Mixed Self-Assembled Monolayers of Mercaptoundecanoic Acid and Thiolactic Acid for the Construction of an Enzymatic Biosensor for Hydroquinone Determination

Rosana Mossanha, Maria Karolina Ramos, Cleverson Siqueira Santos, and Christiana Andrade Pessoa

Universidade Estadual de Ponta Grossa (UEPG), Department of Chemistry, 84030-000 Ponta Grossa, Paraná, Brazil

A horseradish peroxidase (HRP) biosensor was constructed using binary self-assembled monolayers (SAM) of 11-mercaptoundecanoic acid (MUA) and thiolactic acid (TLA) on gold surface. The advantages of using mixed SAM for the enzyme immobilization is that the long carbon chain molecules act as a support for the enzyme while the short chain molecules favor the electron transfer process. In order to obtain this modified surface, the gold electrode was incubated in a solution containing different proportions of MUA and TLA and the best concentration ratio of these molecules was 0.5 and 1.0 mmol L\(^{-1}\), respectively. The preparation steps and the biosensor response were monitored by electrochemical techniques. The biosensor proposed was applied to determine hydroquinone in a 0.10 mol L\(^{-1}\) phosphate buffer solution containing H\(_2\)O\(_2\) 0.3 mmol L\(^{-1}\). The Au-SAM\(_{\text{mix}}\)-HRP electrode, in the presence of hydrogen peroxide, catalyzes the oxidation of hydroquinone to the corresponding quinone, which is electrochemically reduced back to hydroquinone at −0.08 V vs Ag/AgCl. The analytical curve was linear for hydroquinone concentrations from 5.0 to 30 μmol L\(^{-1}\) and the detection limit was 1.26 μmol L\(^{-1}\). The lifetime of this biosensor was 15 days. The modified electrode showed good reproducibility, sensitivity and stability for the determination of hydroquinone.

© 2015 The Electrochemical Society. [DOI: 10.1149/2.0281507jes] All rights reserved.

The modification of electrode surfaces using the self-assembled monolayers (SAM) technique is more convenient, because they form spontaneously, easy to handle mechanically and relatively stable in electrolyte solutions.\(^1\) The main interest in developing mixed self-assembled monolayers (SAM\(_{\text{mix}}\)) is related to the development of attractive methods to promote different arrangements on the electrode surface, which enables the control of specific reaction sites. In this way, SAM\(_{\text{mix}}\) can be configured as an appropriate platform for the immobilization of biomolecules\(^2\) which have been frequently applied in electroanalysis for the development of biosensors,\(^3,4\) since well-organized and compact monolayers present advantages like selectivity, sensitivity and reduced overpotentials in electrocatalytic reactions. The SAM\(_{\text{mix}}\) can be obtained by combining the properties of alkanethiols with different carbon chain lengths or different functional groups.\(^5\)

Several methods for the preparation of mixed SAMs are reported in the literature, being the most commonly used the co-adsorption of different thiols,\(^6,7\) and also by electrochemical substitution modification.\(^8,9\) The SAM\(_{\text{mix}}\) obtained through co-adsorption of different functional groups was described by Ngunjiri et al.,\(^10\) formed by molecules octadecanethiol and 11-mercaptoundecanoic acid for immobilization of proteins. Another example, Ji et al.\(^11\) developed the SAM\(_{\text{mix}}\) formed by thioctic acid (T-COOH) and thioctic acid amide (T-NH\(_2\)) which was used to immobilize tyrosinase to construct an biosensor showed that mixed SAMs improved protein adsorption and activity without the use of crosslinking agents.

