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Chemically Glycosylation Improves the Stability of an Amperometric Horseradish Peroxidase Biosensor

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Graphical Abstract

Highlights

- A sensor based on a 4-mercaptobenzoic acid SAM on gold nano-particles was created
- Horseradish peroxidase (HRP) was immobilized onto the SAM as sensing element
- We employed two forms of HRP: native and chemically glycosylated with lactose
- Modification of HRP with lactose improved operational and storage stability

Abstract

We constructed a biosensor by electrodeposition of gold nano-particles (AuNPs) on glassy carbon (GC) and subsequent formation of a 4-mercaptobenzoic acid self-assembled monolayer (SAM). The enzyme horseradish peroxidase (HRP) was then covalently immobilized onto the SAM. Two forms of HRP were employed: non-modified and chemically glycosylated with lactose. Circular dichroism (CD) spectra showed that chemical glycosylation did neither change the tertiary structure of HRP nor the heme environment. The highest sensitivity of the biosensor to hydroquinone was obtained for the biosensor with HRP-lactose ($414 \text{ nA } \mu\text{M}^{-1}$) compared to $378 \text{ nA } \mu\text{M}^{-1}$ for the one employing non-modified HRP. The chemically glycosylated form of the enzyme catalyzed the reduction of hydroquinone more rapidly than the native form of the enzyme. The sensor employing lactose-modified HRP also had a lower limit of detection ($74 \text{ } \mu\text{M}$) than the HRP biosensor ($83 \text{ } \mu\text{M}$). However, most importantly, chemically glycosylation improved the long-term stability of the biosensor, which retained 60% of its activity over a four-month storage period compared to only 10% for HRP. These results

highlight improvements by an innovative stabilization method when compared to previously reported enzyme-based biosensors.

Keywords: Biosensor, Glycosylation, Gold Nanoparticles, Horseradish Peroxidase, Phenolic Compound

Abbreviations: Horseradish Peroxidase (HRP), Gold Nano-Particles (AuNPs), Glassy Carbon (GC), Detection Limit (LOD), Circular Dichroism (CD), 4-Mercaptobenzoic Acid (4-MBA), Lactose (Lac), Self-Assembled Monolayer (SAM), 2,2'-Azino-Bis(3-ethylbenzthiazoline-6-sulphonic Acid) (ABTS), 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA), Cyclic Voltammetry (CV)

1. Introduction

The major issue when working with enzymes in biosensor applications is their frequently rapid loss of activity. They are structurally and functionally sensitive with a typical lifetime of only 2-8 weeks [1, 2]. When enzymes interact with the electrode surface they can unfold and subsequently lose activity. This becomes detrimental to the performance of biosensors. In general, the physicochemical environment to which the protein is exposed (e.g., supporting matrix) could cause enzyme stability issues. This affects the optimum pH, thermal stability, and therefore the kinetic. It also decreases the long-term storage stability of enzyme-based biosensors. Research has been conducted to increase enzyme stability in biosensors by changing the process of immobilization (e.g., covalent, adsorption, cross-linking, and entrapment) [3-5] or by changing the support matrix (e.g., polymers, mesoporous silica, nanoparticles, self-assembled monolayers (SAM), and nanotubes) [3, 6-9]. However, these strategies only produced limited success and consequently much emphasis should be given to the development of new strategies for the effective long-term stabilization of protein biosensors. A new method of protein

stabilization was introduced by Solá and Griebenow [10], who studied the effect of covalent chemical glycosylation on the kinetics, thermodynamic properties, and stability of α -chymotrypsin. By changing the glycan size and the degree of glycosylation the authors showed that protein thermostability increased at increasing glycosylation degree, regardless of the glycan size. In contrast, prevention of protein aggregation depended on the size of the attached glycan [10]. Since then, chemical protein glycosylation has emerged as one of the most promising approaches to engineer proteins with improved stability by changing glycosylation parameters (e.g., glycosylation degree, glycan size, and glycan structural composition) [11]. Herein, we extended this concept to enzyme biosensors and tested whether protein glycosylation could be employed to stabilize a model enzyme in biosensor construction and application.

Progress has been made in biosensor construction using nanomaterials. For example, the sensitivity and performance of biosensors improved by using nanomaterials during their construction [12]. In the last few years, gold nanoparticles have been used since their size, shape, and morphology can be modified by changing the electrochemical deposition conditions (e.g., time, concentration, and potential). This enables the creation of a wide range of electrodes in a rapid and reproducible manner [12]. The gold nanoparticles offer the advantage of acting as nanoscale electrodes that electrically communicate between the enzyme and bulk electrode material [13]. Nanoparticles can be modified with a wide range of biomolecules and chemical ligands providing a route to functionalize the electrode surface. We chose such a system in this investigation.

The accurate determination of phenols and their derivative compounds is a major concern in medical and environmental analysis, since these chemicals are highly toxic, causing detrimental effects to human health [14]. Their toxicity affects different organs and tissues, (e.g.,

lung, liver, and kidney) [15]. In addition, some phenols are considered to be endocrine-disrupting chemicals (EDCs), which constitute a wide group of environmental pollutants that can mimic hormones [16].

Because of the severity of the impact of phenols on environment and health, several amperometric enzyme biosensors have been developed to detect phenols. The enzymes utilized in these sensors typically involve polyphenol oxidases (PPO) (e.g., tyrosinase and laccase) [13, 16-22] or peroxidases (e.g., HRP). Some limitations of tyrosinase- and laccase-based biosensors include that they can only monitor phenolic compounds with at least one free *ortho*-position or free *para*- and *meta*-position [23]. Moreover, HRP reacts faster and with a wider range of phenolic compounds and shows higher sensitivity compared to PPO-based electrodes [24]. Thus, we selected HRP to construct our enzyme-based biosensor to allow for fast and effective phenol measurements. Enzyme-based biosensors ideally combine the specificity of an enzymatic reaction with the high sensitivity of the amperometric signal transduction. This provides a simple and effective way to detect analytes. The main advantage in the use of redox enzymes is the low applied potential, which leads to good sensitivity [4].

In case of the HRP-based biosensor, the first step in the enzymatic mechanism is the oxidation of the enzyme by hydrogen peroxide, producing an intermediate named compound I (Fig. 1) [4]. The second step consists in the reduction of the oxyferril iron ($\text{Fe}^{4+}=\text{O}$) by two different donor substrate molecules (e.g., a phenol), which are oxidized to a radical. Then, the electrode reduces the formed radical [4]. The current produced by this reduction process (step 3) is proportional to the phenol concentration in the solution [25].

The main aim of our study was to investigate the effect of the covalent modification of the model enzyme HRP with lactose on its function and stability in a biosensor. Due to the

complexity of electrode construction and characterization, however, we were only able to compare two biosensors: HRP and Lac₂-HRP. The electrode was constructed in three steps. First, electrodeposition of AuNPs was performed on the glassy carbon electrode surface. Second, a monolayer was formed by 4-mercaptobenzoic acid (4-MBA), and third, HRP or glycosylated HRP was covalently linked to the 4-MBA SAM (Fig. 2).

2. Experimental

2.1 Chemicals

Peroxidase, type II from horseradish (E.C.1.11.1.7), potassium phosphate (99.7%), sodium hydroxide, 2,2'-azino-bis(3-ethylbenzthiazole-6-sulfonate), 4-mercaptobenzoic acid (99%), gold (III) chloride trihydrate (99.9+%), potassium ferricyanide (III) (99.9+%), 4-methoxyphenol (99%), catechol (99.9+%), dopamine hydrochloride (99%), 4-nitrophenol (99+%), and hydroquinone (98.5%) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30%) was purchased from Thermo Scientific (Fair Lawn, NJ). Platinum gauze (52 mesh, woven from 0.1 mm diameter wire, 99.9 % purity) and platinum wire (0.5 mm diameter, 99.9 % purity) was purchased from Alfa Aesar (Ward Hill, MA). Plain microcloth and alumina micropolish (0.05 µm) was purchased from Buehler (Lake Bluff, IL). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and N-hydroxysulfosuccinimide (99%) were purchased from Proteochem (Denver, CO). Glassy carbon electrode (3.0 mm diameter) and Ag/AgCl (3M NaCl) electrodes were obtained from Bioanalytical Systems (West Lafayette, IN) and were used for electrochemical measurements as working and reference electrode, respectively. Glassy carbon plates (SPI-Glass grade 22) were purchased from SPI Supplies (West Chester, PA) and were used for SEM, EDS and XPS measurement.

