Optimization of Hydrogen Peroxide Detection for a Methyl Mercaptan Biosensor

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Abstract: Several kinds of modified carbon screen printed electrodes (CSPEs) for amperometric detection of hydrogen peroxide (H₂O₂) are presented in order to propose a methyl mercaptan (MM) biosensor. Unmodified, carbon nanotubes (CNTs), cobalt phthalocyanine (CoPC), Prussian blue (PB), and Os-wired HRP modified CSPE sensors were fabricated and tested to detect H₂O₂, applying a potential of +0.6 V, +0.6 V, +0.4 V, −0.2 V and −0.1 V (versus Ag/AgCl), respectively. The limits of detection of these electrodes for H₂O₂ were 3.1 μM, 1.3 μM, 71 nM, 1.3 μM, 13.7 nM, respectively. The results demonstrated that the Os-wired HRP modified CSPEs gives the lowest limit of detection (LOD) for H₂O₂ at a working potential as low as −0.1 V. Os-wired HRP is the optimum choice for establishment of a MM biosensor and gives a detection limit of 0.5 μM.

Keywords: methyl mercaptan; hydrogen peroxide; amperometric sensor; screen printed electrode
1. Introduction

Methyl mercaptan (MM) is one of the volatile sulfur compounds (VSCs), which are known to be involved in halitosis (bad breath) [1,2] and periodontal diseases [2], and the predominant causative factor of noticeable oral malodor [3]. MM is also present in several other cases such as the bottle storage of wines [4]; wood-pulp mills, sewage treatment plants and factories producing jet fuel, pesticides and plastics [5]; and even in the atmosphere and on the ocean surface [6]. Consequently, MM detection is important in the dental, medical, food, environment and atmosphere fields.

A low-cost, sensitive and specific sensor for detecting MM could be an interesting alternative to conventional MM monitoring methods such as the use of a halimeter, an expensive device, in the dental field [7,8]. Biosensors to monitor MM have been described by Mitsubayashi et al. [9–12]. In their work, monoamine oxidase A (MAO-A) or flavin-containing monoxygenase (FMO) was used to catalytically oxidize MM, and the oxygen consumption induced by this reaction was monitored. Coupled with this system, a substrate regeneration cycle with ascorbic acid was carried out. However, a sensor for the detection of O₂ depletion, which has the initially high current background of the oxygen electrode [13], is less sensitive than one for H₂O₂ measurement. To solve this problem and seek sensitive detection methods, our objective was to develop a MM biosensor coupled with sensitive hydrogen peroxide detection. Alcohol oxidase (AOX) is known to catalytically oxidize MM with production of formaldehyde, sulfide and H₂O₂ [14] according to the reaction:

\[
\text{H}_3\text{C-SH} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{[\text{AOX}]} \text{H}_2\text{C}=\text{O} + \text{H}_2\text{S} + \text{H}_2\text{O}_2
\]

Hydrogen peroxide generated during enzyme-catalyzed reactions can be electrochemically detected on modified/unmodified carbon matrices [15–27]. In this work, unmodified carbon nanotube (CNT), cobalt phthalocyanine (CoPC), Prussian blue (PB), and Os-wired HRP modified screen printed electrode (CSPE) sensors were fabricated and tested to detect H₂O₂. Our aim was to seek the most sensitive and optimal detection method of H₂O₂ for a MM amperometric biosensor.

2. Experimental

2.1. Reagents

Hydrogen peroxide (H₂O₂, 30%, w/w), disodium hydrogen phosphate (Na₂HPO₄), potassium chloride (KCl), sodium chloride (NaCl), acetic acid (CH₃COOH), sodium acetate trihydrate (CH₃COONa·3H₂O), potassium hexacyanoferrate (III) (K₃Fe(CN)₆), o-phenylenediamine (99.5%), bovine serum albumin (BSA, ≥96%) were obtained from Sigma-Aldrich (Lyon, France). The concentration of diluted H₂O₂ solutions was determined by the classic potassium permanganate titration method. Sulfuric acid (95%) (H₂SO₄) and NaH₂PO₄·2H₂O were purchased from Prolabo (Briare, France). Hydrochloric acid (HCl) (37%) was obtained from Carlo Erba Reagenti (Milan, Italy). Peroxidase redox polymer (Os-wired HRP) was purchased from Bioanalytical Systems, Inc. (Gloucestershire, UK). Carbon pastes used for screen printed electrodes (Electrodag PE-410, 423SS and 6037SS) were obtained from Acheson (Plymouth, UK). A glycerolphthalic paint (Astral, France) was used as insulating layer. Transparent PVC sheets (200 mm × 100 mm × 0.5 mm) (SKK, Denzlingen,
Germany) were used as screen-printing substrates. All chemicals were used without any further purification. All solutions were prepared using Milli-Q water.