The determination of phenolic compounds - hydroquinone (HQ, 1, 4-dihydroxybenzene) is of great importance in the environmental control, protection and health benefits due to their genotoxicity, carcinogenicity and toxic kinetic effect on humans and persistency in the environment.\(^12\) This study proposes the use of binary SAM to develop a biosensor for hydroquinone. The SAM\(_{\text{mix}}\) exhibits an architecture formed by a self-assembled monolayer of mixed alkanethiols with short (thiolactic acid-TLA) and long (11-mercaptoundecanoic-MUA) chains. Monolayers of carboxylic acids are widely used in surface modifications since the carboxylic functional group favors hydrophilic character to the system and thus helps to improve the solubility favoring the binding of biomolecules.\(^8\) The idea is the existence of an island-like shape, which means that the MUA acts as a barrier for electron transfer while the TLA alkanethiol molecules are deposited in such a way that the electrons can be freely transferred to ions in solution, allowing electrochemical reactions to occur, like a “pore” on the SAM\(_{\text{mix}}\) modified surface. Thus, the mixed monolayers were used for the immobilization of the enzyme horseradish peroxidase (HRP).

Experimental

Materials.— Thiolactic acid (TLA), Horseradish Peroxidase Type VI-A, N-(3-Dimethylaminopropyl)-N’-ethycarbodiimide
hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Mercapto-
toundecanoic acid (MUA) were obtained from Sigma-Aldrich (USA), K3[Fe(CN)6], K4Fe(CN)6.3H2O, CH3CH2OH, H2SO4, H2O2 was
acquired from Vetec (BRAZIL). Phosphate buffered saline solution
0.1 mol L−1 was prepared by mixing Na2HPO4, NaH2PO4 and NaCl
used as supporting electrolyte. The pH adjustment was performed by
NaOH solutions 0.5 mol L−1. All chemicals used were of analytical
grade and used as received. Solutions were prepared with deionized
water.

Electrode pre-treatment.— Before carrying out the deposition of the
corresponding SAMs, the gold electrode was pretreated. Firstly,
the gold electrode (0.0401 cm2) was polished with alumina powder.
Secondly, the gold electrode was cleaned in a "piranha" solution, which is a mixture of 30% H2O2 and concentrated H2SO4
(1:3, v/v). Finally, the electrodes were electrochemically cleaned by
cycling the potential between −0.2 and 1.6 V in 0.5 mol L−1 H2SO4
at scan rate 0.05 V s−1. The characteristic voltammogram of a clean
gold electrode was obtained after approximately 100 cycles. This
surface treatment was the most appropriate to produce a clean and
reproducible surface.20

Preparation of SAMs and SAMmix on gold surface.— For the SAM
preparation, the electrode was firstly immersed in 10.0 mmol L−1
TLA for 3h or 10.0 mmol L−1 MUA for 24h, both ethanol solutions.
The modified electrode was rinsed with pure ethanol, and then with
distilled water. For the binary SAMmix, the electrode was immersed in
ethanol solution containing both the precursors TLA and MUA.
The thiol concentration effect was evaluated in the range of 0.25 to
1.0 mmol L−1 in order to form a stable and orderly surface. The
immersion time for SAMmix was 24h. The modified electrode was
also rinsed with pure ethanol, and then with distilled water before the
electrochemical experiments.

Au-SAMmix-HRP biosensor preparation.— For the biosensor
preparation, Au-SAMmix electrode was immersed for 1h in a solution
containing N-hydroxysuccinimide (NHS) 5.0 mmol L−1 and N-(3-
dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) 2.0 mmol L−1 in
phosphate buffer solution (PBS) 0.1 mol L−1. After that, HRP en-
zyme (55 units) was dropped on the surface. The NHS/EDC acts as a coupling agent between the HRP enzyme and -COOH end groups of
SAMmix. When not in use, the Au-SAMmix-HRP biosensor was stored
in the PBS 0.1 mol L−1 pH 6.5, at 22°C.

Results and Discussion

Formation of SAM and SAMmix of TLA and MUA.— The forma-
tion of SAM containing a defined mixture of different molecular
structures forming the so-called mixed SAM,21 favors the stability
and anchoring of biomolecules. The molecules used in this study have
different carbon chain structures, but both terminal groups, which are
carboxylic acids. The TLA has only three carbon atoms, being chosen
as short chain molecule and the MUA has eleven carbon atoms and
was used as a long chain molecule. The combination with a short-
chain molecule makes this study interesting because it is expected that
the TLA enables the increase of the conductivity, facilitating the
electronic transfer due to the formation of “islands” while the MUA
provides stability to the SAMmix monolayers.