2.2 Glycosylation of HRP with Lactose

Horseradish peroxidase (HRP), which has four solvent-accessible lysine residues, was modified with succinimidyl activated lactose (mono-(lactosylamido)-mono-(succinimidyl) suberate (ss-mLac) from Carbomer (San Diego, CA) at a 1:2 molar ratio. The succinimidyl group selectively reacts with primary amines (i.e., solvent accessible lysine residues and amino-terminus) of HRP. 200 mg of HRP was mixed with 5.62 mg of ss-mLac in 0.1 M sodium borate buffer at pH 9.0 for 3 h at 4°C under constant stirring. Non-reacted lactose was removed by dialysis using a dialysis membrane with a cut-off of 8,000-10,000 Da with nanopure water for 24 h at 4°C. Then Lac₂-HRP was frozen in liquid N₂ and lyophilized for 48 h. The degree of protein modification was determined by colorimetric titration of unreacted amino groups with 2,4,6-trinitrobenzene sulfonic acid [26].

2.2.1 Activity Assay

Enzyme activity was determined photometrically by monitoring the oxidation reaction of 5 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) by 0.5 µM HRP in 25 mM potassium phosphate buffer (PBS) with 5 mM H₂O₂ at 414 nm using a Shimadzu 2450 UV/Vis spectrophotometer [27]. The residual activity was calculated with respect to the specific activity of freshly prepared HRP solution.

2.2.2 Circular Dichroism (CD) Spectroscopy

CD spectra were acquired with an OLIS DSM-10 UV-Vis CD spectrophotometer at 25°C in the near-UV region (250 – 320 nm) using a 1.0 cm path length quartz cell. The protein concentration was adjusted to 0.6 mg ml⁻¹ in 0.1 M PBS at pH 7.0. Each spectrum was obtained

by averaging six scans at 2 nm resolution and solvent reference spectra were subtracted from protein CD spectra.

2.3 Electrochemical Measurements

All the electrochemical measurements were performed using a PARSTAT 2273 from Princeton Applied Research (Oak Ridge, TN). A conventional three-electrode cell was used with glassy carbon as the working electrode, an Ag/AgCl (3M NaCl) reference electrode, and a Pt mesh as the counter electrode. The cyclic voltammetry experiments were carried out in a solution of 2 mM of $K_3Fe(CN)_6$ in 0.1 M PBS at pH 7.0 from -0.2 V to 0.7 V with a scan rate of 50 mV s⁻¹. All chronoamperometric measurements were performed applying a constant potential of +30 mV s⁻¹.

2.4 Biosensor Preparation

The glassy carbon electrodes were polished with alumina powder (0.05 μ m) on a polishing microcloth pad until a mirror-like surface was obtained. To remove the alumina from the electrode surface, the electrode was immersed in an ultra sonication bath for 15 min in nanopure water. Finally, a constant potential of +1.80 V was applied for 10 s in 0.1 M NaOH to assure a reproducible and activated glassy carbon electrode surface [28].

The surface area of each glassy carbon electrode was calculated using chronocoulometry in 1.1 mM ferricyanide, 0.1 M KCl at pH 3.0. The potential was stepped up from an initial value E_i of 0.5 V for 250 ms to E_s of -0.1 V for 200 ms and finally stepped back to E_i . A linear Anson plot was used to obtain the slope $2nFACD_0^{1/2}/\pi^{1/2}$ from linear regression, where n is the number of electrons transferred, F is the Faraday constant (96,485 C mol⁻¹), A is the electrode area (cm²),

C is the ferricyanide concentration (mol cm^{-3}), and D_0 is the diffusion coefficient of ferricyanide ($7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$). The surface area of all electrodes used was on average $0.071 \pm 0.004 \text{ cm}^2$.

The formation of gold nanoparticles was accomplished by immersing the electrode in a $100 \text{ mg l}^{-1} \text{ HAuCl}_4$ solution at a constant potential of -200 mV s^{-1} for 60 s [13]. The solution was purged with N_2 for 10 min before electrodeposition. To obtain the surface area of the gold electrodeposited at the GC electrode, cyclic voltammetry (CV) was performed using $0.05 \text{ M H}_2\text{SO}_4$ in the potential window of -0.2 V to 1.5 V at 50 mV s^{-1} . The determination of the electrodeposited AuNPs/GC electrode area is based on the electrochemically induced deposition of an oxygen monolayer. The integration of the reduction peak area, of the oxidized gold layer, can be used to calculate the roughness factor using equation 1, where Q_e is the experimental charge associated with the reduction peak (μC), Q_t is the theoretical charge of $400 \mu\text{C cm}^{-2}$, and A is the geometric area of the electrode. The roughness factor obtained varied between 0.30 to 0.38 [13].

$$\text{RF} = Q_e / Q_t A \quad \text{Equation 1}$$

The formation of the SAM at the gold modified glassy carbon surface was obtained by immersing the electrode in a 1 mM 4-MBA ethanolic solution for 24 h at room temperature [29]. The surface coverage can be determined using equation 2:

$$\Gamma = I_p 4RT / n^2 F^2 A v \quad \text{Equation 2}$$

where Γ represents the surface coverage (mol cm^{-2}), I_p is the peak current (A), R is the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), T is the temperature (K), n is the number of electrons transferred, F is the Faraday constant, A is the electrode area (cm^2), and v is the scan rate (V/s).

The surface coverage obtained from a cyclic voltammogram in 0.5 M KOH from -0.2 to 1.2 V (vs. Ag/AgCl) varied from 2.05×10^{-10} to $2.45 \times 10^{-10} \text{ mol cm}^{-2}$. To immobilize the enzyme at the

4-MBA monolayer, we first activated the carboxylic acid groups by immersing the electrode for 20 min in a solution of 20 mM EDC/ 25 mM NHS in 0.1 M MES, 0.5 M NaCl at pH 6.0 [30]. Then we immersed the electrode in a solution of HRP or Lac₂-HRP with a concentration of 1 mg ml⁻¹ in 100 mM PBS, 150 mM NaCl at pH 7.2 for 2 h.

2.5 Surface Analysis

Scanning electron microscopy (SEM) was performed using a JEOL JSM-7500F field emission scanning electron microscope at 15 kV. XPS was performed using a PHI 5600ci spectrometer with an Al K x-ray source at 15 kV and 300 W. The pass energy used was 187.85 eV for the survey analysis and 29.35 eV for the high-resolution studies. Binding energies were corrected to the aliphatic hydrocarbon C1s signal at 285.0 eV. The EDS spectra were measured using a JEOL JSM-6480LV with an EDAX detector. Glassy carbon plates for XPS, SEM, and EDS analysis were prepared following the same steps that were carried out to prepare the biosensors. The GC plates were washed with nanopure water between each step.

3. Results and Discussion

Advances in biosensor construction and biotechnology encouraged the discovery and development of new strategies to improve the stability of proteins within a manifold of applications. Due to the well-documented benefits, provided by the modification of proteins with glycans in improving protein stability [11, 31-33], chemical protein glycosylation could be an excellent stabilization strategy for biosensor applications. Herein, we explore this concept by chemically glycosylating the model protein HRP with the disaccharide lactose.

3.1 HRP Glycosylation

3.1.1 Degree of Modification and Activity Assay

As described in the methods section, HRP was covalently glycosylated with succinimidyl-activated lactose (mLac). The TNBSA method was used to determine the amount of covalently attached mLac [26]. Our results demonstrate that 1.7 ± 0.6 lysine residues of HRP were glycosylated on average and the conjugate is thus referred to as Lac₂-HRP. In order to determine if the modification process affected HRP enzymatic activity, we measured its activity using an established colorimetric assay prior to and after glycosylation. The relative activity of the glycoconjugate was with $97.0 \pm 0.2\%$ practically the same as for the unmodified enzyme confirming that glycosylation did not cause a significant activity drop.

3.1.2 Circular Dichroism Spectroscopy

We performed circular dichroism (CD) spectroscopy to explore possible effects of the glycosylation process on the tertiary structure (Fig. 3A) and heme-binding pocket (Fig. 3B) of the enzyme. In the near UV-region we observed the same spectrum for the native and glycosylated enzyme, demonstrating that the modification process did not significantly affect the tertiary structure. Small variations between the native enzyme and the glycosylated enzyme were observed in the heme spectral region (Fig. 3B). This result is consistent with the small activity loss of the enzyme.

