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Modified Laccase-Gold Nanoparticles-Tetrathiafulvalene-SPCEs Based Biosensor to Determine W(VI) in Water

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Abstract: It was developed an amperometric biosensor to determine tungsten in water, based on the inhibition of laccase enzyme, by tungsten ions using pyrocathecol as a substrate. The enzyme was immobilized with a proper mixture containing, bovine serum albumin, and glutaraldehyde, for a cross-linking process over screen-printed carbon electrodes, previously modified with tetrathiafulvalen and gold nanoparticles. Optimized experimental conditions are: pyrocatechol in cell 0.040 mM in a phosphate buffer pH 6.5 and applied potential +350 mV. The repeatability and reproducibility, in terms of relative standard deviation values, of de developed biosensor were 3.3 % (n=3), and 2.2 % (n = 5) respectively, and detection limit was 1.8×10^{-7} mol L^{-1}. Additionally it was determined the kinetics of the systems by means of Michaelis-Menten *Km* apparent constants, calculated using Lineweaver-Burk plots, with and without tungsten. Kinetic study resembles to be competitive inhibition. A recovery study was performed with spiked blanks with a tungsten certified reference standard, traceable to NIST, giving as a result 102.3 ± 6.7 %; tap water samples analyzed presented a mean concentration of 1.75 µM, and recovery of the tungsten certified reference standard on the tap water samples gave 98.8 ± 3.1 %.

Keywords: Biosensor, Cathecol, Gold nanoparticles, Laccase, Screen printed carbon electrodes, Tetrathiafulvalen, Tungsten, Water.

1. Introduction

Tungsten is a transition metal, found along with chromium, molybdenum and seaborgium, element 106 of the periodic table [1]. It is a valuable metal because of its great strength at high temperatures and its high electric and heat conductivity. Tungsten and its alloys have been used for many years in a wide range of applications from daily household necessities to highly specialized components of modern science and technology, such as heating elements for furnaces, filaments for electric lamps, spacecraft shielding, drill tips, edging of cutting tools, medical equipment and small bore ammunition [1].

Tungsten in its metal form is not present in nature; rather, the tungstate anion persists and is thermodynamically stable under most environmental conditions. Polymerization of tungstate with itself and other common oxyanions (e.g. molybdate, phosphate, and silicate) can create a variety of polymer species [2-3].

Tungsten's release into environmental systems may occur as a result of natural or anthropogenic activities [4]. Natural deposits of tungsten ore can

release soluble and mobile tungstate into groundwater. Additionally, there is increasing interest in some anthropogenicuses of tungsten, which could become sources in the environment, including industrial, civilian, recreational and military applications [1-2, 4-6].

Although there have been several studies of the behavior and effects of tungsten in laboratory animals, little attention has been given to the problem of developing a biologically realistic model of the kinetics of tungsten in the human body [7]. Some tungsten compounds, which exert adverse biological effects on humans and animals, had been reported, nevertheless effects of tungsten on environmental systems have not been investigated extensively and published data are fragmentary [4, 8].

In Fallon, Nevada, childhood leukemia cluster affected this zone, supposed an investigation by the United States Centers for Disease Control and Prevention (CDC) that revealed through urine analysis, that residents were exposed to elevated levels of tungsten (VI) [9]. While it is inherently difficult to directly link these childhood leukemia clusters to environmental exposure to elevated tungsten concentrations, either via inhalation or through consumption of tungsten contaminated drinking waters, many studies have shown that this metal can be toxic and may indeed be carcinogenic [7, 10]. This investigation led to a study by the U.S. Geological Survey of tungsten in ground waters of the Carson Desert region of northwest Nevada, which showed concentrations of this metal ranging from 0.27–742 μg kg^{-1} [5, 9-10].

Several methods to determine tungsten in water and other environmental samples have been published; among them are spectrophotometry [11], flame atomic absorption spectrometry (FAAS) [12], inductively coupled plasma atomic emission spectrometry (ICP-AES) [13], inductively coupled plasma mass spectrometry (ICP-MS) [14], X-ray fluorescence (XRF) [15], spectrofluorometry [16], polarography [17], voltammetry [8] and amperommetry [18]. The sensitivity for tungsten by some of these techniques was lower than the necessary to analyze the low tungsten concentration present in water.

