studied generated a significant signal.

On the other hand, the stability of *AF*/AuNPs–SPCE biosensor was evaluated by measuring As(III) everyday with the same biosensor, and after their use was stored at 4ºC in PBS of pH = 7. The amperometric response obtained with *AF*/AuNPs–SPCE under M1 conditions showed good stability, therefore the same biosensor could be used for at least six successive days. On the other hand, when the stability of *AF*/AuNPs–SPCE under M2 conditions was studied, the amperometric response showed an abrupt decrease from the third day, and was not able to regenerate the enzymatic activity of the bacteria. However, for M2 conditions, repeteability was calculated by measuring consecutively 15 times with the same electrode, washing it after each measurement with PBS of pH = 7. The RSD obtained was 7%, however the response showed a continuous decrease, and it was not possible to recovery its

electrochemical activity. It can be deduced that the construction characteristics generated a stable biosensor, but such high pH caused irreversible and progressive damage to the cell (Fig. S4a and Fig. S4b).

3.6. Application of the AF/AuNPs–SPCE biosensor in analysis of total arsenic on Loa river water samples

The optimized method using the *AF/AuNPs–SPCE* electrochemical biosensor was applied to the determination of total arsenic in the previously reduced Loa river water samples. On the other hand, previously a study was carried out to choose the most suitable reducer or mixture of reducers and their optimal concentration, and that they did not present interference in the subsequent determination of As (III). The chosen procedure consisted of adding potassium iodide (0.018mol L^{-1}) and ascorbic acid (0.284mol L^{-1}) to the sample and incubating at room temperature for 30min with stirring (Fig. S5a and Fig. S5b). The results obtained were compared with measurements made by an external analytical laboratory which determined total arsenic by ICP−MS, reporting 12µmol L^{-1} for the same sample. The determination of total As in water samples were made by the standard addition method and by calibration curve method, applying M1 and M2 conditions, respectively. The electrode was washed and the PBS solution was changed after each addition of As(III) for the recovery of enzymatic activity. For M1 the sample was added to electrochemical cell (250µL), and then different concentrations of As(III) standard solutions were added: 40; 66.74; 93.45; 120.13, and 173.52µmol L^{-1} (Fig. 6a). For arsenic determination under M2 conditions a calibration curve was prepared with As(III) concentration range

from 6.67 to 26.69µmol L^{-1}. The analyses were performed for each sample, obtaining values of total arsenic of 13.73 ± 1.65µmol L^{-1} for M1 conditions and 12.67 ± 2.45µmol L^{-1} for M2 conditions (Fig. 6b). These similar results indicate that there is no significant matrix effect. All the results are shown in Table 2, including values for total arsenic obtained for the same sample under different methods.

4. Conclusions

A simple electrochemical biosensor for As(III) determination has been developed through immobilization of the *A. faecalis* bacteria by cross–linking with glutaraldehyde over a commercial AuNPs–SPCE, without aid of a redox mediator. The bacteria coated on the electrode surface generated a direct electron transfer by arsenite oxidase activity. In this work, the amperometric response has been reported for two different experimental conditions: E_{app} 300mV, pH = 7 (M1), and E_{app} 700mV, pH = 12 (M2) in PBS 0.1mol L^{-1}. In both cases the developed biosensor exhibited a good linear relationship with As(III) concentration. However, enzymatic activity from Aro under these conditions has not been reported before, therefore more studies are required for this association. The LOD were 6.61 and 1.84µmol L^{-1} for M1 and M2 respectively. Lower limits could be obtained if Aro is used directly. The biosensor showed a good selectivity under the two methodologies. With M1 conditions the useful lifetime of biosensor was at least six days, and under M2 conditions the same electrode could be used at least 15 times without loss of activity. The usefulness of AF/AuNPs–SPCE has been demonstrated by the analysis of total arsenic in the Loa River samples, with an error in accuracy of less than 15, and 4% for the M1 and M2

procedures, respectively and response time of five minutes. Finally the principal advantage of *AF*/AuNPs–SPCE electrochemical biosensor is the simple procedure and its capacity of being applied to natural water samples contaminated with high arsenic concentrations, providing information essential for the future development of biosensors by direct immobilization of bacteria, considering that the North Zone of Chile is a rich environment for the growth of a wide variety of extremophiles with electrochemical potential. On the other hand, the biosensor only detect As(III), so if the sample is previously reduced, total arsenic can be determined and thus perform redox speciation, which is very important given the difference in toxicity between As(III) and As(V).

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Table captions:

Table 1: Analysis of arsenic in real water samples using different electrodes

Table 2: Analysis of total As in Loa River samples with *AF*/AuNPs–SPCE biosensor under M1 and M2 conditions and other methodologies (n = 3; α = 0.05). ICP–MS determination was perfomed by the Servicio Nacional de Minería y Geología (Sernageomin. Chile).

Figure Captions:

Scheme 1. Schematic illustration of the biosensor preparation steps. **Step 1**: Addition of *Alcaligenes faecalis*. **Step 2**: Addition of glutaraldehyde. **Step 3**: Determination of As(III).