3.2 Surface Analysis Characterization of the Biosensor

X-ray photoelectron spectroscopy (XPS) was used to study the electrode surface during biosensor assembly, and to confirm protein immobilization (Fig. 4). Two intense peaks at 87 eV and 83 eV corresponding to the Au 4f_{5/2} and Au 4f_{7/2} (Fig. 4B) orbitals were observed in high-

resolution spectra as the result of the gold nanoparticle formation on the GC electrode. Their intensity decreased upon SAM formation and protein immobilization. The appearance of an S $2p_{1/2}$ signal at 165 eV, corresponding to the sulfur atom, is indicative of the 4-MBA monolayer formation (Fig. 4C). This signal shifted to 168 eV due to the presence of a sulfite anion upon activation of the monolayer with the EDC/sulfo-NHS, confirming the formation of the reactive intermediate. The XPS spectral shift was expected because of more electronegative atoms attached to the sulfur atom [34]. We also used the high-resolution spectra of N 1s to corroborate this intermediate (Fig. 4D). The appearance of an N 1s signal at 400 eV is attributed to the protonated amine of EDC. The second peak at 403 eV indicates the presence of the nitrogen of the Sulfo-NHS. Due to the electronegativity of the ester oxygen, the latter nitrogen peak appears at a higher binding energy [35]. After the covalent immobilization of the protein, a diminished intensity was observed for the S $2p_{1/2}$, Au $4f_{5/2}$ and Au $4f_{7/2}$ binding energy regions. In contrast, the peak in the N 1s region increased in intensity confirming the presence of HRP on the surface of the GC electrode (Fig. 4D). In conclusion, the XPS data support our ideas of the electrode construction (see Fig. 2).

To confirm the XPS data, we also investigated the chemistry of each modification step by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). The SEM image of the glassy carbon surface was without significant features (Fig. 5A). In contrast, after gold electrodeposition the SEM image clearly shows AuNPs, which had a mean size of around 100 nm (Fig. 5B). Since the hydrodynamic radius of HRP is only about 3 nm, each AuNPs possesses sufficient available surface area to be modified with many HRP molecules [36]. Formation of the SAM did not produce drastic changes in the surface morphology, which is

expected since the size of 4-MBA is very small when compared to the size AuNPs (Fig. 5C). Presumably due to high accumulation of HRP in certain areas, deposition of HRP on the GC plate resulted in some visible precipitation spots (Fig. 5D).

We performed EDS to characterize the material deposition in the individual steps (Fig. S1, Tab. S1). After each biosensor construction step, we determined the abundance of gold (Au), sulfur (S), and nitrogen (N) atoms. Gold deposition caused an increase in the amount of Au atoms from 0.00 to 67.80% (Fig. S2, all percentages are for weight). The high amount of oxygen initially found on the GC plate, possibly due to oxidation, diminished from 86.17 to 17.92% upon Au electrodeposition. Significant increases in oxygen (23.12 to 51.56%) and nitrogen (12.12 to 24.64%) atoms during the two subsequent steps are consistent with the SAM formation and protein deposition.

3.3 Electrochemical Characterization by Cyclic Voltammetry

The measurement of the electrochemical response of a biosensor to $\text{K}_3\text{Fe}(\text{CN})_6$ / $\text{K}_4\text{Fe}(\text{CN})_6$ by CV is a valuable tool for testing the barrier created after each construction step. The CV for bare GC showed an anodic peak at 250 mV and a cathodic peak at 200 mV both arising from $\text{Fe}(\text{CN})_6^{4-}$ and $\text{Fe}(\text{CN})_6^{3-}$, respectively, and corresponding to the reversible behavior of the electroactive species (Fig. 6). We observed an increase in both the cathodic and anodic current when the gold nanoparticles were electrodeposited on the GC. AuNPs promote the electron transfer between the electroactive species in solution and the electrode surface by acting as nanoscale electrodes.

A decrease in the current response was observed, after the electrode modification with 4-MBA, cause by the insulating effect of the molecules. Finally, the 4-MBA-AuNPs-GC modified

surface was used to attach HRP and Lac₂-HRP. An additional decrease in the current was observed, confirming the protein deposition on the surface.

3.4 Cyclic Voltammetry in the Presence of Hydroquinone

The electrocatalytic response of both biosensors to hydroquinone was studied using CV (Figs. 7A and 7B). No obvious reduction peak was observed for the biosensors in the presence of 0.1 M PBS at pH 7.0, but after adding 400 μ M of hydroquinone a pair of oxidation/reduction peaks appeared, representing the expected electrochemical behavior of hydroquinone. In the presence of hydroquinone and H₂O₂, a dramatic enhancement in the cathodic/anodic current was observed, suggesting an increase of the electrocatalytic response of the biosensor towards hydroquinone in the presence of H₂O₂. The same behavior was observed for both the native and glycosylated enzyme biosensor.

To elucidate whether the current was controlled by diffusion of hydroquinone to the electrode, we performed CV of the HRP and Lac₂-HRP biosensor in 0.1 M PBS at pH 7.0 after addition of 400 μ M hydroquinone and 200 μ M H₂O₂ (Figs. 8A and 8B). The inset in Fig. 8A and Fig. 8B demonstrates that the peak current increased linearly with the square root of the scan rate from 10 mV s⁻¹ to 900 mV s⁻¹ (both biosensors obtained an R² = 0.99). These results demonstrate the reaction between HRP and Lac₂-HRP with hydroquinone is a diffusion-controlled process at these scan rates [9]. Thus, the oxidized and reduced forms of hydroquinone are in solution and are not irreversibly adsorbed on the biosensor surface [37].

3.5 Reduction Potential of Hydroquinone

The applied potential has an important influence on the sensor response. It contributes to both the sensitivity and selectivity of the biosensors. We studied the effect of the applied potential on the amperometric response of both biosensors at 10 to 300 μM of hydroquinone using a constant concentration of 200 μM H_2O_2 . The sensitivity increased when the applied potential changed from 50 mV to 30 mV (Fig. 9). The maximum sensitivity for both biosensors was found to be at 30 mV, demonstrating that at this potential the hydroquinone present in the system was efficiently catalyzed by HRP. At more negative potentials a plateau was observed, suggesting that HRP can be inactivated by the formation of compound-III (Fe^{+6}) [3, 4, 38]. A potential of 30 mV vs. Ag/AgCl was accordingly used in subsequent experiments. The current produced by the biosensor was corrected for the area of the electrode. The biosensor sensitivity was determined from the slope of the linear plot of the corrected current *versus* the hydroquinone concentration.

3.6 Influence of H_2O_2 on the Biosensor Response

The catalytic cycle of HRP leads to H_2O_2 consumption and water generation. For this reason, the study of the H_2O_2 concentration is important when optimizing the biosensor sensitivity [3, 38]. Inactivation of HRP by high concentrations of H_2O_2 has to be avoided. The sensitivity of HRP and Lac₂-HRP biosensors towards H_2O_2 was studied at increasing concentrations of hydroquinone (Fig. 10). The sensitivity reached a maximum at 200 μM of H_2O_2 for the HRP and 300 μM of H_2O_2 for the Lac₂-HRP biosensor and then diminished. The latter can be attributed to the gradual inactivation of HRP by H_2O_2 . Although we found that the sensitivity of the Lac₂-HRP biosensor was slightly larger at 300 μM of H_2O_2 than at 200 μM , the

latter concentration was further used in experiments to compare the stability of both biosensors under the same experimental conditions.

3.7 Thermal Dependence of the Biosensor Sensitivity and Determination of the Activation Energy

The thermal stability of the HRP and Lac₂-HRP biosensors was studied from 15-75°C in 0.1 M PBS at pH 7.0 using 10 to 300 µM of hydroquinone at a constant concentration of 200 µM H₂O₂ and a potential of 30 mV (Fig. 11). The sensitivity of both biosensors rose at increasing temperature and reached a maximum at 35°C. Subsequently it decreased, likely due to denaturation of the enzyme. The Lac₂-HRP biosensor had a superior sensitivity showing that glycosylated-HRP catalyzed the oxidation of hydroquinone more efficiently. At higher temperatures (45°C and 50°C) the Lac₂-HRP biosensor also showed higher sensitivity, suggesting that the glycosylation of the enzyme helped maintain its tertiary structure. Previous studies investigated the effect of glycosylation on the unfolding and refolding of proteins in solution, and found that the glycosylation slows the protein unfolding process without affecting the refolding rates significantly, thus increasing the thermodynamic stability of the native state of the enzyme [39].

