Effect of Nanoparticles on Modified Screen Printed Inhibition Superoxide Dismutase Electrodes for Aluminum

Miriam Barquero-Quirós 1,* and María Julia Arcos-Martínez 2

1 Department of Chemistry, University of Costa Rica, CELEQ, San Pedro de Montes de Oca, San José 11501-2060, Costa Rica
2 Department of Chemistry, Faculty of Sciences, University of Burgos, Plaza Misael Bañuelos s/n, Burgos 09001, Spain; jarcos@ubu.es
* Correspondence: miriam.barquero@ucr.ac.cr; Tel.: +506-2511-2442; Fax: +506-2511-2446

Abstract: A novel amperometric biosensor for the determination of Al(III) based on the inhibition of the enzyme superoxide dismutase has been developed. The oxidation signal of epinephrine substrate was affected by the presence of Al(III) ions leading to a decrease in its amperometric current. The immobilization of the enzyme was performed with glutaraldehyde on screen-printed carbon electrodes modified with tetrathiofulvalene (TTF) and different types of nanoparticles. Nanoparticles of gold, platinum, rhodium and palladium were deposited on screen printed carbon electrodes by means of two electrochemical procedures. Nanoparticles were characterized through scanning electronic microscopy, X-rays fluorescence, and atomic force microscopy. Palladium nanoparticles showed lower atomic force microscopy parameters and higher slope of aluminum calibration curves and were selected to perform sensor validation. The developed biosensor has a detection limit of 2.0 ± 0.2 µM for Al(III), with a reproducibility of 7.9% (n = 5). Recovery of standard reference material spiked to buffer solution was 103.8% with a relative standard deviation of 4.8% (n = 5). Recovery of tap water spiked with the standard reference material was 100.5 with a relative standard deviation of 3.4% (n = 3). The study of interfering ions has also been carried out.

Keywords: superoxide dismutase biosensor; aluminum; tetrathiofulvalene; screen-printed electrodes; nanoparticles

1. Introduction

Aluminum toxicity has been shown in vivo and in vitro, but complexity of its interactions with human organism makes it very difficult to assign the responsibility in Alzheimer’s disease. It can be considered as the combined effect of oxidant action, participation on amyloid cascade, neuronal degeneration [1] and accumulation in neurofibrillary tangles. Aluminum presence favors T protein link through phosphate bridge [2] and alters homeostatic ion equilibrium [3], and it has shown a strong effect on reactive oxygen species (ROS) production on living organisms, due to iron accumulation in oxidative stress [4]. Markedly, ROS impaired enzymes such as superoxide dismutase (SOD) and catalase react with radical species such as O$_2$•$^-$, OH$^-$, and ONNO$^-$•. Although O$_2$•$^-$ radical is not so reactive itself, in the presence of Fe$^{2+}$, Fenton reaction can turn it into hydroxyl radical, which is the most potent radical. These findings show that O$_2$•$^-$ is involved in cellular damage. Biosensors have employed superoxide dismutase (SOD), an enzyme that scavenges superoxide to measure superoxide anion accordingly with the reactions [5]:

\[
\text{Cu}^{2+} + \text{SOD} + \text{O}_2\bullet^- \rightarrow \text{Cu}^{1+} + \text{SOD} + \text{O}_2
\]
\[ \text{Cu}^{1+} - \text{SOD} + \text{O}_2\cdot^- + 2\text{H}^+ \rightarrow \text{Cu}^{2+} - \text{SOD} + \text{H}_2\text{O}_2 \]

O$_2$•$^-$ affects cytochrome c (Cyt c) by oxidizing Fe$^{2+}$ to Fe$^{3+}$ and reducing itself to H$_2$O$_2$. O$_2$•$^-$ radical levels were found to be elevated in homogenized cancerous brain tissue compared to normal human brain tissue [6]. O$_2$•$^-$ is formed in living biological systems by the donation of an electron to molecular oxygen, through oxidation of semiquinone-type radicals formed in the mitochondrial electron transport chain. In its presence, free radical scavengers, enzymes such as superoxide dismutase and glutathione peroxidase (GSH-Px), decrease their antioxidant status, and lipid peroxide levels are increased.

Aluminum administration to laboratory animals induces SOD dysfunction and damage on target organs [7–10]. It has been found that zinc [11], selenium [12] and therapies used against Alzheimer's disease [13–16] have a protective role against aluminum induced toxicity, improving SOD function. Aluminum presence in humans is associated with oxidative stress [17,18].

Biosensors constructed with screen printed electrodes (SPCE) offer low detection limits, easier assembly of metallic nanoparticles (NPs), good reproducibility, low contamination and excellent biocompatibility with enzymes and antibodies [18]. The metal NPs' small size, high chemical strength and high chemical and thermal stability allow them, when acting as enzyme-carrier materials, to improve the efficiency of immobilized enzymes, facilitating reaction kinetics, and supplying a larger surface area, leading to higher enzyme loading per unit mass of particles. This fact allows achieving enhanced device sensitivity and reduced mass transfer resistance [19]. Besides, NPs increased electric conductivity and electron transfer between redox enzyme center and electrode [20,21].

Different types of NPs such as metal, metal oxides, semiconductors, polymers and composite-metal NPs have been used to assembly miniaturized electrochemical sensors and biosensors. Gold NPs (AuNPs), due to their unique properties, relatively low cost and ease of preparation, are the most used in many biochemical applications [22,23]. AuNPs can be synthesized by different chemical methods and applied to electrochemical detection of As(III) [24]. AuNPs use in amperometric biosensors and electrochemical techniques enhances detection sensitivity [25–27].