Along with the monolayer formation, the cyclic voltammograms
presented lower faradaic currents compared to the unmodified gold
surface due to the immobilization of carboxylic groups which blocks
the electron transfer of the negative redox ions [Fe(CN)6]3−/4 to the
electrode surface (Fig. 1a). For Au-TLA, a pair of peaks was observed
(Epa = 0.35 V and Epc = 0.17 V) similar to the clean gold electrode
(Epa = 0.31 V and Epc = 0.17 V) however, with decrease in the peak
current, due to a slow kinetic electron transfer process compared to
the Au electrode, which is an indication that the TLA was immo-
ibilized on the gold surface. As long chains require longer time for
organization and orientation on the metal surface,22 the Au electrode
was left immersed for a period of 24 h in the MUA thiol solution. For
Au-MUA electrode, the electroanalytical parameters were impossible to determine due to lack of oxidation and reduction peaks in all immersion times studied. This is probably due to the high surface coverage, which makes the electron transfer between the species in solution and the metal surface difficult to occur due to the high degree of packing of long-chain monolayer.\textsuperscript{25} When using gold electrodes modified with short chains of 2 to 4 carbons, the electron transfer occurs through the SAM, producing voltammetric profiles very similar to those observed with unmodified gold surface.\textsuperscript{24} In the case of long chains as MUA, the electron transfer is partially blocked, providing no voltammetric profile. For the electrode SAM\textsubscript{mix}, when there was mixing between the TLA and the MUA, the incubation time chosen for the monolayer formation was 24 h. SAM\textsubscript{mix} voltammograms presented an intermediate behavior when compared to the isolated monolayers of thiols (E\textsubscript{pa} = 0.45 V and E\textsubscript{pc} = −0.05 V).

Such results were confirmed by electrochemical impedance spectroscopy using [Fe(CN)\textsubscript{6}]\textsuperscript{3−/4−} redox couple as a probe. Electrochemical impedance spectroscopy is extensively used to evaluate the structural integrity of the monolayer and also to determine the electron transfer resistance across the SAM modified electrode.\textsuperscript{25} In Figure 1b, the impedance spectra of the electrodes is observed to show two frequency regions, the semicircle (higher frequencies) corresponding to the electron transfer process while the line (lower frequencies) represents the diffusion process.\textsuperscript{26,27} As discussed previously by the CV, the impedance frequency regions, the semicircle (higher frequencies) corresponding to the electron transfer process while the line (lower frequencies) represents the diffusion process.\textsuperscript{26,27}

Using the EIS data obtained in the presence of redox couple [Fe(CN)\textsubscript{6}]\textsuperscript{3−/4−}, it was possible to estimate the apparent rate constant, which can be expressed by equation 1.\textsuperscript{28}

\[
k\text{app} = \frac{RT}{F^2R_{ct}C^*}
\]

In eq. 1, \(R\) is the gas constant, \(T\) is the temperature, \(F\) is the Faraday constant, \(R_{ct}\) is the value of the charge transfer resistances and \(C^*\) is the concentration of the redox couple in the bulk solution.

The Au-SAM\textsubscript{max} electrode (\(k\text{app} = 1.60 \times 10^{-15} \text{ cm s}^{-1}\)) showed intermediate value in comparison to Au-TLA (\(k\text{app} = 6.88 \times 10^{-9} \text{ cm s}^{-1}\)) and Au-MUA (\(k\text{app} = 1.15 \times 10^{-11} \text{ cm s}^{-1}\)). These \(k\text{app}\) values are in agreement with the cyclic voltammetry experiments, which showed the improvement of the reversibility of Au-SAM\textsubscript{electrode}, when compared to Au-MUA in the presence of the probe molecule. These values also agree with those obtained in the literature.\textsuperscript{29} For \(k\text{app}\), the effect is inverse and it decreases as the alkyl chain increases, because long chains make the electron transfer more difficult.

The coverage of surface with monolayers was determined by impedance measurements across the charge transfer resistance, by equation 2.\textsuperscript{28}

\[
\theta = 1 - \frac{R_{ct Au}}{R_{ct Au mod}}
\]

In eq. 2, \(R_{ct Au}\) and \(R_{ct Au mod}\) are the value of the charge transfer resistances derived from the Nyquist diagram of the bare gold electrode and gold modified with SAM, respectively.