Among electroanalytical techniques, the chronoamperometry is getting more significance in the development of amperometric biosensors based on enzyme immobilization onto electrode surfaces that can retain their bioactivity and is one of the main issues in several fields ranging from environmental analysis to clinical diagnosis [19]. In the specific case of laccase-based biosensors, an extensive research effort has been addressed to incorporate laccase on electrode surfaces by different immobilization strategies in order to design biosensors with a wide range of applications. Many references show that its retained bioactivity mostly depends on the chosen immobilization method [20-24].

Chemically modified electrodes (CMEs) represent a modern approach to electrodic systems. Thus a proper alteration of electrode surfaces can achieve characteristics to solve many electroanalytical problems, and may be the basis for a new device with better electrochemical distinctiveness.

The use of tetrathiafulvalen (TTF) as a mediator and the fact to be screen-printed is based in its non-solubility in water, so it may avoid risks of dissolution when working in aqueous solutions. Besides, TTF oxides losing one electron at +0.34 V, and a second electron at +0.78 V vs. Ag/AgCl in acetonitrile solution [24]. Thus, TTF based SPCEs have been built by screen-printed of a mixture of mediator and carbon ink. Then gold nanoparticles have been deposited and laccase enzyme have been cross-linked to TTF modified SPCEs ($SPC_{TTF}Es$) with glutaraldehyde (GA) and bovine serum albumin (BSA) to obtain a biosensor for tungsten detection [25-27].

The purpose of this work was to obtain a sensitive biosensor for the quantitative determination of W(VI), based in the inhibitor effect of this metal over the enzymatic oxidation of cathecol by laccase enzyme. So far it has not published anything like in the literature. To achieve this, laccase $SPC_{TTF}Es$ based biosensors are presented and the effects of gold nanoparticles (AuNPs), are also described.

2. Materials and Methods

2.1. Reagents

Laboratory-made screen-printed electrodes were made-up using different commercial inks: carbon ink (C10903P14, Gwent Electronic Materials, Torfaen, UK), dielectric ink (D2071120D1, Gwent Electronic Materials, Torfaen, UK), Ag/AgCl ink (SS Electrodag 6037, Acheson Colloiden, Scheemda, Netherlands) and Ag ink (Electrodag 418, Acheson Colloiden, Scheemda, Netherlands).

Tetrathiafulvalen (TTF) was acquired from Acros Organics (Geel, Belgium). Glutaraldehyde (GA), bovine serum albumin (BSA), Rhus vernicifera laccase enzyme (LAC), and pyrocatechol from Fluka Analytical, (St. Louis, MO, USA).

Phosphate salts for buffer preparation, Na_2HPO_4 and KH_2PO_4 used as supporting electrolyte were from Merck, (Darmstadt, Germany), NaOH and H_3PO_4 to adjust pH from J.T. Baker, (Deventer, Netherlands).

Tungsten standard solutions 2 mg L^{-1} were prepared daily from a 100 mg L^{-1} ammonium tungstate CertiPUR traceable to NIST (Merck, Darmstadt, Germany) SRM stock solution, and store at 4 °C when not in use. Tungsten metal purity 99.99 % in 2 % v/v HNO_3, certified value 1000 ± 4 mgL^{-1} (High-Purity Standards, CRM traceable to NIST, SRM against 3163, lot 080331, USA) was used to spiked blanks samples and tap water samples for recovery studies.

All chemical reagents used were analytical grade, without any further purification. All solutions were prepared with ultrapure water from a TKA

System, 0.05 µS/cm (18 MΩ (Gen-Pure TKA, Niederelbert, Germany).

2.2. Apparatus and Software

A DEK 248 screen-printing machine (DEK, Weymouth, UK) was used for the preparation of the screen printed transducers.

Cyclic voltammetric and chronoamperometric measurements were performed with an electrochemical potentiostat Autolab 128N with GPES software (Eco Chemie, Utrecht, The Netherlands). Buffer pH was adjusted with a Mettler-Toledo pHmeter S47-K (Columbus, Ohio, USA).

3. Methodology

3.1. Laboratory-made $SPC_{TTF}Es$ Preparation

Depending of the nature and characteristics of the electrodes, they can be easily built by using diverse kinds of inks. In this way, the screen-printed electrodic system based on three-electrode configuration (working, reference and counter electrode) has been manufactured by sequential layer deposition according to previously described procedures [28-30]. Carbon ink and, well-mixed and immediately printed, carbon ink with TTF (5 %w/w) were used to prepare the working electrodes, [25-26].