Due to their inertness, platinum NPs (PtNPs) are the principal metal NPs alternative for anodic current measurement and have been applied to formaldehyde [28], neurotransmitters [29], glucose [30], uric acid [31], and As(III) determination [32].

Glassy carbon electrodes modified with palladium nanoparticles (PdNPs) have been applied to catecholamines determination [33]. PtNPs and PdNPs/methylthiophene (PMT) sensors have been applied to dopamine (DA) and AA determination [34].

Rhodium NPs’ (RhNPs) main application has been as precursors for the preparation of catalytic [35,36] and for catalysis and sensing of cytochrome c [37] and H$_2$O$_2$ [38], as well as for biosensing of α-ketoglutarate [39].

Due to sensitivity and specificity joint benefits produced by modified SPCEs with metallic NPs and enzymes, this research work was conducted with the goal to compare the effect of NPs of Au, Pt, Pd and Rh deposited by distinct electrochemical procedures on sensitivity of amperometric inhibition SOD by aluminum, with TTF as mediator using epinephrine (EPI) as substrate.

Most developed biosensors that immobilize SOD enzyme in a carrageenan gel are based on H$_2$O$_2$ amperometric detection [40] and were successfully applied as a tool for antioxidant capacity assessment to evaluate red and white wines [41], fresh herbs and fruits, olives, tea [42–44], algae [45], phytoterapeutic preparations [46], drugs containing salicylic and ascorbic acid, and β-carotene [40,47]. This developed biosensor enables measurement of antioxidant capacity of healthy and diseased tissues in vitro [40], and was also applied to determination of total antioxidant capacity of berries [48].

The others SOD based biosensors that employ modified solid electrodes are shown in Table 1.
Table 1. SOD based biosensors applied to samples of biological interest.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Electrode</th>
<th>Potential Modification</th>
<th>Modification</th>
<th>Range</th>
<th>LOD</th>
<th>Sample/Analyte</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV ¹</td>
<td>SPCE</td>
<td>−0.8 to +0.8 V</td>
<td>Pyrrole/SAMs</td>
<td>$0.5 \times 10^{-9}$ M</td>
<td>$0.5 \times 10^{-9}$ M</td>
<td>cultured human keratinocytes NO₃⁻ [49]</td>
<td></td>
</tr>
<tr>
<td>Amperometry</td>
<td>CFME ²</td>
<td>+0.25 V</td>
<td>cysteine/AuNPs</td>
<td>$(13-104) \times 10^{-9}$ M</td>
<td>-</td>
<td>$\text{O}_2$⁻</td>
<td>[50]</td>
</tr>
<tr>
<td>Amperometry</td>
<td>SPCE</td>
<td>−0.1 V</td>
<td>porous Pt-Pd/nafion</td>
<td>$(16-1536) \times 10^{-6}$ M</td>
<td>$0.13 \times 10^{-6}$ M</td>
<td>Cell culture medium/$\text{O}_2$⁻</td>
<td>[51]</td>
</tr>
<tr>
<td>CV ¹ Chronoamperometry</td>
<td>Gold electrode</td>
<td>−0.2 to +0.5 V</td>
<td>Au/Cys/SOD ³, Au/GNP/Cys/SOD ¹⁰ and Au/GNP/Cys/SOD/Chit ¹¹</td>
<td>0.5-4 Gy</td>
<td>0.03 $\times 10^{-6}$ M</td>
<td>thallium 201/water</td>
<td>[52]</td>
</tr>
<tr>
<td>Amperometry</td>
<td>GC CFME ²</td>
<td>0.2 V</td>
<td>NTA/HT ⁷</td>
<td>$10^{-7}$ to $10^{-4}$ M</td>
<td>21 $\times 10^{-6}$ M</td>
<td>brain tissue/$\text{O}_2$⁻</td>
<td>[53]</td>
</tr>
<tr>
<td>CV ¹</td>
<td>Carbon paste electrode Electrochem</td>
<td>0-0.3 V</td>
<td>cytochrome c in solution and Fe(III)-protoporphyrin immobilized</td>
<td>(1-6) $\times 10^{-3}$ M</td>
<td>$0.3 \times 10^{-3}$ M</td>
<td>Xantine</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>System Carbon paste electrode</td>
<td></td>
<td>Protoporphyrin and cytochrome both immobilized</td>
<td>(1-8) $\times 10^{-3}$ M</td>
<td>$0.2 \times 10^{-3}$ M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cronoamperometry</td>
<td>Composite electrode</td>
<td>−0.3 V</td>
<td>PiPd-PDARGO ⁶</td>
<td>(0.016-0.24) $\times 10^{-3}$ M</td>
<td>2 $\times 10^{-6}$ M</td>
<td>DMEM ⁵/$\text{O}_2$⁻</td>
<td>[55]</td>
</tr>
<tr>
<td>Amperometry</td>
<td>GC ⁸</td>
<td>−0.3 V</td>
<td>MWCNT ⁴</td>
<td>(0.01-0.3) $\times 10^{-3}$ M</td>
<td>1 $\times 10^{-6}$ M</td>
<td>Wines, berry juice/$\text{O}_2$⁻</td>
<td>[56]</td>
</tr>
<tr>
<td>Amperometry</td>
<td>SPC TTF ¹²</td>
<td>0.2 V</td>
<td>SOD/PdNP ¹³</td>
<td>(1.0-60) $\times 10^{-5}$ M</td>
<td>2 $\times 10^{-6}$ M</td>
<td>Al(III) water samples</td>
<td>This article</td>
</tr>
</tbody>
</table>