2.2. Instrument

CSPEs were produced in the laboratory using a semi-automatic DEK 248 screen-printing system (DEK, Weymounth, UK). The working electrode was a 4 mm diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved wire and the Ag/AgCl reference electrode was a 5 mm × 1.5 mm straight wire (Figure 1).

Figure 1. Screen printed electrode.

Cyclic voltammetry (CV) measurements, PB electrodeposition, PPD electropolymerisation and amperometric measurements were carried out on an AUTOLAB PGSTAT100 (Metrohm, Switzerland), using GPES v4.7 (Metrohm) as informatic interface. All potential values are reported versus Ag/AgCl. Amperometric measurements were performed in a 10 mL glass bath cell with magnetic stirring at room temperature.

2.3. Carbon Screen Printed Electrode Modifications

2.3.1. Preparation of CNT Modified CSPEs

CNT modified CSPEs were prepared as described in the work of Silveira et al. [28]. Briefly, 10 µL of 0.3 mg/mL SWCNT water dispersion were successively deposited on the CSPEs working electrode, drying each layer one by one under vacuum. The electrodes were then washed with water.

2.3.2. Preparation of CoPC Modified CSPEs

Cobalt-phtalocyanine-modified paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK) and modifications were performed on working electrode by the DEK screen-printing system.

2.3.3. Preparation of PB/PPD Modified CSPEs

The PB film was first deposited by covering the CSPEs with a solution containing 2.5 mM FeCl₃, 2.5 mM K₃Fe(CN)₆, 0.1 M KCl and 0.1 M HCl and applying a potential +0.4 V versus Ag/AgCl for 40 s. Then the PB film was activated by covering the electrode by a solution containing 0.1 M KCl and 0.1 M HCl, electrochemically cycling for 20 cycles between −0.05 V and 0.35 V versus Ag/AgCl at a scan rate
of 50 mV·s$^{-1}$. After washing with distilled water, it was dried for 1 h at 100 °C in oven. To improve the stability and selectivity properties of the PB electrodes, the electropolymerisation of a poly- (o-phenylenediamine) (PPD) coat was formed. The PPD layer was deposited by electrochemically cycling the PB modified electrode with potential between $-0.5$ V and $0.7$ V versus Ag/AgCl at a scan rate of 50 mV·s$^{-1}$ in deaerated 0.1 M, pH 5.0 acetate buffer solution containing 0.5 mM o-phenylenediamine under a stream of nitrogen [29].

2.3.4. Preparation of Os Wired HRP (Os-HRP) Modified CSPEs

For the Os-HRP modified CSPEs, 10 μL 0.1 M phosphate buffer solution, pH 7.5, containing 10% (v/v) Os-HRP was deposited on the surface of CSPEs. It was allowed to dry at room temperature for 2 h. It was thoroughly washed with buffer before use.

3. Results and Discussion

3.1. H$_2$O$_2$ Detection with Unmodified CSPEs

The cyclic voltammetry studies were performed in 0.1 M phosphate buffer solution, pH 7.5 to investigate the CSPEs’ electrochemical behavior (Figure 2). CSPEs showed no obvious peak in the absence of H$_2$O$_2$ in the potential range from $-0.2$ V~0.8 V versus Ag/AgCl. In the presence of H$_2$O$_2$, CSPEs started to perform current response at potential around +0.3 V versus Ag/AgCl, indicating the onset potential of the H$_2$O$_2$ electrooxidation.

**Figure 2.** Cyclic voltammograms of CSPEs in 0.1 M phosphate buffer solution, pH 7.5 (a); in presence of 1 mM H$_2$O$_2$ (b). Scan rate 20 mV·s$^{-1}$.