The electrode with the highest surface coverage was the Au-MUA (\(\theta \approx 0.999\)) which has a very dense and homogeneous coverage, so its apparent constant velocity becomes quite low compared to other electrodes. This behavior is in agreement with the literature for long chain monolayers.\textsuperscript{30} The coating of the electrode Au-TLA (\(\theta \approx 0.580\)) while the Au-SAM\textsubscript{max} (\(\theta \approx 0.990\)) was close to the Au-MUA electrode, virtually covering the entire surface, confirming that the long-chain molecules even at lower concentration compared to TLA, make the surface more orderly and compacted.

Thus, the results of cyclic voltammetry and electrochemical impedance spectroscopy revealed that the TLA and the MUA electrochemical behavior is distinct and when they are mixed an intermediate behavior is obtained, indicating that there was a co-adsorption of thiols at Au electrode surface.

**Reductive desorption studies.—** In order to investigate the alkanethiol coverage, the reductive desorptive process in alkaline medium was evaluated. Studies of these processes suggest that desorption occurs primarily in the SAM defect regions and then proceed to areas with higher and more packed organization.\textsuperscript{31} Electrodes modified with SAMs of each type of alkanethiol have their potential swept between −0.6 and −1.4 V at 20 mV s\textsuperscript{−1} in 0.5 mmol L\textsuperscript{−1} NaOH solution and the voltammograms are presented in Fig. 2.

From Figure 2, it was possible to characterize the monolayers by the reduction potential. The SAM organization is influenced by the size of the carbon chain. When the molecule contains a small amount of methyl in its side chains interactions are not sufficient to stabilize the structure.\textsuperscript{32} The TLA has only a methylene group, then the interaction between these side groups is weak and it is not so strongly stabilized as in a long chain molecule. This explains why the reduction peak of the TLA occurred at less negative potentials (−0.89 V) because these interactions are weaker when compared to the MUA. In Fig. 2, the monolayer Au-MUA desorption is shown, which revealed an intense reduction peak at −1.13 V. This molecule contains a large number of methyl groups, so the lateral interactions between the molecules are more intense and then its reduction peak occurs at more negative potentials. For SAM\textsubscript{mix}, it is observed that the desorption voltammograms presented a mixed profile between the two monolayers, however with a slight displacement of the desorption potentials, at −0.99 V and −1.13 V. This is due to the interaction between the long and short chain thiols, which promotes stabilization of the mixed monolayer.\textsuperscript{15}

**Influence of the thiol concentration in the mixed monolayer.—** Experimental studies have shown that the rate of electron transfer of SAM\textsubscript{mix} can be significantly affected due to the relation between the long and short thiol chain on the electrode surface.\textsuperscript{33} SAM\textsubscript{mix} was obtained by immersing the gold electrode in ethanol solution containing five different concentrations of MUA with TLA in the

![Figure 2. Differential pulse voltammograms of different monolayers: MUA (1.0 mmol L\textsuperscript{−1}), TLA (1.0 mmol L\textsuperscript{−1}) and SAM\textsubscript{mix} (0.25 MUA and 1.0 mmol L\textsuperscript{−1} TLA) in NaOH 0.5 mol L\textsuperscript{−1}. Scan rate: 20 mV s\textsuperscript{−1}.](image-url)
range 0.25 to 1.0 mmol L\(^{-1}\). In Fig. 3 the voltammetric behavior of these electrodes is shown.

According to Fig. 3, MUA monolayer has an insulating effect on the surface when its concentration is 1.0 mmol L\(^{-1}\), even in the presence of a short chain molecule. This behavior is associated with a high degree of electron transfer resistance imposed by the long carbon chain in the MUA molecule. However, when its concentration is increased, the oxidation peak potential appeared and the oxidation current value increased, approaching of the pure TLA.