¹ Cyclic voltammetry; ² carbon fiber microelectrode; ³ poly(3,4-ethylenedioxythiophene); ⁴ multiwalled carbon nanotubes, ⁵ Dulbecco’s modified Eagle’s medium; ⁶ PiPd poly dopamine reduced graphene oxide; ⁷ Nitrilotriacetic acid/histidine-tag; ⁸ glassy carbon; ⁹ gold, cysteine, super oxido dismutase; ¹⁰ gold, gold nanoparticles cysteine, super oxido dismutase; ¹¹ gold, gold nanoparticles cysteine, super oxido dismutase, chitosane; ¹² screen printed carbon TTF5% electrode; ¹³ super oxido dismutase, palladium nanoparticles.
Taking into account that TTF allows the rapid electron transfer between SOD and electrode surface can be carried out at lower potential [57], and that pro-oxidant activity of aluminum inhibits SOD activity [58], this study indicates that Al(III) SOD inhibition can be performed at lower potential compared to other aluminum enzymatic determinations. It was shown for the first time using SPC\textsubscript{TTF}Es that Al(III) inhibits SOD enzyme linked with Alzheimer’s disease at low concentrations.

2. Materials and Methods

2.1. Reagents

All solutions were prepared with purified water supplied by TKA Gen Pure, inverse osmosis, with a UV lamp irradiation system.

SOD enzyme (30 KU), EPI, bovine serum albumine (BSA), glutaraldehyde (GA) and hydrogen tetrachloroaurate (III) trihydrate (HAuCl\textsubscript{4}) were obtained from Sigma-Aldrich (Sigma-Aldrich), Steinheim, Germany).

Solutions of platinum, rhodium and palladium 0.1 mM were prepared from ICP solutions of 1000 mg/L (Merck, Darmstadt, Germany).

Titrisol solutions from (Merck, Darmstadt, Germany) were used to prepare stock standard solutions of Al(III), Cu(II), Fe(III), Sn(II), Co(II), Ni(II), Se(IV) Cr(III), Cd(II), Pb(II), W(VI) and V(V). Mo(VI) and Ca(II) solutions were obtained from Inorganic Ventures (Lakewood, NJ, USA). As(V) and Hg(II) solutions were prepared from Atomic Spectroscopy Standards solutions (Perkin Elmer Co, Whaltham, MA, USA).

Al(III) solutions used for spike were prepared from High Purity Standards (Charleston, SC, USA) confirmed against standard reference material SRM 3101.

Britton Robinson (BR) supporting electrolyte solutions were prepared as usual with boric, phosphoric and acetic acids (Merck, Darmstadt, Germany), and the required pH was obtained by adjusting with NaOH solution (Suprapur, Merck, Darmstadt, Germany).

Several inks were used in the fabrication of SPEs, namely Electrotag PF-407 A (carbon ink), Electrotag 6037 SS (silver/silver chloride ink) and Electrotag 452 SS (dielectric ink) supplied by Acheson Colloiden (Acheson Colloiden, Scheemda, The Netherlands).

The working electrode ink was prepared by thoroughly mixing carbon ink with tetrathiofulvalene (C\textsubscript{TTF}) 5%. TTF was obtained from Acros Organics (Acros Organics, Geel, Belgium).

2.2. Equipment

An electrochemical system Autolab PGSTAT Echo Chemie128 N with GPS software was used to record electrochemical measurements (Echo Chemie, Utrecht, The Netherlands).

All pH values were adjusted with a pHmeter (Mettler Toledo, Schwerzenbach, Switzerland).

A S-3700 Hitachi was used to perform scanning electronic microscopy (SEM) of SPCEs. An IXRF Systems model 550\textsuperscript{i} was used to obtain spectra of elements on the SPCE. Atomic force microscopy (AFM) parameters and images were obtained with a NanoScopeQuadrex Digital Instruments Veeco Metrology Group.

SPC\textsubscript{TTF}Es Construction

SPC\textsubscript{TTF}Es were homemade built using a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke. These transducers consisted of three screen-printed electrodes deposited onto polyethylene terephthalate films (HiFi Industrial Film, Dardilly, France). The different inks were screen-printed and cured according to the manufacturer’s specifications. The working electrode ink was prepared by thoroughly mixing carbon ink with TTF (5% v/w) and immediately screen-printed. One electrode is shown in (Figure 1).
water and wiped carefully.

Al(III) concentration influence inhibition process and can be quantitatively evaluated determining the

This mixture was dropped onto the surface electrode and stored at 4 °C before used and between

presence of Al(III), (I) namely ∆(I0-I). Accordingly, with the following working principle proposed in

Scheme 1, a SOD based biosensor, with TTF incorporated in electrode ink, has been developed.

2.3. Nanoparticles Electrodeposition Methods

SPC{TTF}Es modification with nanoparticles (NPs/SPC{TTF}Es) was carried out by both controlled potential cyclic voltammetry scan methods.

(A) Metal plating was carried at two different potentials namely +0.3 and +0.18 V, in a quartz cell containing Au(III), Pt(IV), Rh(IV) or Pd(IV) solutions (0.1 mM) in H2SO4 (0.5 M) [24]. Following electrodeposition process, the NPs/SPC{TTF}Es was removed from platting solution, rinsed with purified water and wiped carefully.

(B) Cyclic voltammetry deposition was performed doing a set of seven successive voltammetric scans between +1.0 and −0.2 V in a quartz cell containing Au(III), Pt(IV), Rh(IV) or Pd(IV) (0.1 mM) in H2SO4 (0.5 M) [59]. Electrodes were prepared by setting two cyclic voltammetric conditions namely CV1 and CV2.

CV1: delay time 60 s, step potential 0.0150 V, scan rate 0.050 V/s.
CV2: delay time 120 s, step potential 0.025 V, scan rate 0.100 V/s.

After nanoparticles deposition, the electrode was rinsed with purified water and wiped carefully.