3.2. Functionalization of $SPC_{TTF}Es$ with Gold Nanoparticles (AuNPs)

$SPC_{TTF}Es$ were modified with gold nanoparticles ($AuNPs/SPC_{TTF}Es$) using a 0.1 mM $HAuCl_4$ solution in 0.5 M H_2SO_4. The deposit was made by applying a potential of + 0.18 V (vs. Ag / AgCl SPE) for 15 s under mechanical agitation [31].

3.3. Immobilization of Laccase Enzyme on $AuNPs/SPC_{TTF}Es$

Mixing proper amounts of crosslinking agent, GA, BSA with LAC enzyme was performed in order to join it to working electrode surface, already prepared with TTF mediator and functionalized with gold nanoparticles. The optimum results were achieved by dropping over different functionalized electrodes, 10 µL of a mixture prepared by 5 µL of LAC (0.11 % w/v), 2.5 µL BSA (1.75 % w/v) and 2.5 µL GA (2.5 % w/v) onto modified electrodes and let them to dry 120 min at 4 °C.

3.4. Measuring Amperometric Procedure

Amperometric measurements were carried out at room temperature, placing the modified electrode in a cell containing 5 mL of supporting electrolyte, pH=6.5 with constant stirring, at an applied potential of + 350 mV vs. Ag/AgCl SPE. Once a stable current was obtained after the addition of substrate or corresponding samples, sequential additions of tungsten standard solutions, were added.

For different prepared biosensors, their performances were evaluated by determining precision, limits of detection and quantification, accuracy, recovery of spiked samples and possible interferences. Also a kinetic study was carried out.

4. Results and Discussion

As it is well know from literature, LAC is a good oxide-reductase enzyme that oxidizes ortho and para phenol groups to ortho and para quinones, and reduces oxygen to water [32-34] (Fig.1). Laccase has not being reported for tungsten determination, but to analyze polyphenols. Nevertheless, previous experiments had shown that tungsten inhibit the activity of this enzyme to catechol as a substrate; this fact is the base of the biosensor proposed in this work.

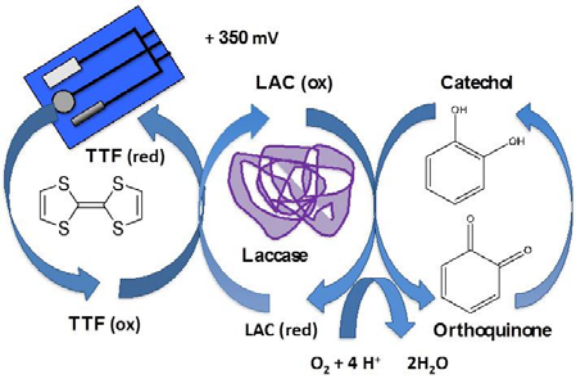

Fig. 1. Schematic representation of possible mechanism of catechol electrochemical reactions at laccase based biosensor-using TTF as a mediator, in the presence of oxygen according to references [32-34].

To obtain a sensitive analytical signal, a group of experiments were carried out considering the influence of three important experimental factors: applied potential (Eap), pH and catechol concentration, changing one at a time in a selected range, and keeping constant the rest. These experiments were done with $LAC/AuNPs/SPC_{TTF}Es$-based biosensors; so there were applied potentials between + 100 mV and + 500 mV at a fixed pH; once a potential of + 350 mV was set, then pH was changed from 5.0 to 9.0, being the best, between 6 and 7, so it was decided to work at pH 6.5. Different catechol solutions were tested ranging from 0.10 mM to 20 mM; the most stable signals were obtained with 0.040 mM catechol in cell. Fig. 2 presents a typical chronoamperogram under the selected conditions.