**HRP enzyme immobilization at the SAM\(_{\text{mix}}\).**— In order to evaluate the HRP enzyme immobilization effect on the electrode monolayer, the SAM\(_{\text{mix}}\) obtained with MUA 0.25 mmol L\(^{-1}\) and TLA 1.0 mmol L\(^{-1}\) was chosen. The HRP enzyme was immobilized by the method of covalent attachment, in which the reaction occurs between the -COOH end groups of the SAM ligands with EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and NHS (N-hydroxysuccinimide ester), which favors a more stable immobilization of the enzyme. Studies of cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed in the presence of 5.0 mmol L\(^{-1}\) of \([\text{Fe(CN)}_6]^{3-/4}\). Scan rate: 50 mV s\(^{-1}\).

Figure 3. Cyclic voltammograms of mixed monolayers obtained at different concentrations of thiols dependence on the ratio MUA/TLA. Electrolyte: KCl solution 0.1 mol L\(^{-1}\) containing 5.0 mmol L\(^{-1}\) of the redox couple \([\text{Fe(CN)}_6]^{3-/4}\). Scan rate: 50 mV s\(^{-1}\).

Figure 4. Characterization by (a) Cyclic voltammetry and (b) electrochemical impedance spectroscopy for the electrode Au-SAM\(_{\text{mix}}\) and Au-SAM\(_{\text{mix}}\)-HRP in the presence of 5.0 mmol L\(^{-1}\) of \([\text{Fe(CN)}_6]^{3-/4}\) in KCl 0.1 mol L\(^{-1}\), pH = 6.5.

Figure 5. Dependence of SAM\(_{\text{mix}}\) and SAM\(_{\text{mix}}\)-HRP in relation to the concentration of H\(_2\)O\(_2\) in the range of 0.4 to 1.0 mmol L\(^{-1}\) and applied potential −0.2 V. Electrolyte PBS 0.1 mol L\(^{-1}\) pH 6.5. Concentration of thiols solutions to prepare the SAM\(_{\text{mix}}\): MUA 0.25 mmol L\(^{-1}\) and TLA 1.0 mmol L\(^{-1}\).
activity, the electrochemical behavior of different SAM\textsubscript{max} biosensors (with different proportions of TLA and MUA) in the presence of H\textsubscript{2}O\textsubscript{2} substrate was evaluated by chronocoulometry technique according to Fig. 6.

The sensitivity of Au-MUA/TLA(0.5:1.0)-HRP electrode obtained by the incorporation of the enzyme HRP covalently bound to the mixed monolayer was observed to be higher (2.38 μA mmol L\textsuperscript{-1}) when compared to other modifications. The mixed SAMs containing the concentration 1.0 mmol L\textsuperscript{-1} MUA presented the lowest sensitivities, because of the energy barrier imposed by the length of the carbon chain, which requires higher energy for the electron transfer to occur, and hence the current decreases due to the electron transfer resistance imposed by the MUA molecules. However, when the ratio between the monolayers is equal Au-MUA/TLA(1.0:1.0)-HRP, an unexpected behavior occurs in the biosensor response, the sensitivity decreases to 1.30 μA mmol L\textsuperscript{-1} while for the Au-MUA/TLA(1.0:0.5)-HRP electrode it was 1.48 μA mmol L\textsuperscript{-1}. For the Au-MUA/TLA(1.0:1.0)-HRP electrode, higher sensitivity was expected but it seems that at a certain concentration of MUA, the molecules are so densely packed that higher immobilization of TLA molecules is prevented.

Repeatability measurements were performed with the Au-MUA/TLA(0.5:1.0)-HRP electrode, which showed the highest sensitivity, by monitoring the biosensor response in relation the H\textsubscript{2}O\textsubscript{2} concentration. It is known that high concentrations of H\textsubscript{2}O\textsubscript{2} can inactivate the enzyme.\textsuperscript{37} Then, the effect of the concentration of H\textsubscript{2}O\textsubscript{2} in response on this Au-SAM\textsubscript{max}-HRP was investigated. It was observed that as the concentration of H\textsubscript{2}O\textsubscript{2} increased there was a linear increase in the biosensor current response from of 30.0 to 300.0 μmol L\textsuperscript{-1}, with sensitivities of 10.35 μA/mol L\textsuperscript{-1} and 1.1 mmol L\textsuperscript{-1} for HRP/Au nanoparticle/Cysteine in silica sol-gel.\textsuperscript{41} In general, the lower the value of $K_{\text{Mapp}}$, the higher the affinity of the enzyme and its substrate, i.e., the higher the biosensor sensitivity.\textsuperscript{17} This $K_{\text{Mapp}}$ value obtained in this work showed that the immobilized HRP on SAM\textsubscript{max} maintained its biocatalytic activity exhibiting higher biological affinity to hydrogen peroxide. Thus the modified electrode is a favorable surface for the attachment of the HRP enzyme.