2.4. SOD Enzyme Immobilization onto AuNPs//SPC{TTF}Es

Enzyme was immobilized by crosslinking polymerization with glutaraldehyde [60] on the surfaces of AuNPs/SPC{TTF}Es, PtNPs/SPC{TTF}Es, PdNPs/SPC{TTF}Es, and RhNPs/SPC{TTF}Es. To carry out the immobilization procedure, superoxide dismutase enzyme solution was prepared by dissolving enzyme in Britton Robinson buffer at pH 7.0. To avoid loss of enzymatic activity, BSA was used in a mixture made of 20 µL of SOD (5.9 mg/mL), 10 µL of BSA (1.69% w/v) and 10 µL of GA (2.5% v/v) [61]. This mixture was dropped onto the surface electrode and stored at 4 °C before used and between measurements. The modified electrode was washed with purified water, before and after use.

3. Results

3.1. Optimization of Experimental Parameters

EPI originates an amperometric signal at NPs/SPC{TTF}E with SOD enzyme immobilized (SOD/PdNPs/SPC{TTF}E), after which a steady-state current is reached. The presence of Al(III) ions produces SOD enzyme inhibition which causes a decrease in the EPI amperometric signal. Al(III) concentration influence inhibition process and can be quantitatively evaluated determining the difference between the steady state current in absence of Al(III), (I0), and the steady state current in the presence of Al(III), (I) namely ∆(I0-I). Accordingly, with the following working principle proposed in Scheme 1, a SOD based biosensor, with TTF incorporated in electrode ink, has been developed.
The parameter $\Delta (I_0-I)$ depends on EPI concentration, applied potential (Eap) and pH solution. Therefore, an optimization of these variables was performed in order to ensure the quality of the results. Because the dependence between $\Delta (I_0-I)$ and Al(III) concentration is linear, substrate response was obtained from pH 5.0 to pH 8.0, and a pH of 5.0 was selected regarding substrate stability to autoxidation. In the same way, substrate response was obtained from +0.20 V to +0.60 V, and a potential of 0.2 V was selected driving substrate oxidation to epinephrinequinone [57]. Then, several aluminum inhibition calibration curves were performed at different potential and pH values and their slopes were compared, in order to obtain Al(III) inhibition effect with pH and Eap. Slope calibration curve with pH was calculated from pH 5.0 to pH 8.0. In the same way, slope calibration curve with potential was calculate from +0.20 V to +0.60 V. Higher slope values were obtained at pH 5.0 and Eap of +0.2 V, so these conditions were chosen to perform Al(III) inhibition calibration curves. Slopes of calibration curves with potential and pH are shown in Figure 2.

![Scheme 1. Oxidation of EPI on SOD/SPC TTFE.](image)

The parameter $\Delta (I_0-I)$ and Al(III) concentration is linear, substrate response was obtained from pH 5.0 to pH 8.0, and a pH of 5.0 was selected regarding substrate stability to autoxidation. In the same way, substrate response was obtained from +0.20 V to +0.60 V, and a potential of 0.2 V was selected driving substrate oxidation to epinephrinequinone [57]. Then, several aluminum inhibition calibration curves were performed at different potential and pH values and their slopes were compared, in order to obtain Al(III) inhibition effect with pH and Eap. Slope calibration curve with pH was calculated from pH 5.0 to pH 8.0. In the same way, slope calibration curve with potential was calculate from +0.20 V to +0.60 V. Higher slope values were obtained at pH 5.0 and Eap of +0.2 V, so these conditions were chosen to perform Al(III) inhibition calibration curves. Slopes of calibration curves with potential and pH are shown in Figure 2.

![Figure 2.](image)

**Figure 2.** (a) Slope values of Al(III) calibration curves with pH; and (b) Slope value of Al(III) calibration curves with applied potential. [EPI] = 1.6 × 10$^{-4}$ M; Britton Robinson buffer pH 5.0, Eap = +0.2 V vs. Ag/AgCl.

Findings indicated that substrate stability improved at low values of pH and potential; furthermore, if applied potentials were higher than +0.6 V, the electrodes showed erratic behavior. Since substrate response increases with concentration, a value of 1.6 × 10$^{-4}$ M for EPI was chosen, as this concentration gives a proper sensibility, and a very stable signal with very low noise. Upper concentrations produced higher noise on amperometric recording of calibration curves. Under the selected conditions, the electrodes showed good performance. Calibration curves of Al(III) using SOD/AuNPs/SPC TTFEs, SOD/PtNPs/SPC TTFEs, SOD/PdNPs/SPC TTFEs, and SOD/RhNPs/SPC TTFEs were obtained under the optimized conditions.
Sensors 2016, 16, 1588

Preliminary experiments showed that modification of electrode surface with NPs increased the sensitivity of the biosensor; therefore, a thorough study of conditions of NPs deposition was carried out. AuNPs, PtNPs, PdNPs and RhNPs were deposited on electrodes surfaces according to methods described in the Experimental Section.

3.2. XRF and SEM for NPs/SPC TTFE Study METHOD A

Two different controlled potentials, +0.18 V and +0.3 V, were applied for 15 s to SPCEs in order to deposit NPs of every metal. X-ray fluorescence emission (XRF) spectra were obtained from surfaces of SPC TTFEs modified with the different type of NPs. Table 2 shows XRF percentage of elements deposited using indicated potentials.

The plating of metals at +0.18 V for 15 s produced a higher percentage of Au, Pd and Rh. Pt deposited percentage was higher at +0.30 V. Since the Eap of +0.18 V applied for 15 s produced a higher percentage for Pd, Rh and Au, and the application of a deposition potential of +0.3 V did not deposit Pd or Au, conditions of Eap of +0.18 V and 15 s of method A were selected to deposit NPs of metals.