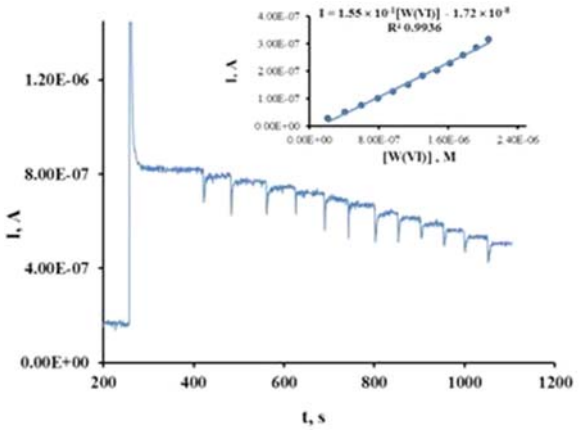

Fig. 2. Chronoamperogram registered using LAC/AuNPs/SPC$_{TTF}$Es based-biosensor under the optimum conditions (applied potential, + 350 mV vs. Ag/AgCl SPE; Phosphate buffer pH 6.5; [cathecol]: 0.04 mM). Inset: calibration curve corresponding to this chronoamperogram.

4.1. Electrodes Characterization and Validation

Electrodes characterization was done by estimating precision, in terms of repeatability and reproducibility, limit of detection and quantification, accuracy and sensitivity.

The reproducibility of the modified electrodes was determined by means of relative standard deviations RSD (%), of the slopes of three independent calibration curves with three different LAC/AuNPs/SPC$_{TTF}$Es and repeatability with the slopes of three independent calibration curves, using the same modified electrode; limit of detection (LOD) and limit of quantification (LOQ) were determined according to IUPAC definition [35], considering the LOD based on three times the standard deviation (3Sy/x) of the calibration curve divided by the slope of the calibration curve. LOQ is ten times the standard deviation (10 Sy/x) divided by the slope. Electrode performance in terms of repeatability as RSD was 3.2 %, reproducibility 2.2 %, sensitivity 0.255 A*M^{-1}, LOD = 0.18 μM and LOQ = 0.62 μM.

Also recovery of spiked blanks with CRM W(VI) standard (1000 ± 4) mgL^{-1} were evaluated obtaining 102.3 ±6.7 % Recovery in tap water was 98.8 ± 3.1 %. Table 1 resume the results of this recoveries.

4.2. Kinetics and Laccase Inhibition

To study kinetic behavior of laccase enzyme with catechol as a substrate, optimum conditions were used in the presence and absence of W(VI). To create Michaelis-Menten plots, successive aliquots of substrate were added in the cell with or without W(VI) until a constant rate was acquire, then with the linear part of it, there were obtained two reciprocal Lineweaver-Burk plots. Then their slopes and intercepts were used to calculate Michaelis-Menten apparent constants. According to Table 2, slopes and *Km* apparent increases with W(VI) concentration, which means that inhibitory effect of W(VI) on the laccase-catechol reaction was confirmed through the higher affinity of the enzyme for the substrate in the absence of this metal. Based on these results and accordingly to Fig. 3 and Table 2, *V max* unchanged, and *Km app.* increases with inhibitor concentration, suggest competitive inhibition of the enzymatic process by the presence of the metal [36-37].

Table 1. Recovery of spiked blanks and tap water at pH 6.5, Eap. + 350 mV vs Ag/AgCl SPE, with W(VI) SRM standard traceable to NIST 1000 ± 4 mg L^{-1}, with LAC/AuNPs/SPC$_{TTF}$Es.

Spiked blanks			
[W(VI)] added	**[W(VI)] found**	**% Recovery**	**[W(VI)] mg L^{-1} Recovery**
M	**M**		1000 ± 4 mg L^{-1}
	4.44×10^{-7}	106.8	1067
	4.41×10^{-7}	106.1	1060
4.16×10^{-7}	3.91×10^{-7}	94.0	940
	4.52×10^{-7}	108.7	1087
	3.99×10^{-7}	95.9	960
Media		102.3	1023
Std. Dev.		6.8	68
RSD %		6.6	6.6
D.F. dilution factors 50.00 mL/2.00 mL and 50.00 mL/5.00 mL			
Spiked tap water			
1.04×10^{-6}	1.03×10^{-6}	99.0	1014
	9.97×10^{-7}	95.6	988
	1.06×10^{-6}	101.6	953
Media		98.8	985
Std. Dev.		3.0	30
RSD %		3.1	3.1
D.F. dilution factors 5.20 mL/0.100 mL and 100.00 mL/1.00 mL			

Table 2. *Km$_{app}$* and Lineweaver-Burk calibration parameters for inhibition kinetics of laccase on catechol enzyme using LAC/AuNPs / SPC$_{TTF}$Es.