Electrochemical behavior of electrodes in the presence of hydroquinone.— Peroxidase is an oxidoreductase that catalyzes the oxidation of a variety of aromatic compounds. In the enzymatic reaction, cosubstrates such as phenolic compounds or aromatic amines are converted mainly into quinones or free radical products, which are electroactive and can be electrochemically reduced on the electrode surface.\textsuperscript{25} (Scheme 1).

The hydroquinone in the presence of hydrogen peroxide is oxidized by peroxidase, forming p-henzoquinone which is reduced electrochemically on the biosensor surface.\textsuperscript{35} The reduction current is proportional to the concentration of phenolic compounds in solution. Therefore, this biosensor was applied to the determination of HQ. In Figure 7, CVs for different configurations of the SAM\textsubscript{max} modified electrode (SAM\textsubscript{max} and SAM\textsubscript{max}-HRP with or without H\textsubscript{2}O\textsubscript{2}) in the presence of HQ are shown.

The SAM\textsubscript{max}-HRP presented higher reduction current response compared to the SAM\textsubscript{max}. However, when hydrogen peroxide was added, the current response decreased (not shown).

$K_{\text{Mapp}}$ was calculated in this work and its value obtained was 0.4 mmol L\textsuperscript{-1}, which is lower than those reported ones: 7.0 mmol L\textsuperscript{-1} for a biosensor composed of single walled nanotubes and chitosan for immobilization of HRP,\textsuperscript{39} 2.6 mmol L\textsuperscript{-1} of Au/graphene/HRP/chitosan biocomposites on glassy carbon electrode and 1.1 mmol L\textsuperscript{-1} for HRP/Au nanoparticle/Cysteine in silica sol-gel.\textsuperscript{41} In general, the lower the value of $K_{\text{Mapp}}$, the higher the affinity of the enzyme and its substrate, i.e., the higher the biosensor sensitivity.\textsuperscript{17} This $K_{\text{Mapp}}$ value obtained in this work showed that the immobilized HRP on SAM\textsubscript{max} maintained its biocatalytic activity exhibiting higher biological affinity to hydrogen peroxide. Thus the modified electrode is a favorable surface for the attachment of the HRP enzyme.

$\text{Peroxidase is an oxidoreductase that catalyzes the oxidation of a variety of aromatic compounds.}$

$\text{In the enzymatic reaction, cosubstrates such as phenolic compounds or aromatic amines are converted mainly into quinones or free radical products,}$

$\text{which are electroactive and can be electrochemically reduced on the electrode surface.}$

$\text{The hydroquinone in the presence of hydrogen peroxide is oxidized by peroxidase, forming p-henzoquinone which is reduced electrochemically on the biosensor surface.}$

$\text{The reduction current is proportional to the concentration of phenolic compounds in solution.}$

$\text{Therefore, this biosensor was applied to the determination of HQ.}$

$\text{In Figure 7, CVs for different configurations of the SAM\textsubscript{max} modified electrode (SAM\textsubscript{max} and SAM\textsubscript{max}-HRP with or without H\textsubscript{2}O\textsubscript{2}) in the presence of HQ are shown.}$

$\text{The SAM\textsubscript{max}-HRP presented higher reduction current response compared to the SAM\textsubscript{max}.}$

$\text{However, when hydrogen peroxide was added, the current response decreased (not shown).}$
added, the biosensor showed an increment in the voltammetric response, evidenced by the increase in the reduction peak current and potential displacement to less negative potentials. This behavior can be explained by the fact that hydrogen peroxide reduction is readily catalyzed by the biosensor in the presence of hydrogen peroxide, where its current is higher than that of the biosensor without \( \text{H}_2\text{O}_2 \). As hydrogen peroxide is the HRP enzyme natural substrate, it is essential for a good response of the biosensor. Therefore, the immobilized HRP in the presence of \( \text{H}_2\text{O}_2 \) exhibits a higher catalytic activity for phenolic components.