For the investigation of H$_2$O$_2$ limit detection, chronoamperometry experiments were carried out with several concentrations of H$_2$O$_2$ injected into the stirred bath cell (Figure 3). For unmodified CSPEs, the H$_2$O$_2$ detection limit was 3.1 μM (S/N = 3) applying a +0.6 V potential versus Ag/AgCl, and the current response slope of the calibration curve was 0.208 μA/mM. To investigate the reproducibility, three parallel measurements with 0.1 mM H$_2$O$_2$ revealed a relative standard deviation (RSD) of 12.1%. The
high RSD observed of unmodified CPSEs is likely related to the marked differences in the real active electrode area, which is difficult to handle and adjust.

**Figure 3.** Amperometric performance of CSPEs for detection of H$_2$O$_2$ at +0.6 V in a stirred 0.1 M phosphate buffer solution, pH 7.5. The upper left inset shows the calibration curve of CSPEs for H$_2$O$_2$ concentration; the lower right inset zooms in the part of the amperometric response of 5 μM H$_2$O$_2$ injected.

### 3.2. H$_2$O$_2$ Detection with CNT/CSPEs

Both CSPEs (Figure 2) and CNT/CSPEs (Figure 4) showed a H$_2$O$_2$ oxidation peak in the cyclic voltammetry experiments for the studied potential range. The onset potential of the H$_2$O$_2$ electrooxidation for CNT/CSPEs was around +0.2 V versus Ag/AgCl, the detection limit was 1.3 μM (S/N = 3) applying a positive potential of +0.6 V versus Ag/AgCl, and the current response slope of CNT/CSPEs for H$_2$O$_2$ was 32.1 μA/mM. To investigate the reproducibility, three parallel measurements with 0.1 mM H$_2$O$_2$ revealed a RSD of 19.7%.

**Figure 4.** Cyclic voltammograms of CNT/CSPEs in 0.1 M phosphate buffer solution, pH 7.5 (a); in presence of 1 mM H$_2$O$_2$ (b). Scan rate 20 mV·s$^{-1}$. 
Both unmodified CSPEs and CNT are intrinsically carbon. Compared to unmodified CSPEs, detections of H$_2$O$_2$ for CNT/CSPEs need lower oxidation potential (CSPEs, +0.3 V; CNT/CSPEs, +0.2 V), and have lower detection limit (CSPEs, 3.1 μM; CNT/CSPEs, 1.3 μM) with higher current response (CSPEs, 0.208 μA/mM; CNT/CSPEs, 32.1 μA/mM). The increased current response may arise from the large electric active area and a thin, porous diffusion layer [30]; the reasons of lower onset oxidation potential and lower detection limit are still controversial [31], because CNT may contain metal impurities derived from the catalysts used for their growth [32,33]. In a sense, CNT/CSPEs could be more favorable than unmodified CSPEs for H$_2$O$_2$ detection.

3.3. H$_2$O$_2$ Detection with CoPC/CSPEs

To investigate the electrochemical behavior of CoPC/CSPEs in phosphate buffer solution, cyclic voltammetry experiments were carried out in the potential range of −0.2 V~0.8 V versus Ag/AgCl (Figure 5). The presence of a well-defined oxidation current peak at around +0.3 V versus Ag/AgCl is consistent with the following reaction [21]:

\[
2\text{Co}^{2+} + \text{H}_2\text{O}_2 \rightarrow 2\text{Co}^{+} + \text{O}_2 + 2\text{H}^+ \\
2\text{Co}^{+} \rightarrow 2\text{Co}^{2+} + 2e^-
\]

The reaction can be described by a chemical-electrochemical (CE) mechanism [21]: H$_2$O$_2$ chemically reduces Co$^{2+}$ to Co$^{+}$ and its subsequent electrochemical re-oxidation is observed as an oxidation peak. Consequently, this peak is used for the quantification of H$_2$O$_2$. For CoPC/CSPEs, the H$_2$O$_2$ detection limit was calculated as 71 nM (S/N = 3) applying a positive potential of +0.4 V versus Ag/AgCl in chronoamperometry experiments, and the slope of the calibration curve was 3.7 μA/mM by. Three parallel measurements with 10 μM H$_2$O$_2$ reveal a RSD of 2.4%, indicating a good reproducibility for this sensor.