[W(VI)] M	***Km/Vm* (Slope)**	***Km* apparent, M**
0	19.56	3.1×10^{-4}
4.95×10^{-6} M	29.747	5.5×10^{-4}
3.06×10^{-5} M	35.888	4.2×10^{-3}

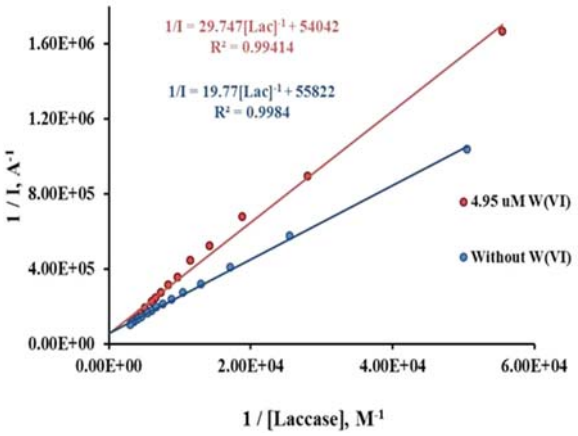

Fig. 3. Lineweaver-Burk graphics for laccase activity on catechol using Lac/AuNPs/ $SPC_{TTF}Es$, with and without W(VI) at pH 6.5 and Eap + 350 mV vs Ag/AgCl SPE.

4.3. Interferences Study

An analysis of possible effects caused by the presence of different metal ions was carried out by preparing standards of different cations to acquire concentrations in cell as 1.0 µM, 10 µM, 0.1 mM and 1.0 mM. Then it was calculated current from baseline to current signals obtained, determining inhibition percentage of the substrate with a number of metals added. Nevertheless, Fig. 4 represents the inhibition % of possible interferences, some of them could be usually present in natural waters, such as Al(III), Fe(III), Ca(II), As(V), Zn(II), Mo(VI), Cu(II), V(V), K(I), Na(I) and Mg(II).

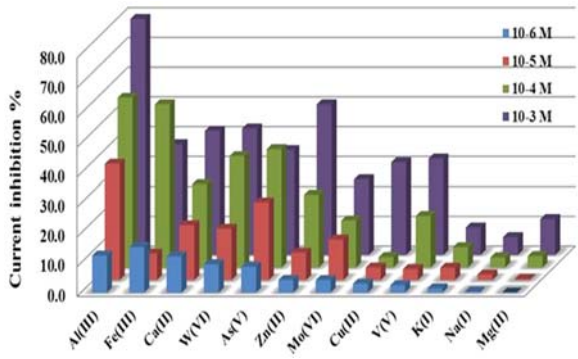

Fig. 4. Inhibition percentage of substrate current from several interferent cations for LAC/AuNPs/$SPC_{TTF}Es$ based biosensor at pH 6.5 and Eap +350 mV vs. Ag/AgCl SPE.

According to Fig. 4, Al(III), Fe(III), Ca(II) and As(V) can interfere with W(IV) measurements, at low and high concentrations and Zn(II) at 0.1 and 1 mM. Ions commonly present in water, such as Na(I), K(I) and Mg(II), cannot be considered as interferences. In agreement with several references, heavy metals in such concentrations can be removed from water samples before use the biosensors [38-39].

5. Conclusions

The use of laccase based biosensors using AuNPs/$SPC_{TTF}Es$ allows sensitive determination of W(VI). The proposed biosensor is based on the laccase enzyme inhibition by the presence of W(VI). This fact can be used to measure the decrease of a substrate current by successive additions of W(VI). According to Lineweaver-Burk plots laccase inhibition by W(VI) is competitive for these modified electrodes.

The biosensor showed excellent figures of merit such as repeatability and reproducibility, with a RSD of 3.3 % and 2.2 % respectively. LOD 1.8×10^{-7} M and LOQ 6.2×10^{-7}, accuracy in spiked blanks with a recovery of 102.3 % with a RSD of 6.6 %.

Some metals such as Al(III), Fe(III), Ca(II) and As(V) may represent significant interferences when W(VI) is present at low concentrations, if they are present in water samples, but there are many methods to remove them from water.

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