**Effect of the hydrogen peroxide in the biosensor response to HQ**—Another parameter that affects the Au-SAM\(_{\text{mix}}\)-HRP electrode response is the hydrogen peroxide concentration since the enzyme peroxidase needs to catalyze the reaction. As high concentrations of \( \text{H}_2\text{O}_2 \) inactivate the enzyme, a study on the effect of its concentration in the biosensor response to HQ was investigated. Thus, concentrations from 0.003 to 0.38 mmol \( \text{L}^{-1} \) were investigated using 0.12 mmol \( \text{L}^{-1} \) hydrogen peroxide solution, in PBS (0.1 mol \( \text{L}^{-1} \), pH 6.5) according to Fig. 8.

As can be seen in Fig. 8, as the \( \text{H}_2\text{O}_2 \) concentration increased, an increase in current values of the biosensor was observed until \( \text{[H}_2\text{O}_2\text{]} = 0.3 \text{ mmol L}^{-1} \) peroxide, from which a decrease in the current occurred probably due to enzyme denaturation. Thus, this concentration was chosen (\( \text{[H}_2\text{O}_2\text{]} = 0.3 \text{ mmol L}^{-1} \)) since it ensures good voltammetric results and also avoids the enzymatic inactivation.

**Differential pulse voltammetry of Au-SAM\(_{\text{mix}}\) electrode and Au-SAM\(_{\text{mix}}\)-HRP electrode.**—The electrochemical behavior of hydroquinone using the Au-SAM\(_{\text{mix}}\) electrode and the Au-SAM\(_{\text{mix}}\)-HRP electrode was investigated by differential pulse voltammetry, in the potential range of \( +0.4 \) to \( -0.6 \text{ V} \) vs. \( \text{Ag/AgCl} \). Figure 9 shows the voltammograms obtained using these electrodes: (a) Au-SAM\(_{\text{mix}}\) electrode and (b) Au-SAM\(_{\text{mix}}\)-HRP electrode in 0.3 mmol \( \text{L}^{-1} \) hydroquinone and 0.3 mmol \( \text{L}^{-1} \) hydrogen peroxidase solution in PBS (0.1 mol \( \text{L}^{-1} \), pH 6.5).

As can be seen, for the Au-SAM\(_{\text{mix}}\)-HRP electrode a higher response of the reduction current of quinone to hydroquinone was observed compared with the Au-SAM\(_{\text{mix}}\) electrode. The Au-SAM\(_{\text{mix}}\) electrode surface when modified with peroxidase presented an increase in the analytical signal of the resultant peak since the organized arrangement of the thiols and the enzyme facilitates the analyte response to the electrode surface. In addition, the sensitivity of the Au-SAM\(_{\text{mix}}\)-HRP electrode (1889.80 \( \mu \text{A mol L}^{-1} \)) was much higher than that of the Au-SAM\(_{\text{mix}}\) electrode (290.53 \( \mu \text{A mol L}^{-1} \)).