**Figure 5.** Cyclic voltammograms of CoPC/CSPEs in 0.1 M phosphate buffer solution, pH 7.5 (a); in presence of 0.1 mM H$_2$O$_2$ (b). Scan rate 20 mV·s$^{-1}$. 
3.4. $H_2O_2$ Detection with PPD/PB/CSPEs

PB is known to have a high solubility in neutral and basic solutions [34,35], consequently the PB-modified electrodes may have stability problems under our conditions. To improve the stability and selectivity of the PB electrodes a PPD coat is formed. In order to assess the effect of the PPD coat on the stability of the PB modified electrodes, cyclic voltammetry experiments were carried out in 0.1 M phosphate buffer pH 7.5 (not shown in this paper).

Without PPD layer coating, PB dissolved in solution, resulting in a CV current decrease in the scanning process. After 20 scan cycles, the PB/CSPEs CV’s performance were similar to the unmodified CSPEs (Figure 2). With PPD layer coating, the response of redox peaks of PB reduced slightly even after 50 scan cycles, indicating that the PPD layer stabilized the PB. To investigate the electrochemical performance of PPD/PB/CSPEs $H_2O_2$ was added to the solution (Figure 6).

**Figure 6.** Cyclic voltammograms of PPD/PB/CSPEs in 0.1 M phosphate buffer solution, pH 7.5 (a); in presence of 0.1 mM $H_2O_2$ (b) and 1 mM $H_2O_2$ (c). Scan rate 20 mV·s$^{-1}$.

In the phosphate solution, the redox peaks correspond to the reduction of Prussian blue and oxidation of Prussian white as [36]:

$$Fe_4^{(III)}[Fe^{(II)}(CN)_6]_3 + K^+ + e^- \leftrightarrow K_4Fe_4^{(II)}[Fe^{(II)}(CN)_6]_3$$

Prussian blue \hspace{1cm} Prussian white

In the presence of $H_2O_2$, the electrocatalytical reductive reaction of PB towards to $H_2O_2$ can be described as:

$$K_4Fe_4^{(II)}[Fe^{(II)}(CN)_6]_3 + H_2O_2 \rightarrow Fe_4^{(III)}[Fe^{(II)}(CN)_6]_3 + K^+ + OH^-$$

Prussian white \hspace{1cm} Prussian blue

For PPD/PB/CSPEs, the $H_2O_2$ detection limit was 1.3 $\mu$M (S/N = 3) for a cathode potential of $-0.2$ V and the current response slope was $-33$ $\mu$A/mM. Three parallel measurements with 10 $\mu$M $H_2O_2$ revealed a RSD = 4.1%. The lower RSDs observed of CoPC/CSPEs and PPD/PB/CSPEs than for unmodified CPSEs and CNT/CSPEs are likely related to the better control of mediator deposition.
3.5. H$_2$O$_2$ Detection with Os-HRP/CSPEs

The Os-HRP/CSPEs show a couple of stable and well-defined redox peaks at around +30 mV and +70 mV at a scan rate of 20 mV·s$^{-1}$ (Figure 7 curve a). In the presence of H$_2$O$_2$ (Figure 7 curve b), the electrocatalytical reductive reaction process of Os-HRP towards to H$_2$O$_2$ can be described by Scheme 1.

**Figure 7.** Cyclic voltammograms of Os-HRP/CSPEs in 0.1 M phosphate buffer solution, pH7.5 (a---); in presence of 0.1 mM H$_2$O$_2$ (b-----). Scan rate 20 mV·s$^{-1}$.

![Cyclic voltammograms](image)

**Scheme 1.** The electrocatalytical reductive reaction process of Os-HRP towards H$_2$O$_2$.

![Scheme 1](image)

For Os-HRP/CSPEs, the H$_2$O$_2$ detection limit was 13.7 nM (S/N = 3) at a cathode potential −0.1 V versus Ag/AgCl (Figure 8). According to the Michaelis-Menten equation, the calculated apparent K$_{M,p}$, from the curve fitting is 53.5 μM. This low K$_{M,p}$ value indicates Os-HRP’s high affinity and high effective conversion for the H$_2$O$_2$ substrate and a favorable electron-transfer rate with the osmium mediator. A linear range was obtained until 25 μM. Three parallel measurements with 1 μM H$_2$O$_2$ revealed a RSD of 1.3%, which indicates a good reproducibility. This may be ascribed to the application of a low cathode potential to avoid the interferences with electro-active species. In addition, Os-HRP/CSPEs can be the most specific in detection of H$_2$O$_2$ because of the specificity of the reaction between HRP and H$_2$O$_2$. 