Table 2. XRF percentage of element deposited on SPC TTFEs by method A and B.

<table>
<thead>
<tr>
<th>Element</th>
<th>Method A</th>
<th></th>
<th>Method B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>XRF%</td>
<td>XRF%</td>
<td>CV1</td>
<td>CV2</td>
</tr>
<tr>
<td>(+0.18 V, 15 s)</td>
<td>(+0.30 V, 15 s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pd</td>
<td>0.136</td>
<td>0.00</td>
<td>0.557</td>
<td>0.632</td>
</tr>
<tr>
<td>Pt</td>
<td>0.223</td>
<td>1.48</td>
<td>2.74</td>
<td>2.71</td>
</tr>
<tr>
<td>Rh</td>
<td>0.693</td>
<td>0.380</td>
<td>4.49</td>
<td>2.95</td>
</tr>
<tr>
<td>Au</td>
<td>1.42</td>
<td>-</td>
<td>1.87</td>
<td>2.23</td>
</tr>
</tbody>
</table>

The inhibition calibration curves for Al(III) are shown in Figure 3, where the lowest slope value corresponds to SPC TTFE without NPs deposited and the highest corresponds to SOD/AuNPs SPC TTFE. The other metal NPs modified SPC TTFE tested showed lower linear adjustment than SOD/AuNPs SPC TTFE.

SEM images of AuNPs obtained by method A deposited on SPC TTFEs are presented in Figure 4.

Figure 3. Calibration plots using: (●) SOD/AuNPs/SPC TTFE; (■) SOD/PtNPs/SPC TTFE; (▲) SOD/RhNPs/SPC TTFE; (♦) SOD/PtNPs/SPC TTFE and (●) SPC TTFE performed with NPs/SPC TTFEs prepared by method A, [EPI] 1.6 × 10⁻⁴ M, Britton Robinson buffer pH 5.0, Eap = +0.2 V vs. Ag/AgCl.
3.3. XRF and SEM for NPs/SPC-TTFEs Study METHOD B

XRF percentages for every metal deposited with method B are shown in Table 1. AuNPs, PtNPs, PdNPs, and RhNPs were deposited on SPC-TTFEs according to method B and modified with immobilized SOD. SOD/SPCEs modified with metallic NPs showed the best linear adjusted Al(III) calibration curve at CV1 conditions for PtNPs and at CV2 conditions for PdNPs (Figures 5 and 6). Regressions with the best linear fit performed by methods A and B showed that the highest slope corresponds to SOD/PdNPs/SPC-TTFEs (Figure 7). SEM image of PdNPs/SPC-TTFEs at CV2 conditions is shown in Figure 8, where it is observed that PdNPs are deposited in a regular form on SPC-TTFEs for the CV2 conditions.

![SEM image of AuNPs/SPCTTFE deposited at 0.18 V for 15 s.](image)

**Figure 4.** SEM image of AuNPs/SPCTTFE deposited at 0.18 V for 15 s.

**Figure 5.** Calibration curves of Al(III) inhibition for (▲) SOD/RhNPs/SPC-TTFEs; (■) SOD/PdNPs/SPC-TTFEs; (♦) SOD/PtNPs/SPC-TTFEs and (•) SOD/AuNPs/SPC-TTFEs performed with NPs/SPC-TTFEs prepared under CV1 condition, [EP] 1.6 × 10⁻⁴ M, Britton Robinson buffer pH 5.0, Eap = +0.2 V vs. Ag/AgCl.
Figure 6. Calibration curves of Al(III) inhibition for (■) SOD/PdNPs/SPC TTFEs; (♦) SOD/PtNPs/SPC TTFEs; (▲) SOD/RhNPs/SPC TTFEs and (•) SOD/AuNPs/SPC TTFEs performed with NPs/SPC TTFEs prepared under CV2 conditions, [EPI] = 1.6 × 10^{-4} M, Britton Robinson buffer pH 5.0; Eap = 0.2 V vs. Ag/AgCl.

Figure 7. Calibration curves of Al(III) for (■) SOD/PdNPs/SPC TTFEs; (▲) SOD/RhNPs/SPC TTFEs prepared method B; CV2 conditions; (♦) SOD/PtNPs/SPC TTFEs, prepared by method B and CV1 conditions and (•) SOD/AuNPs/SPC TTFEs, prepared by method A, [EPI] = 1.6 × 10^{-4} M, Britton Robinson buffer pH 5.0, Eap = +0.2 V vs. Ag/AgCl.
3.4. AFM Analysis of SPC_{TTF}Es Prepared by Methods A and B

AuNPs deposit on SPC_{TTF}E increases roughness of SPC_{TTF}E compared with control electrode, as can be observed in AFM images of the surfaces of AuNPs/SPC_{TTF}Es obtained by deposition of AuNPs at 0.18 V for 15 s (Figure 9). The highest slope presented for SOD/AuNPs/SPC_{TTF}E using method A is afforded by lower AFM parameters of AuNPs at 0.18 V for 15 s, than when the CV1 or CV2 conditions are used. AFM images of PdNPs/SPC_{TTF}Es obtained by deposition of PdNPs at CV1 and CV2 conditions are shown in Figure 10. Analysis of AFM parameters confirmed that metal deposited was nanometric size. Table 3 shows the most important parameters of NPs/SPC_{TTF}Es and SPC_{TTF}Es control electrode obtained through tapping mode and Roughness Kurtosis (RKu) and Skewness (RSk) coefficients. PdNPs/SPC_{TTF}Es, obtained using CV2 conditions, showed lower Roughness Average (RA), Roughness Mean Square (RMS) and height of the highest peak above mean line in the profile (Rmax) than other SPC_{TTF}Es modified with metallic NPs, indicating that PdNPs/SPC_{TTF}Es prepared by CV2 condition present a more homogeneous surface [62]. AFM image of PdNPs prepared by CV2 method showed lower values of RA and RMS when compared to surface prepared by CV1 method.