The temperature effect can be analyzed by the Arrhenius equation:

$$\ln i = \ln i_0 - E_a/RT \quad \text{Eq. 3}$$

where i_0 is a collection of currents, R is the gas constant (8.314 J mol⁻¹ K⁻¹), T is the temperature and E_a is the activation energy [40-42]. To calculate the E_a , we measured the current produced at concentrations of hydroquinone and H₂O₂ of 130 µM and 200 µM, respectively, in the temperature region from 5°C to 35°C (Inset Fig. 11.) We constructed an Arrhenius plot by

plotting the reciprocal of the temperature *versus* the logarithm of the current [40] and calculated E_a from the slope of the plot. The E_a values obtained for HRP and Lac₂-HRP biosensors were -10.14 kJ mol⁻¹ and -3.40 kJ mol⁻¹, respectively. The lower E_a of the Lac₂-HRP biosensor indicates that the glycosylated-HRP biosensor possesses a higher enzymatic activity than the non-modified HRP biosensor. Although we found that the sensitivity of the biosensor was greatest at 35°C, we performed the experimental work described below at room temperature to prolong the useful lifetime of the biosensors.

3.8 Response of the Biosensors to Hydroquinone

The amperometric response after successive addition of hydroquinone, using both biosensors was evaluated using the optimized conditions identified: applied potential of 30 mV, 200 µM H₂O₂ in 0.1 M PBS at pH 7.0 under continuous stirring. Fig. 12A shows the typical current-time response of the HRP and Lac₂-HRP biosensors after the addition of successive aliquots of hydroquinone every 100 s. Both biosensors displayed a broad linear range and excellent sensitivity.

The inset in Fig. 12A shows the complete chronoamperometric response after many subsequent substrate additions. At high substrate concentrations the biosensor response reaches a plateau, as it has been reported repeatedly in the literature [8, 18]. This could be due to two events. First, at high substrate concentrations we could have the onset of substrate inhibition. Second, during the experiment the end product is accumulating and this can lead to subsequent product inhibition. This behavior is reversible when the electrode is immersed in a new buffer after the experiment, thus the process does not inactivate the enzyme. Fig. 12B shows the calibration curve for both biosensors, showing a linear response from 5 to 300 µM of hydroquinone. Table 1 summarizes the characteristics of the calibration plot, as well as the

detection limits calculated according to the $3S_b/m$ criteria, where m is the slope of the linear range of the respective calibration curve, and S_b is the standard deviation of the lowest concentration of the calibration curve [6]. The lowest detection that we obtained was with the Lac₂-HRP biosensor (0.74 μ M) compared to 0.83 μ M with the HRP biosensor. The response time was 15 s for the HRP and 18 s for the Lac₂-HRP biosensor.

To investigate the substrate specificity of the HRP biosensors, the response of both developed sensors to the phenol substrates catechol, dopamine, 4-methoxyphenol, and 4-nitrophenol was investigated (Fig. S2). Both sensors displayed a very similar behavior. It is apparent that the various phenols produce a response with the exception of 4-nitrophenol, but the magnitude of the response depends on the specific molecule used. This is in agreement with the literature, which describes that HRP catalyzes the oxidation of a broad range of substrates [4, 6, 23, 25, 42-45]. The current produced by catechol and hydroquinone is about equal in agreement with previous data [4, 6, 23, 44-45]. Dopamine and 4-methoxyphenol produce substantially less current, again in agreement with previous research data [4, 23, 44]. A final addition of hydroquinone was done to confirm that the diminished current intensity is not a result of the inactivation of the protein during the experiment.

3.9 Operational Stability, Reproducibility, and Long-term Stability of the Biosensors

Operational and long-term stability are important factors in biosensor performance. The operational stability of the HRP and Lac₂-HRP biosensors was examined by means of 60 repetitive measurements using 100 μ M hydroquinone and 200 μ M H₂O₂ under constant stirring (Fig. 13). The HRP biosensor showed less consistent relative standard deviation values than the Lac₂-HRP biosensor (Table 2). These results suggest that the chemical glycosylation improved operational stability.

The long-term stability of the biosensors was investigated over a 130-day period. When not in use, the biosensors were stored in 0.1 M PBS at pH 7.0 and 4°C. It was found that the current response remained nearly at 60% of its initial value for 100 days (Fig. 14). Table 3 provides a comprehensive literature review on HRP biosensors. It is evident that our biosensor constructs are superior to most reported in the literature. The functional efficacy of enzymes depends on the conformational stability of their native state. Enzymes adopt a tertiary structure that minimizes exposure of hydrophobic residues in aqueous solution. This hydrophobic core is also stabilized by several types of atomic interactions within the protein core (e.g., electrostatic interaction, hydrogen bond, Van der Waals interaction, charge-charge interaction) [11]. Therefore, any physical or chemical phenomenon present during biosensor construction, which disrupts these forces, can cause protein structural changes and activity loss. The stabilization of HRP by the attachment of glycans is likely largely due to so-called dielectric screening [10, 11, 46]. Glycans on the surface of the protein displace water molecules and thus reduce dielectric coupling of water vibrations and vibrations of polar protein groups. This in turn reduces protein dynamics and increases protein thermodynamic stability [10, 11, 46]. Glycans could also prevent interactions of the protein molecules with each other and with the electrode surface, which could also lead to irreversible events. Regardless of the specific stabilization mechanism, which was not elucidated in this work, the glycosylated enzyme-based biosensor displayed excellent long-term stability when compared to the biosensor employing non-modified HRP.

3.10 Comparison with Other HRP Biosensors

Biosensor characteristics, such as, sensitivity, linear range, detection limit, and stability were compared with the literature data presented in Table 3. The constructed Lac₂-HRP and HRP

biosensors exhibited improved analytical performance in terms of sensitivity, linear range, and detection limit when compared with all other reported biosensors. We believe that this makes the developed biosensor platform and the use of glycosylated enzymes in such applications attractive. It is important, however, to establish the generality of this in future investigations employing other enzymes.

4. Conclusions

In this paper, we have presented a promising method to stabilize enzymes for biosensor applications. The formation of a monolayer on the gold nanoparticle modified glassy carbon electrode provided a suitable, simple, and low cost platform that could effectively be used to immobilize Lac₂-HRP and HRP. The experimental results clearly show that the immobilized Lac₂-HRP biosensor possesses high catalytic activity and outstanding enzyme activity retention, for a prolonged period of time. The developed biosensor showed excellent analytical characteristics toward hydroquinone with respect to linear range, sensitivity, detection limits, repeatability, and stability in comparison with previously reported HRP biosensors. Future studies will include the investigation of the possible role of the glycan (e.g., glycan size, glycosylation degree) on sensor sensitivity, linear range, and stability. The results should then be transferred to the development of an enzyme sensor employing an enzyme with greater substrate specificity since the latter is not pronounced in HRP. To our knowledge, this study demonstrates for the first time the use of a chemically glycosylated enzyme to promote stability in an enzyme-based biosensor application.

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Supplementary information is available. It contains Table S1: Quantitative EDS results after each biosensor construction step; Fig. S1: The atom distribution after each biosensor construction step; Fig. S2: Chronoamperometric response of the HRP and Lac₂-HRP biosensors to hydroquinone (a), catechol (b), dopamine (c), 4-methoxyphenol (d), 4-nitrophenol (e), and hydroquinone (f).

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Fig. 1. Detection of phenolic compounds with an HRP-based biosensor. The substrate molecules labeled AH_2 represent the phenol molecules and AH^* the oxidized phenol molecules produced during the catalytic cycle.

Fig. 2. Schematic representation for the biosensor construction.