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Figure 8. Amperometric performance of Os-HRP/CSPEs sensor in detection of \( \text{H}_2\text{O}_2 \) at \(-0.1 \text{ V}\) in a stirred 0.1 M phosphate buffer solution, pH 7.5. The upper right inset shows the calibration curve of Os-HRP/CSPEs for \( \text{H}_2\text{O}_2 \) concentration; the lower left inset zooms in on the part of the amperometric response of 0.01 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) injected.

The comparison of the amperometric analytical behavior to \( \text{H}_2\text{O}_2 \) of the five kinds of electrodes developed in this study is summarized in Table 1. It shows that the Os-HRP/CSPEs are the most sensitive electrodes with a low reduction potential applied for \( \text{H}_2\text{O}_2 \) detection. This is due to the specific, sensitive and rapid turnover of Os-HRP to \( \text{H}_2\text{O}_2 \).

| Type of Electrodes | Potential Applied (V) | LOD (S/N = 3) | RSD (Tested Concentration of \( \text{H}_2\text{O}_2 \)) |
|--------------------|----------------------|---------------|-----------------------------------------------|
| CSPEs              | +0.6                 | 3.1 \( \mu \text{M} \) | 12.1\% (0.1 \text{ mM}) |
| CNT/CSPEs          | +0.6                 | 1.3 \( \mu \text{M} \) | 19.7\% (0.1 \text{ mM}) |
| CoPC/CSPEs         | +0.4                 | 71 \( \text{nM} \)  | 2.4\% (10 \( \mu \text{M} \)) |
| PPD/PB/CSPEs       | −0.2                 | 1.3 \( \mu \text{M} \) | 4.1\% (10 \( \mu \text{M} \)) |
| Os-HRP/CSPEs       | −0.1                 | 13.7 \( \text{nM} \) | 1.3\% (1 \( \mu \text{M} \)) |

Interference of electro-active species [25,37–39] is often encountered when using amperometric biosensors and applying a high potential in real samples. The decrease of the applied potential can be effective to avoid a lot of electrochemical interferences. With this consideration, PPD/PB/CSPEs and Os-HRP/CSPEs are used to combine with alcohol oxidase (AOX) in bovine serum albumin matrix to detect methyl mercaptan (MM) applying a low potential in the aqueous phase. The limit of detection of AOX/PPD/PB/CSPEs to MM is 10 \( \mu \text{M} \); of AOX/Os-HRP/CSPEs to MM is 0.5 \( \mu \text{M} \). For AOX/Os-HRP/CSPEs, the calibration curve of the response to MM is linear in the concentration range 0–15 \( \mu \text{M} \) with a good correlation with the classical analytical method. We are working on the stability of the biosensor which is the crucial point to improve its accuracy and reliability. Consequently, Os-HRP/CSPEs are combined with alcohol oxidase an optimum method which gives the best sensitivity in methyl mercaptan detection.
4. Conclusions

Five kinds of modified carbon screen printed electrodes applied for H$_2$O$_2$ amperometric detection for MM biosensors were presented in this work. In comparison, and despite a worse reproducibility, CNT/CSPEs are a better choice than unmodified CPSEs in H$_2$O$_2$ detection resulting from their lowest detection limit, lowest onset oxidation potential and highest current response of CNT/CSPEs. However, the applied potential of +0.6 V versus Ag/AgCl is too positive to avoid the interference of electro-active species. CoPC/CSPEs and PPD/PB/CSPEs are also a good choice in H$_2$O$_2$ detection because of their low applied potential, low detection limit and good reproducibility. Os-HRP/CSPEs display the lowest detection limit and the best operational reproducibility towards H$_2$O$_2$. With the cathode potential applied and the use of HRP, Os-HRP/CSPEs can avoid the interference of electro-active species and be specific for H$_2$O$_2$ detection. The Os-wired HRP modified screen printed electrode is the optimum method we used to combine with alcohol oxidase in a methyl mercaptan biosensor, usable in both aqueous and gaseous phase detection.

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