**Table 3.** AFM parameters of modified SPC_{TTF}Es with metallic NPs.

<table>
<thead>
<tr>
<th>NPs/SPC_{TTF}Es</th>
<th>Method/Conditions</th>
<th>RA (nm)</th>
<th>RMS (nm)</th>
<th>Rmax (nm)</th>
<th>RKu</th>
<th>RSk</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC_{TTF}E</td>
<td>-</td>
<td>16.8</td>
<td>21.5</td>
<td>131</td>
<td>3.53</td>
<td>−0.183</td>
</tr>
<tr>
<td>AuNPs/SPC_{TTF}E</td>
<td>A/0.18 V</td>
<td>31.3</td>
<td>39.2</td>
<td>218</td>
<td>3.34</td>
<td>−0.537</td>
</tr>
<tr>
<td></td>
<td>B/CV1</td>
<td>33.8</td>
<td>41.4</td>
<td>234</td>
<td>2.62</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>B/CV2</td>
<td>34.6</td>
<td>44.5</td>
<td>303</td>
<td>3.19</td>
<td>−0.00292</td>
</tr>
<tr>
<td>PdNPs/SPC_{TTF}E</td>
<td>B/CV1</td>
<td>24.8</td>
<td>34.6</td>
<td>216</td>
<td>4.72</td>
<td>0.0604</td>
</tr>
<tr>
<td></td>
<td>B/CV2</td>
<td>14.7</td>
<td>18.2</td>
<td>106</td>
<td>2.69</td>
<td>0.0939</td>
</tr>
<tr>
<td>PtNPs/SPC_{TTF}E</td>
<td>B/CV1</td>
<td>106</td>
<td>140</td>
<td>864</td>
<td>3.63</td>
<td>0.157</td>
</tr>
<tr>
<td></td>
<td>B/CV2</td>
<td>106</td>
<td>140</td>
<td>864</td>
<td>3.60</td>
<td>0.141</td>
</tr>
<tr>
<td>RhNPs/SPC_{TTF}E</td>
<td>B/CV1</td>
<td>22.0</td>
<td>28.1</td>
<td>173</td>
<td>3.30</td>
<td>−0.0733</td>
</tr>
<tr>
<td></td>
<td>B/CV2</td>
<td>25.6</td>
<td>33.9</td>
<td>204</td>
<td>3.65</td>
<td>−0.189</td>
</tr>
</tbody>
</table>
Figure 9. (a) AFM image for SPC$_{TTF}$ control electrode; and (b) AFM image for AuNPs/SPC$_{TTF}$ prepared by plating at 0.18 V for 15 s.

Figure 10. (a) AFM image of PdNPs/SPC$_{TTF}$ prepared under method B and CV1 conditions; and (b) AFM image of PdNPs/SPC$_{TTF}$ prepared under method B and CV2 conditions.

It was observed that PdNPs/SPC$_{TTF}$s modified by means of method B and CV2 conditions showed lower AFM values than the other NPs/SPC$_{TTF}$s. Then, it was decided to analyze the response of EPI at SOD/NPs/SPC$_{TTF}$s in presence of aluminum.

3.5. Inhibition Behavior of Al(III) on SOD Enzyme

Michaelis Menten Km apparent constants were estimated by Lineweaver–Burk plot. It was obtained in presence and absence of Al(III) with SPC$_{TTF}$s modified with AuNPs, PtNPs, RhNPs and PdNPs. Modified electrodes were prepared under the best conditions for each NP deposition method used, namely 0.18 V for method A and CV2 conditions for method B. Figure 11 shows amperometric recording of SOD/PdNPs/SPC$_{TTF}$s (obtained by method B and CV2 conditions).
3.6. Validation of SOD/PdNPs/TTF/SPCE Based Biosensor

SOD/PdNPs/SPCTTFEs were selected to perform validation of the developed biosensor through estimation of their performance parameters.

3.6.1. Limit of Detection

The limit of detection under the optimum working conditions (2.0 ± 0.2 μM) was calculated from the standard deviation (Sy/x) of five Al(III) inhibition calibration curves according to the criteria 3 Sy/x [63], and its RSD was 7.9%. Analogous to LOD, quantification limit (LOQ) was estimated under optimal conditions from the standard deviation of five Al(III) inhibition calibration curves using the criteria 10 Sy/x, and its value was 6.7 ± 0.5 μM, with a RSD of 7.9%.

3.6.2. Precision

This parameter is usually calculated in terms of reproducibility and repeatability. Repeatability was assessed using the same electrode surface. In this way, successive calibrations for Al(III) were tested with SOD/PdNPs/SPCTTFEs prepared under CV2 conditions. The electrodes were conditioned in a Britton Robinson buffer solution, pH 5.0, stirring for 5 min between experiments. The RSD...
obtained for the slopes of the first two graphs was 5.1%, but, in the third measurement, a decrease in the biosensor sensitivity and a RSD increase, reaching 15%, were observed. Because the electrodes are disposables, the reproducibility is a better estimate of performance. Likewise, the reproducibility of the amperometric signal was checked using the slopes of five regression lines carried out with different electrode surfaces, RSD slope value estimated was 7.0%.