- Fig. 3.** CD spectra for HRP (—) and Lac₂-HRP (----) in the near-UV region (A) and heme absorption region (B).
- Fig. 4.** XPS survey and high resolution spectra of the Au 4f, S 2p and N 1s regions acquired after each principal step of biosensor construction; bare GC (—), AuNPs (···), 4-MBA monolayer (---), EDC-sulfo-NHS intermediate (— · —), and HRP (— · —). All spectra were background corrected and vertically displaced for ease of visualization.
- Fig. 5.** Scanning electron micrographs of the bare GC electrode (A), after electrodeposition of AuNPs (B), after 4-MBA monolayer formation (C), and after HRP immobilization (D).
- Fig. 6.** Cyclic voltammograms of the bare GC electrode (—), after electrodeposition of AuNPs (···), 4-MBA monolayer formation (----) and after the immobilization of HRP (— · —) (A) and Lac₂-HRP (— · —) (B) in the presence of 2 mM K₃Fe(CN)₆ in 0.1 M PBS, pH 7.0, scan rate of 50 mV s⁻¹.
- Fig. 7.** HRP (A) and Lac₂-HRP (B) biosensors in the presence of 0.1 M PBS at pH 7.0 (—), with 400 μM hydroquinone (···), and with 400 μM hydroquinone/200 μM H₂O₂ (----) at a scan rate of 50 mV s⁻¹.
- Fig. 8.** Cyclic voltammograms for HRP (A) and Lac₂-HRP (B) biosensors at scan rates of 10, 50, 100, 150, 200, 250, 300, 350, 400, 500, 600, 700, 900 mV s⁻¹ in 0.1 M PBS, pH 7.0.
- Fig. 9.** Sensitivity of the HRP (●) and Lac₂-HRP (○) biosensor at different reduction potentials for hydroquinone. Conditions: 10-300 μM hydroquinone, 200 μM H₂O₂, 0.1 M PBS, pH 7.0, constant stirring.
- Fig. 10.** Effect of the H₂O₂ concentration on the sensitivity of the HRP (●) and Lac₂-HRP (○) biosensor. Conditions: applied potential 30 mV, 0.1 M PBS, pH 7.0, 10-300 μM hydroquinone, constant stirring.

Fig. 11. Effect of the temperature on the sensitivity of the HRP (●) and Lac₂-HRP (○)

biosensor. Conditions: applied potential of 30 mV, 0.1 M PBS, pH 7.0, 10-300 μ M hydroquinone, constant stirring. Inset: Arrhenius Plot.

Fig. 12. Chronoamperometric response of the HRP and Lac₂-HRP biosensor upon successive addition of 25 (a), 50 (b), 75 (c), 100 (d), 125 (e), 150 (f), 200 (g), 225 (h), 250 (i), 275 (j), 300 (k) and 325 (l) μ l of 1.0 mM hydroquinone every 100 s (A). Inset: chronoamperometric response of HRP and Lac₂-HRP biosensors until protein saturation. Calibration curve of the HRP (●) and Lac₂-HRP (○) biosensor (B).

Conditions: applied potential 30 mV, 200 μ M H₂O₂, 0.1 M PBS at pH 7.0, constant stirring.

Fig. 13. Current responses obtained with the HRP (●) and Lac₂-HRP (○) biosensors.

Conditions: applied potential 30 mV, 100 μ M hydroquinone, 200 μ M H₂O₂, 0.1 M PBS, pH 7.0, constant stirring.

Fig. 14. Relative activity obtained during storage at 4°C of the HRP (●) and Lac₂-HRP (○)

biosensors. Conditions: applied potential 30 mV, 100 μ M hydroquinone, 200 μ M H₂O₂, 0.1 M PBS, pH 7.0, constant stirring.

Table 1.

Analytical characteristics of the HRP and Lac₂-HRP biosensor using hydroquinone as substrate.

Biosensor	Linear Range (μ M)	R ²	Sensitivity (nA μ M ⁻¹ cm ⁻²)	Limit of Detection (μ M)	Response Time (s)
HRP	5-300	0.9932	378 $\pm$ 35	0.74 $\pm$ 0.1	18
Lac ₂ -HRP	5-300	0.9921	414 $\pm$ 20	0.83 $\pm$ 0.2	15

Table 2.

Relative standard deviation (R.S.D.) obtained from repeatability measurements of the HRP and Lac₂-HRP biosensor.

	HRP	Lac ₂ -HRP
Number of Measurements	RSD (%)	RSD (%)
10	8.16	5.32
15	8.26	5.65
30	7.96	5.30
60	13.04	6.48

Table 3.

Properties of phenol biosensors based on HRP.

Biosensor	Sensitivity (nA μ M ⁻¹ cm ⁻²)	LOD (μ M)	Linear Range (μ M)	Useful Lifetime	
HRP	378	0.74	5-300	5 weeks	hy
HRP-Lac ₂	414	0.83	5-300	17 weeks	hy
HRP-Graphite	0.6	4.0	10-20	-	
	1	0.5	10-20	-	2 ch
HRP-MPA-Au	-	2.0	1-25	4 weeks	
HRP-ConA-MPS-	160	0.6	6.0-48.0	4 weeks	
PAH/PSS/PAH-Au	3.9	2.0	6.0-72.0	4 weeks	hy
HRP-MWCNT-PPY-Au	8	6.42	16-240	4 weeks	hy
	1	3.52	16-44	4 weeks	
HRP-Met-MWCNT	-	0.5	1-150	300 determination 10 h continuous used	
HRP-MWCNT-GC	-	0.38	1-100	2 weeks	dic

MPA = mercaptopropionic acid, MPS= 3-mercapto-1-propanesulfonic acid, ConA = concanavadin A, PAH= poly(allilamine hydrochloride), PSS= poly(sodium-p-styrene-sulfonate), PPY = polypyrrole, Met = methylene Blue

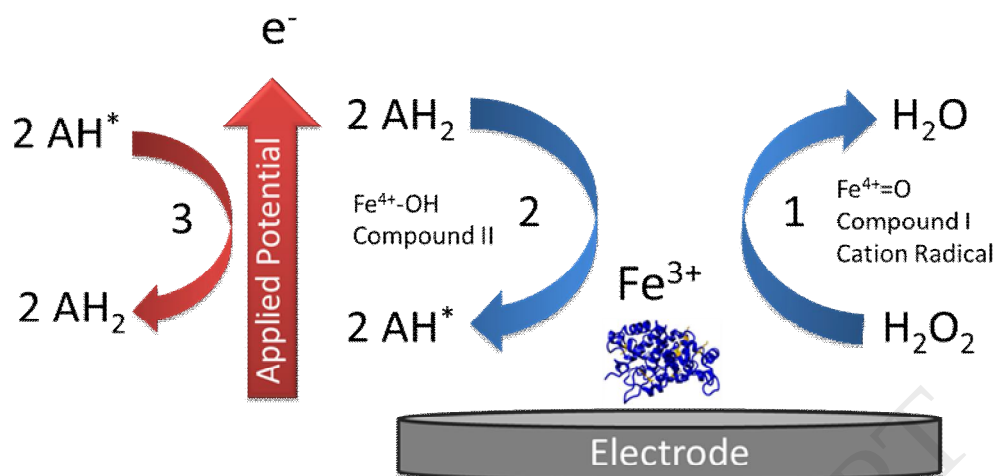


Fig. 1.