Fig. 1. (A) Amperometric response of 200µmol L^{-1} As(III) obtained with different electrodes with and without immobilized *A. faecalis*. (B) Cyclic voltammograms of the $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ 5mmol L^{-1} (KCl 0.1mol L^{-1}) system obtained with SPCE, AuNPs–SPCE, and AF/AuNPS–SPCE electrodes. (C) Nyquist diagrams obtained with SPCE, AuNPs–SPCE and AF/AuNPS–SPCE electrodes. Conditions: *AF* 5µL; GA 13µmol; incubation time 15min (electrode preparation). PBS 0.1mol L^{-1} (pH = 7.0); E_{app} 300 mV (amperometric response).

Fig. 2. Effect of the potential on the amperometric response of 200µmol L^{-1} As(III) PBS 0.1mol L^{-1} (pH = 7). Conditions: *A. faecalis* 5µL; GA 13µmol; incubation time 15 min (electrode preparation).

Fig. 3. Pathway of electron transfer on the surface electrode by the enzyme Aro of *A. faecalis* (A) Described by Male et al. [13] applying a potential of 300mV (B) Approach applying a potential of 700mV

Fig. 4. Effect of pH on the amperometric response of 200µmol L^{-1} As(III) PBS 0.1mol L^{-1}. Potentials applied were 300 and 700mV. Conditions: *A. faecalis* 5µL; GA 13µmol; incubation time 15min (electrode preparation).

Fig. 5. Calibration curves of As(III) solutions with *AF*/AuNPs–SPCE biosensor. (A) As(III) solutions from 6.67 to 200µmol L^{-1}. Carried out applying 300mV (PBS 0.1mol L^{-1} pH = 7). (B) As(III) solutions from 6.67 to 66.74µmol L^{-1}. Carried out applying 700mV (PBS 0.10mol L^{-1}. pH = 12). Conditions: A. *faecalis* 5µL; GA13µmol; incubation time 15 min.

Fig. 6. Loa River water analysis of total As (previously reduced) with *AF*/AuNPs–SPCE biosensor. (A) Carried out applying 300mV (PBS 0.1mol L^{-1} pH = 7) and quantified through standard addition method. (B) Carried out applying 700mV (PBS 0.1mol L^{-1} pH = 12) and quantified through curve calibration method. Conditions: A. *faecalis* 5µL; GA 13µmol; incubation time 15min.

Supplementary material

Fig. S1. Influence of the amount of *A. faecalis* on the amperometric response of 200µmol L^{-1} As(III) using *AF*/AuNPs–SPCE biosensor. Conditions: Eapp 300mV; PBS 0.1mol L^{-1} (pH = 7); GA 13µmol; incubation time 15 min.

Fig. S2. Influence of the amount of GA (*A. faecalis* 5µL; incubation time 15min.) (A) and influence of incubation time (*A. faecalis* 5µL; GA 13µmol.) (B) on the amperometric response of 200µmol L^{-1} As(III) using *AF*/AuNPs–SPCE biosensor. Conditions: Eapp 300mV; PBS 0.1mol L^{-1} (pH = 7).

Fig. S3. Study of interference of different metals on *AF*/AuNPs–SPCE biosensor with As(III) solution of 66.74µmol L^{-1}. Conditions: A. *faecalis* 5µL; GA 13µmol; incubation time 15min. (A) Carried out applying 300mV. PBS 0.1mol L^{-1} (pH = 7). (B) Carried out applying 700mV. PBS 0.1mol L^{-1} (pH = 12).

Fig. S4. (A) Study of stability of *AF*/AuNPs–SPCE biosensor applying 300mV, PBS 0.1mol L^{-1} (pH = 7) in a 66.74µmol L^{-1} As(III) solution. (B) Study of repeatability of AF/AuNPs–SPCE biosensor applying 700mV, PBS 0.1mol L^{-1} (pH = 12) in a 1.84µmol L^{-1} As(III) solution. Conditions: A. *faecalis* 5µL; GA 13µmol; incubation time 15min.

Fig. S5. Study of interference of different reducing agents: $Na_2S_2O_4$ 0.004mol L^{-1} KI 0.018mol L^{-1}; ascorbic acid ($C_6H_8O_6$) 0.284mol L^{-1}; and cysteine ($C_3H_7NO_2S$) 0.413mol L^{-1}. (A) Applying 300 mV (PBS 0.10mol L^{-1} pH = 7) and (B) applying 700mV (PBS 0.1mol L^{-1} pH = 12). Conditions: A. *faecalis* 5µL; GA 13µmol; incubation time 15min.

Highlights

► A electrochemical biosensor for As(III) detection based on the catalytic activity of *Alcaligenes faecalis* was prepared.

► *Alcaligenes faecalis* was immobilized on a gold nanoparticle-modified screen–printed carbon electrode.

► *AF*/AuNPs–SPCE biosensor showed greater stability at pH 7.0 using the same biosensor for at least six successive days.

► The method was applied to the determination of total arsenic in Loa river water samples after reduction, with satisfactory results.

► *AF*/AuNPs–SPCE biosensor only detect As(III), so if the sample is previously reduced, total arsenic can be determined and thus perform redox speciation.

Declaration of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.