3.6.3. Accuracy

The accuracy of the developed method was tested by a recovery study in which a known amount of Al(III) standard reference material (SRM), SRM High Purity Standards solution (Lot Number 1121015, (1000 ± 3) mg·L⁻¹) was spiked to a buffer solution. The aluminum average concentration quantified by the developed procedure, 1038 ± 50 mg·L⁻¹ (n = 5; α = 0.05), matches the certified value of the sample considering the associated uncertainty. The mean recovery percentage obtained was (103.8 ± 4.8)%. Recovery values are shown in Table 5. SRM was spiked to tap water replicates, SRM aluminum average concentration found was 1005 ± 34 mg·L⁻¹ (n = 3; α = 0.05). Mean recovery percentage obtained was (100.5 ± 3.4)%. Recovery values are shown in Table 6.

### Table 5. Recovery of Al(III) SRM (1000 mg/L ± 3 mg/L) spiked to buffer solution.

<table>
<thead>
<tr>
<th>Added SRM (M)</th>
<th>Found SRM (M)</th>
<th>Found SRM (mg/L)</th>
<th>SRM (mg/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.30 × 10⁻⁵</td>
<td>1.235 × 10⁻⁵</td>
<td>9.50</td>
<td>950</td>
<td>95.0</td>
</tr>
<tr>
<td>-</td>
<td>1.366 × 10⁻⁵</td>
<td>10.51</td>
<td>1051</td>
<td>105.1</td>
</tr>
<tr>
<td>-</td>
<td>1.370 × 10⁻⁵</td>
<td>10.51</td>
<td>1051</td>
<td>105.3</td>
</tr>
<tr>
<td>-</td>
<td>1.374 × 10⁻⁵</td>
<td>10.58</td>
<td>1058</td>
<td>105.8</td>
</tr>
<tr>
<td>-</td>
<td>1.402 × 10⁻⁵</td>
<td>10.79</td>
<td>1079</td>
<td>107.9</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>Mean</td>
<td>1038</td>
<td>103.8</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>SD</td>
<td>50.3</td>
<td>5.0</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>RSD</td>
<td>4.8</td>
<td>4.8</td>
</tr>
</tbody>
</table>

### Table 6. Recovery of SRM (1000 mg/L ± 3 mg/L) spiked to tap water.

<table>
<thead>
<tr>
<th>Added SRM (mg/L)</th>
<th>Found SRM (mg/L)</th>
<th>SRM (mg/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.170</td>
<td>0.180</td>
<td>10.44</td>
<td>104.0</td>
</tr>
<tr>
<td></td>
<td>0.171</td>
<td>9.90</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>0.169</td>
<td>9.82</td>
<td>98.2</td>
</tr>
<tr>
<td>-</td>
<td>Mean</td>
<td>1005</td>
<td>100.5</td>
</tr>
<tr>
<td>-</td>
<td>SD</td>
<td>34</td>
<td>3.4</td>
</tr>
<tr>
<td>-</td>
<td>RSD</td>
<td>3.4</td>
<td>3.4</td>
</tr>
</tbody>
</table>

3.7. Study of Interferences on SOD/PdNPs/SPC<sub>TTF</sub>E Biosensors

Interference study was performed comparing the percentage of inhibition showed by the developed SOD based biosensor in the presence of aluminum and other foreign ions. Three concentration levels of possible interfering ions, namely 1 × 10⁻³ M, 1 × 10⁻⁴ M, and 1 × 10⁻⁶ M, were tested. Regarding Al(III), LOD value obtained for SOD/PdNPs/SPC<sub>TTF</sub>E is meaningful at 1 × 10⁻⁴ M. As can be seen in Figure 12, the highest interference effect was found for Sn(II), Cd(II) and Mo(VI) for concentrations tested; however, these cations should usually not be present in water.
Figure 12. Inhibition percentage of current at SOD/PdNPs/SPC TTF Es, (method B and CV2 conditions) in presence of: Al(III), Cu(II), As(V), Zn(II), Hg(II), Sn(II), Co(II), Cd(II), Pb(II), W(VI), Se(IV), V(V), Mo(VI), Ni(II), Ca(II), Fe(III) and Cr(III) at three concentration levels; [EPI] = 1.6 × 10^{-4} M; Britton Robinson buffer pH 5.0, Eap = +0.2 V vs. Ag/AgCl.

4. Discussion

SOD biosensor was developed looking for effect of metallic NPs on sensibility of slopes of Al(III) calibration curves. One initial hypothesis was that NPs generation methodology influence sensitivity of biosensor. For these reasons, two different methodologies to deposit NPs were tested, direct deposit at 0.18 V produced the highest slope for AuNPs, CV2 deposit methodology produced the highest slope for PdNPs, followed for RhNPs and CV1 deposit methodology produced the highest slope for PtNPs. Criteria used for slope selection were linearity and sensibility. Regarding all optimum slopes values obtained with SOD/NPs/SPC TTF Es biosensors under the two methodologies, the highest slope value was obtained for SOD/PdNPs/SPC TTF Es based biosensor. NPs’ physical characteristics are also modified by deposit methodologies. Although SEM were performed on every NPs/SPC TTF Es prepared by means of the above-mentioned methodologies and NPs were visualized, AFM is a more appropriate instrument for NPs/SPC TTF Es surface characterization. AuNPs/SPC TTF Es prepared by method A showed lower AFM parameters, namely RA, RMS and Rmax, than CV deposition methodologies. PdNPs/SPC TTF Es prepared by CV2 showed lower AFM parameters than PdNPs/SPC TTF Es prepared by CV1. Although PtNPs/SPC TTF Es prepared by CV2/CV1 showed similar AFM values and slopes, best linearity was obtained for CV2 condition. RhNPs/SPC TTF Es prepared under CV2 condition showed much better linearity than CV1. All RKu values are near 3, providing evidence that the obtained values are closer to a normal distribution, and the surface is named Mesokurtic, for kurtosis minor 3, surface is flat and called Platykurtic. When kurtosis is greater than 3, surface owns more peaks than valleys. RSk measures the profile of symmetry about mean line. If the height distribution is asymmetrical and the surface has more peaks than valleys, skewness is positive, while in the opposite case, skewness is negative.