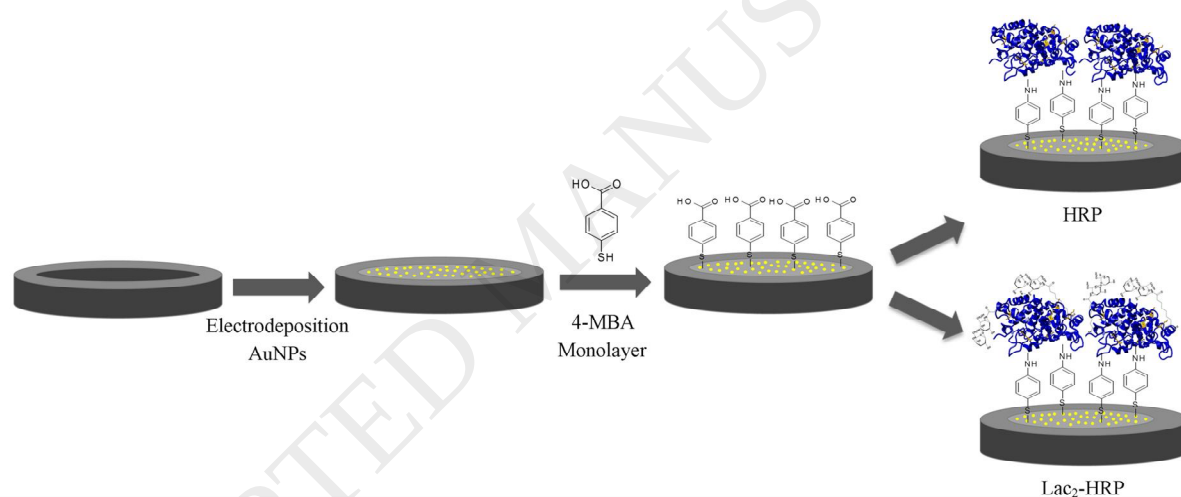
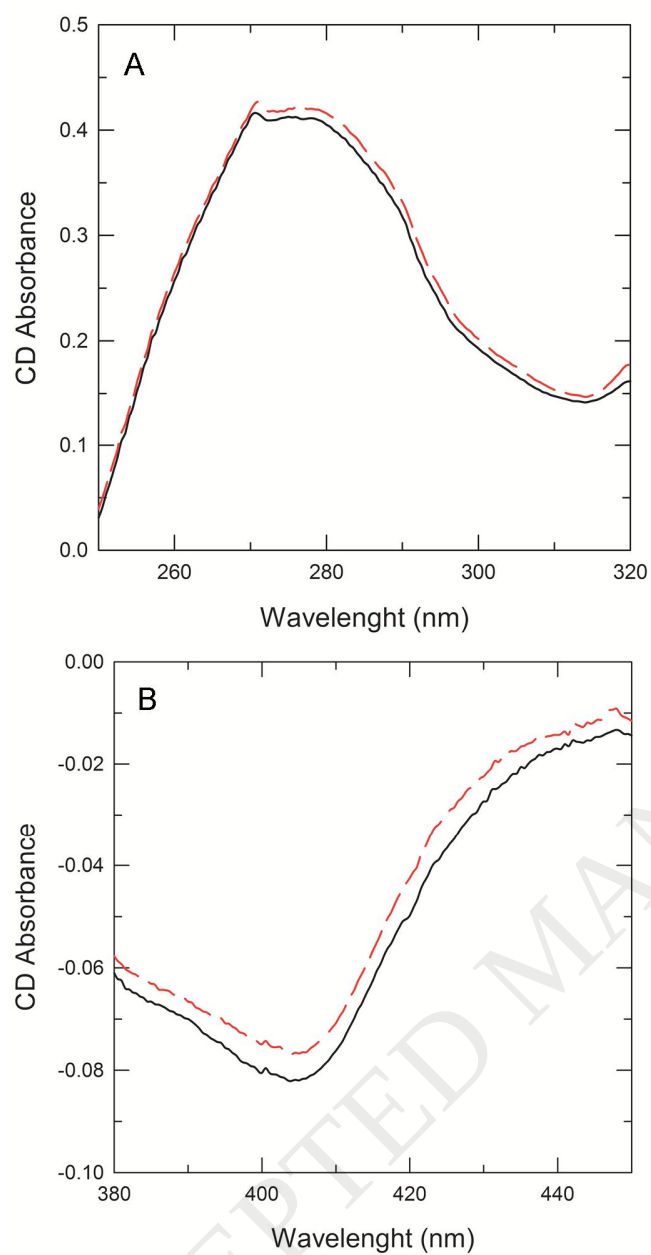
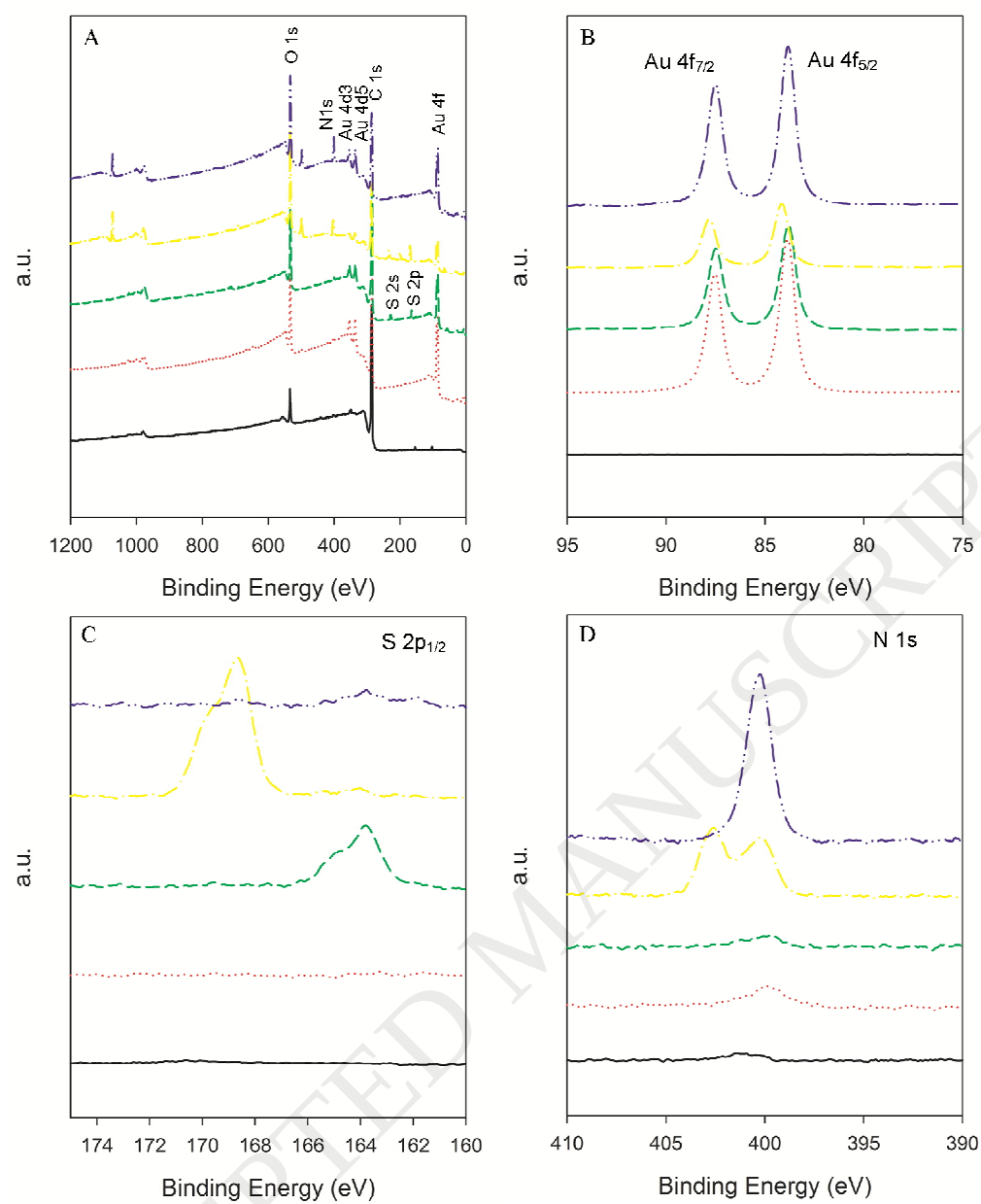


Fig. 2.