**Determination analytical voltammetric for biosensor Au-SAM\(_{\text{mix}}\)-HRP.**—Based on the high electrocatalytic activity of the SAM\(_{\text{mix}}\) film and efficient HRP immobilization on the mixed monolayer differential pulse voltammetry, measurements were carried out in the presence of hydroquinone using the Au-SAM\(_{\text{mix}}\)-HRP electrode in the range of 5.0 to 30.0 \( \mu \text{mol L}^{-1} \) in the presence of \( \text{H}_2\text{O}_2 \). The differential pulse voltammetry technique offers an improved sensitivity of the electrochemical response and detection limit. Thus, the analytical performance and voltammograms (Fig. 10) of the Au-SAM\(_{\text{mix}}\)-HRP electrode was obtained employing DPV for the construction the analytical curve (inset Fig. 10) for hydroquinone. Under the optimum conditions, the analytical curve obtained was linear from 5.0 to 30.0 \( \mu \text{mol L}^{-1} \) of hydroquinone and the corresponding equation was \( \Delta \text{I} = -0.00861 + 2387.53 \text{ [HQ]} \) (\( r = 0.994 \)), where \( \Delta \text{I} \) is the resultant peak current and [HQ] is the Hydroquinone concentration in \( \mu \text{mol L}^{-1} \). The standard deviation for the calibration curve was estimated at \( \text{SB} = 0.00101 \). This data was used to calculate the detection limit and quantification limit of the biosensor which were \( DL = 1.26 \mu \text{mol L}^{-1} \) and the \( QL = 4.23 \mu \text{mol L}^{-1} \), respectively. The DL presented by this biosensor, was compared with other sensors and biosensors described in the literature, as shown in Table I.
Repeatability and stability of the Au-SAMmix-HRP biosensor.—The repeatability of the biosensor Au-SAMmix-HRP was performed on a series of 10 measurements (n = 10) in a solution containing the same HQ concentration 0.3 mmol L$^{-1}$. The biosensor stability was evaluated after 100 consecutive cycles by cyclic voltammetry, with 0.99% RSD. The biosensor was stored in a refrigerator at 4°C in PBS solution and only a slight decay of the cathodic current peak was observed, at the SAMmix-HRP electrode, the peroxidase, in the presence of hydrogen peroxide, which is electrochemically reduced back to hydroquinone when varying the concentration of one of the molecules. This study revealed that the binary SAM of carboxylic acids on gold electrodes increase the stability of the biosensor with TLA monolayer. The results obtained by cyclic voltammetry and electrochemical impedance spectroscopy showed that the electrode surface changes dramatically when varying the concentration of one of the molecules. This study revealed that the binary SAM of carboxylic acids on gold electrodes can be applied as a promising platform for anchoring enzymes in the development of new biosensors. The HRP enzyme was efficiently immobilized on the biosensor Au-MUA/TLA(0.5:1.0)-HRP for H$_2$O$_2$ detection. Michaelis–Menten kinetics, K$_M$ of 0.4 mmol L$^{-1}$ was obtained, indicating that the electrode architecture employed presents advantages for the fabrication of enzymatic biosensor. The Au-SAMmix-HRP electrode, the peroxidase, in the presence of hydrogen peroxide, catalyzes the oxidation of hydroquinone to the corresponding quinone, which is electrochemically reduced back to hydroquinone at −0.08 V vs Ag/AgCl. The resulting Au-SAMmix-HRP exhibited an excellent electrocatalytic activity toward the hydroquinone, which presents a wide liner range from 5.0 to 30.0 mmol L$^{-1}$, with good sensitivity, detection limit 1.26 μmol L$^{-1}$ and limit of quantification 4.23 μmol L$^{-1}$.

Acknowledgments
We thank CNPq, nBioNet/CAPES and Fundação Araucária (Brazil), for financial support.

References

Table I. Comparisons with other modified electrodes sensing for HQ.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Modification</th>
<th>Linear range ($\mu$mol L$^{-1}$)</th>
<th>DL ($\mu$mol L$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>Copper hexacyanoferrate/platinum layer</td>
<td>10 – 100</td>
<td>2.2</td>
<td>44</td>
</tr>
<tr>
<td>Gold</td>
<td>3-mercapto-1-propanesulfonic acid and LBL self-assembly of Con A and HRP</td>
<td>6.0 – 72.0</td>
<td>2.0</td>
<td>18</td>
</tr>
<tr>
<td>Gold</td>
<td>SAMmix-HRP</td>
<td>5.0 – 30.0</td>
<td>1.26</td>
<td>This work</td>
</tr>
<tr>
<td>GCE</td>
<td>CuS nanocrystals and chitosan</td>
<td>4.5 – 450.0</td>
<td>1.5</td>
<td>45</td>
</tr>
<tr>
<td>GCE</td>
<td>Polyvinylferrocene/polypyrrole and HRP</td>
<td>1.6 – 15</td>
<td>0.6</td>
<td>35</td>
</tr>
</tbody>
</table>