SOD/NPs/SPC TTF Es based biosensor was based on Al(III) inhibition of SOD, and Km inhibition were estimated for SOD/NPs/SPC TTF Es under method A and CV2 condition. It was established that Al(III) exerts its inhibitory action at low concentration. Inhibitory effect for SOD/NPs/SPC TTF Es was confirmed by means of Km app values with Al(III) increasing concentrations. This fact is in accordance with theoretical considerations regarding enzymatic behavior in presence of inhibitors. However, the last measurement for SOD/RhNPs/SPC TTF Es, displayed an unusual behavior.

SOD/PdNPs/SPC TTF Es were selected to perform validation of the developed biosensor. The selection was based on the Al(III) inhibition calibration curves of SOD enzyme, that clearly showed higher sensibility by modifying SPC TTF Es with PdNPs prepared under method B and CV2
conditions than the other metallic NPs. Al(III) inhibition on SOD/NPs/SPC$_{TTF}$Es could be used with analytical purposes, but at first it is necessary to perform developed biosensor validation. This goal was achieved by performance parameters estimation. Precision was established through reproducibility of calibration curves slope, as SPC$_{TTF}$Es are disposable, and this reproducibility is a good precision estimate. LOD and LOQ values allowed quantification of low Al(III) concentrations. Recovery percentage of certified SRM afforded accuracy of SOD/NPs/SPC$_{TTF}$Es based biosensor and indicated that developed SOD/PdNPs/SPC$_{TTF}$Es biosensor can be applied to Al(III) determination in aqueous solutions. Validation results suggest that the fabrication procedure of the SOD/PdNPs/SPC$_{TTF}$Es based biosensor is reliable and allows reproducible amperometric responses to be obtained with different electrodes constructed using the method described in this work.

A weakness of biosensor SOD/PdNPs/SPC$_{TTF}$Es is its response to interfering ions, but these toxic ions should not be naturally present in water. Ca(II) and Fe(III) usually found in water do not interfere. Al(III) showed inhibition on SOD enzyme at all tested concentrations.

5. Conclusions

A novel amperometric biosensor based on SOD/PdNPs/C$_{TTF}$/SPCEs was developed, validated and applied to Al(III) determination in aqueous matrices. The biosensor was based on inhibitory effect of Al(III) on SOD enzyme and presents fast response, very good reproducibility, stability, and low LOD. Michaelis Menten constants were calculated from Lineweaver–Burk plots and showed increasing values with Al(III) concentration in accordance with theory of enzymatic inhibition.

SOD enzyme immobilization was easily and rapidly achieved by crosslinking using glutaraldehyde and allowed obtaining a good reproducibility value of biosensor.

Modification of SPC$_{TTF}$Es with different types of NPs improves biosensor performance. A study of electrolytic generation conditions of NPs of Au, Pt, Rh and Pd onto SPC$_{TTF}$ E surface was carried out and results showed that SPC$_{TTF}$Es modified with PdNPs by means of cyclic voltammetry under method B and CV2 conditions (delay time 120 s, step potential, 0.025 V, scan rate 0.1 V/s) gave a higher sensibility on amperometric inhibition of Al(III) calibration curves.

SEM images showed presence of the metallic NPs deposited on SPC$_{TTF}$Es. XRF study was conducted to evaluate percentages of every metal deposited on SPC$_{TTF}$Es. In addition, AFM study showed roughness, characteristic of SPC$_{TTF}$Es and NPs/SPC$_{TTF}$Es surfaces and provided useful information about morphology and surface homogeneity. It was also found that PdNPs/SPC$_{TTF}$Es deposited by method B and CV2 conditions had lower RA, RSM and Rmax than the others metallic NPs.

Biosensor validation was performed under optimized conditions: pH 5.0, applied potential of 0.2 V and a concentration of EPI of $1.6 \times 10^{-4}$ M. The recovery value obtained using certified material, supported the feasibility of SOD/PdNPs/SPC$_{TTF}$Es based biosensor for Al(III) determination.

Developed biosensor presents LOD similar to other developed Al(III) biosensors, but it has the advantage of using a lower applied potential of only +0.2 V. The possibility of using this biosensor at low potentials results in a much higher selectivity compared with the others Al(III) biosensors.

Acknowledgments: Authors would like to acknowledge funding from the Vice-presidency for Research at the University of Costa Rica (Project 804-B5-117), Ministerio de Ciencia e Innovación (MICINN Spain) and Fondo Europeo de Desarrollo Regional (FEDER) (Projects: TEC-TEC20013-40561-P and MUSSEL RTC-2015-4077-2).

Author Contributions: Miriam Barquero-Quirós and María Julia Arcos-Martínez conceived and designed the experiments; Miriam Barquero-Quirós performed the experiments; Miriam Barquero-Quirós and María Julia Arcos-Martínez analyzed the data; Miriam Barquero-Quirós wrote the paper; and María Julia Arcos-Martínez contributed SPC$_{TTF}$Es manufacturing.

Conflicts of Interest: The authors declare no conflict of interest.

References


43. Campanella, L.; Bonanni, A.; Tomassetti, M. Determination of the antioxidant capacity of samples of different types of tea, or of beverages based on tea or other herbal products, using a superoxide dismutase biosensor. *J. Pharm. Biomed. Anal.* 2003, 32, 725–736. [CrossRef]


© 2016 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).