**Fig. 3**

**Fig. 4.**

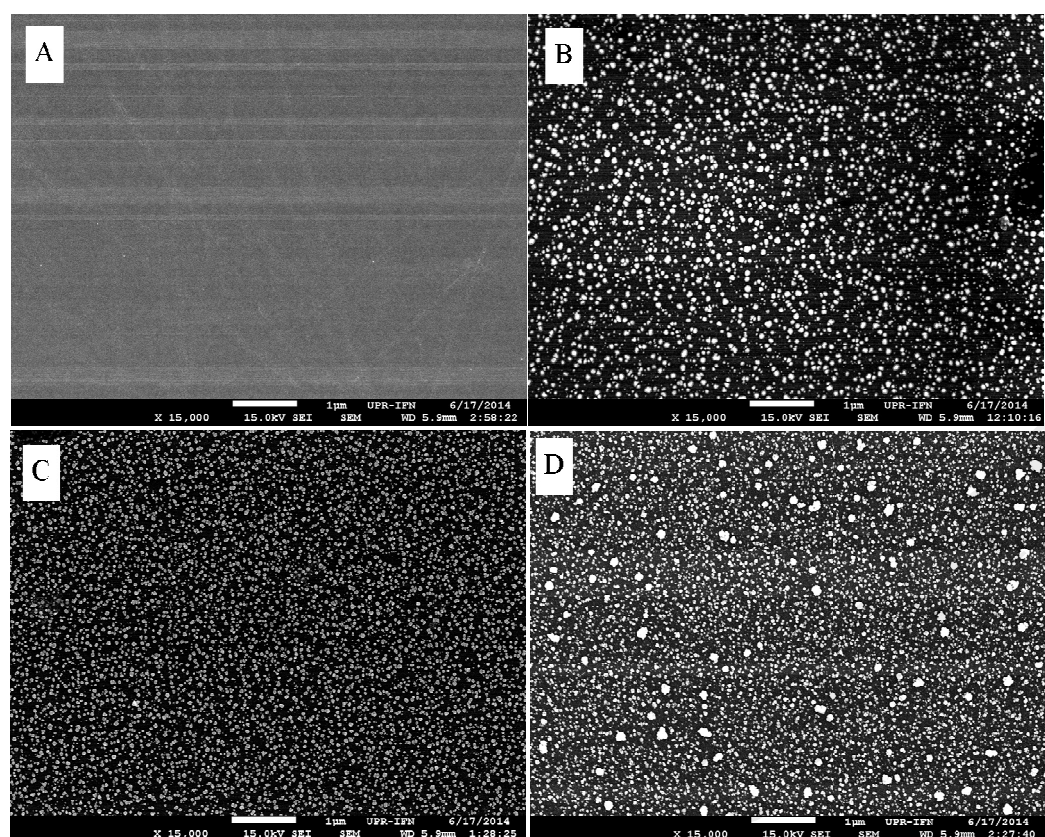
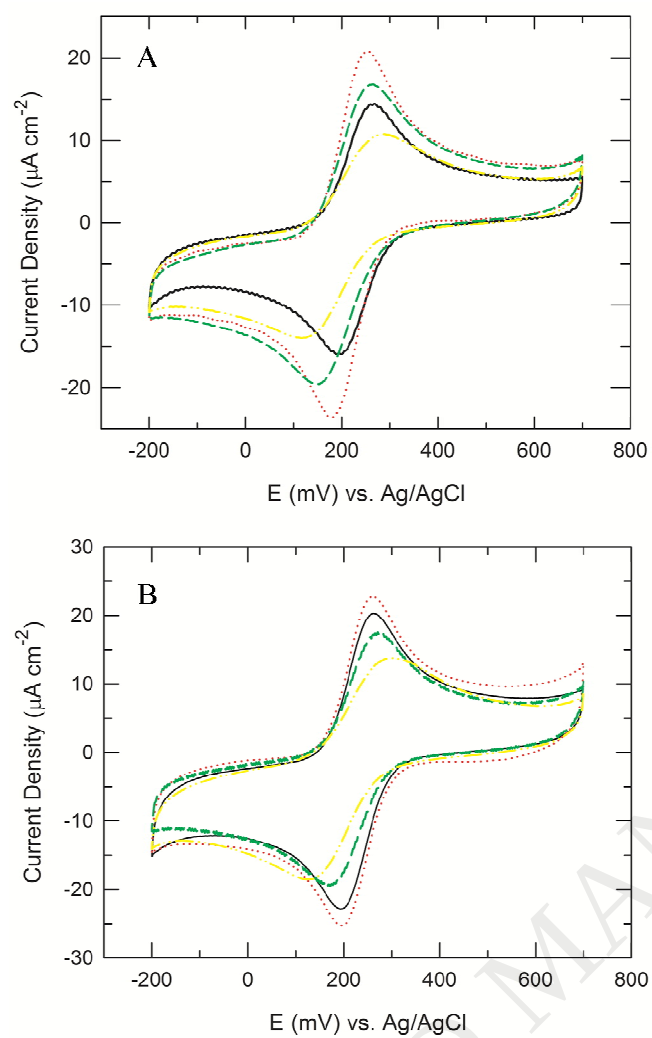
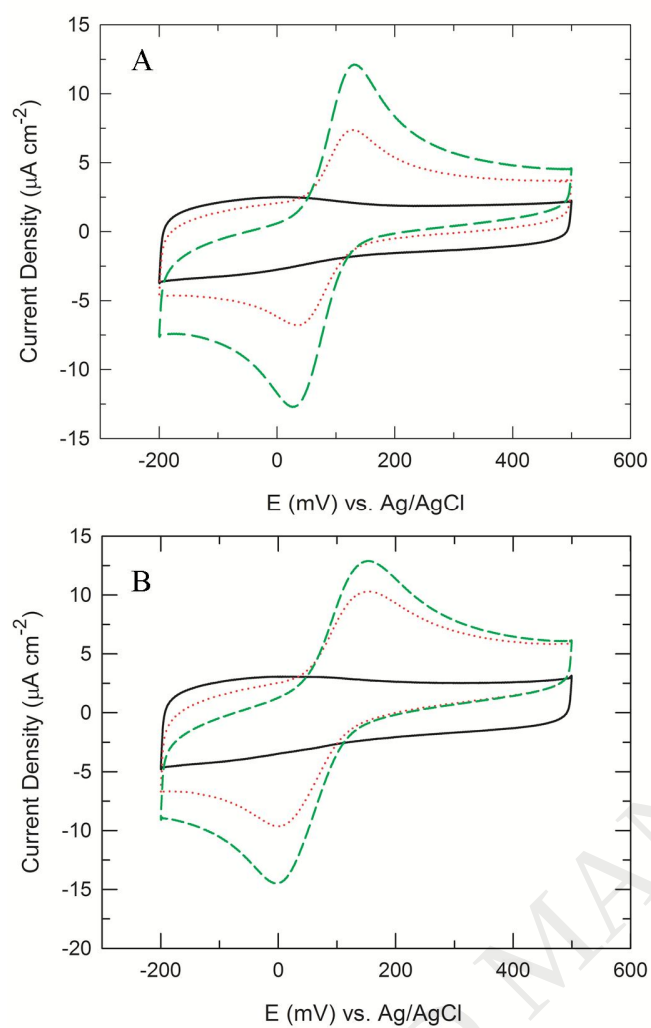
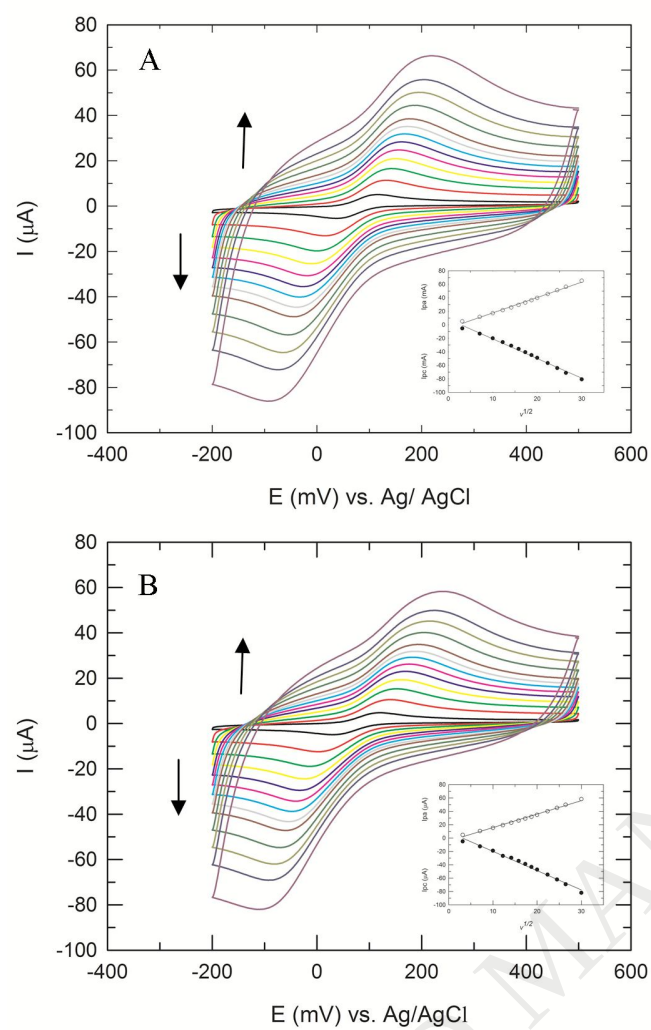
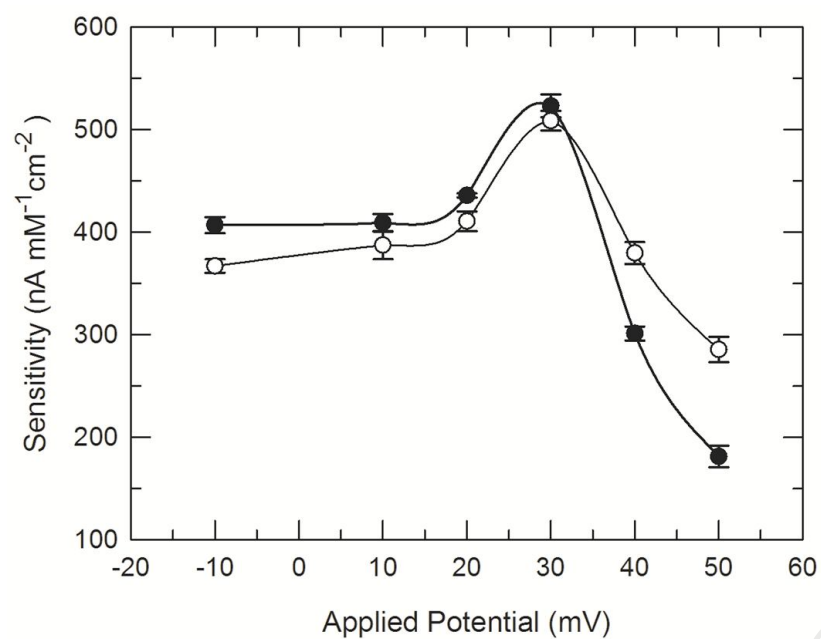
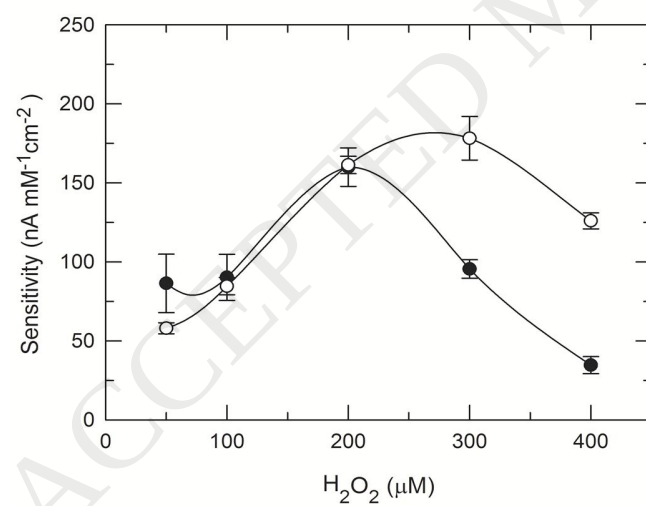


Fig. 5.

**Fig. 6.**

**Fig. 7.**

**Fig. 8.**

**Fig. 9.****Fig. 10.**

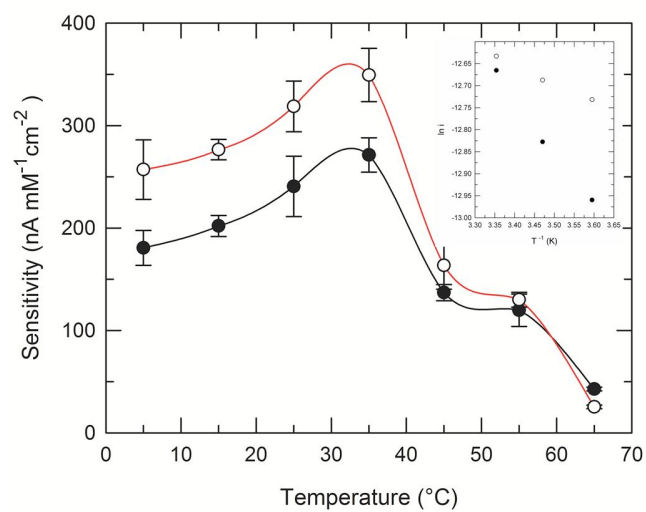
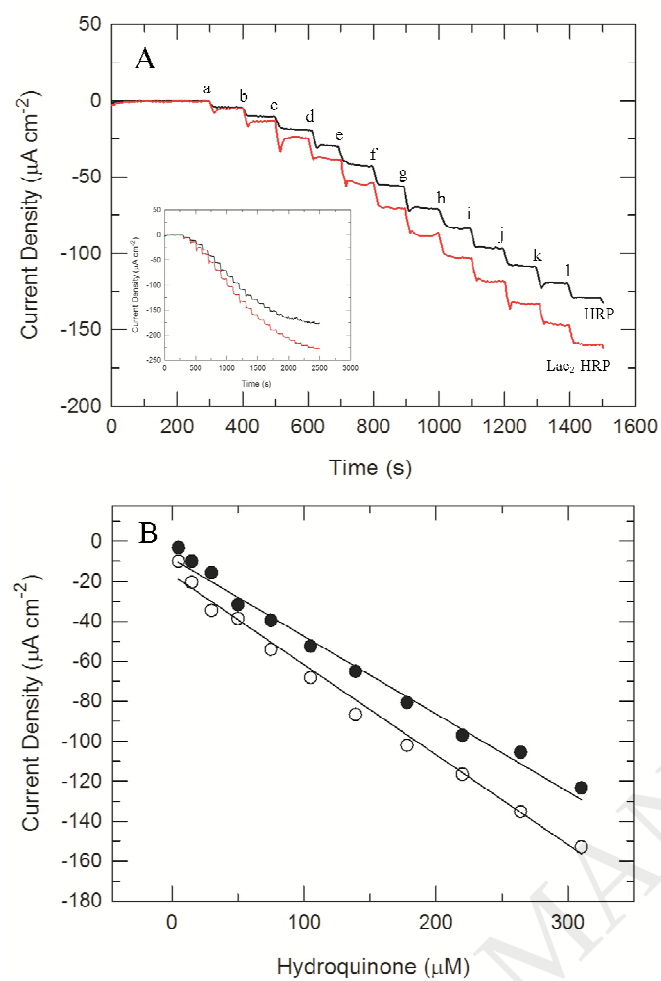
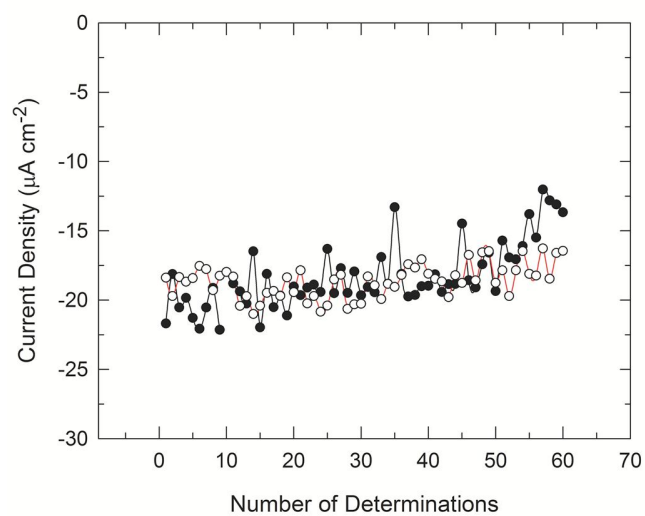
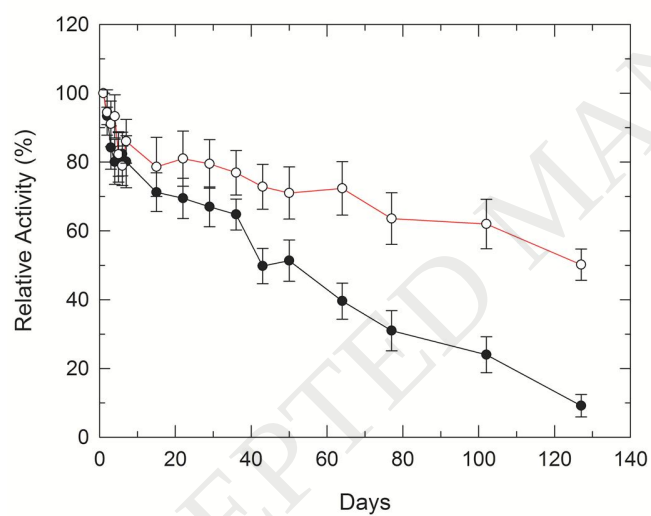


Fig. 11.

**Fig. 12.**

**Fig. 13.****Fig. 14.**