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Electrochemical determination of hydrogen peroxide with cytochrome *c* peroxidase and horse heart cytochrome *c* entrapped in a gelatin hydrogel

Karolien De Wael [a,*], Qamar Bashir [b], Sandra Van Vlierberghe [c], Peter Dubruel [c], Hendrik A. Heering [b], Annemie Adriaens [a]

[a] Department of Analytical Chemistry, Ghent University, Krijgslaan 281 S12, B-9000 Ghent, Belgium
[b] Leiden University, Leiden Institute of Chemistry, Einsteinweg 55, NL-2333 CC Leiden, The Netherlands
[c] Polymer Chemistry & Biomaterials Research Group, Ghent University, Krijgslaan 281 S4-Bis, B-9000 Ghent, Belgium

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ABSTRACT

A novel and versatile method, based on a membrane-free enzyme electrode in which both the enzyme and a mediator protein are entrapped in a gelatine hydrogel was developed for the fabrication of biosensors. As a proof of principle, we prepared a hydrogen peroxide biosensor by successfully entrapping both horse heart cytochrome *c* (HHC) and *Saccharomyces cerevisae* cytochrome *c* peroxidase (CCP) in a gelatin matrix which is immobilized on a gold electrode. This electrode was first pretreated with 6-mercaptohexanol. The biosensor displayed a rapid response and an expanded linear response range from 0 to 0.3 mM ($R = 0.987$) with a detection limit of 1×10^{-5} M in a HEPES buffer solution (pH 7.0). This method of encapsulation is now further investigated for industrial biosensor applications.

1. Introduction

Mediated and direct electrochemistry between redox proteins and electrodes have received a lot of interest in the last few decades [1–5]. It can provide us information about the mechanism of the processes in biological systems and establish a foundation for fabricating a new generation of electrochemical biosensors in which membranes are no longer a necessity. The entrapment of proteins in a hydrogel without a membrane results in a faster response or a faster diffusion of analyte to the enzyme. In addition, an increased enzyme stability is obtained next to a better resistance to dehydration/rehydration which has his benefits concerning the storage of the biosensor. The electrochemistry of the enzymes is realized by immobilizing them in a film or sol–gel which is modified on an electrode surface. This film or matrix provides an ideal environment for biomolecules and facilitates the electron transfer between enzyme and electrode. Our strategy is to create an open hydrogel for the predictable response of quasi-freely diffusing enzyme, mediator and substrate (analyte). This method is now a model system for new biosensor applications in industry.

Gelatin is chosen as a matrix for the encapsulation of the proteins. A gelatin hydrogel is a three-dimensional hydrophilic polypeptide network, which can provide a desirable water-rich buffering environment for the entrapped proteins because of its attractive properties of film-forming ability, biocompatibility, non-toxicity, high mechanical strength and low cost. Because of the hydrophilic groups or domains, the hydration and native configuration of the encapsulated biomolecules is ensured [6]. This hydrophilic polymer network is able to swell in water by absorbing a large amount of water (one tenth to one thousand times their dry weight) [7]. Depending on the nature of the enzyme, the gelatin can be charged to trap specific enzymes. Because gelatine is a protein itself, charge and specifically reactive groups can be altered/introduced/removed by genetic modification to accommodate a variety of enzymes.

In the past, we described the use of gelatin as matrix for horse heart cytochrome *c* (HHC) [6]. It is a model system for biological electron transfer [8] and for bioelectrocatalysis [9]. In addition, this protein acts as a mediator for cytochrome *c* peroxidase (CCP). CCPs are heme-containing enzymes that catalyze the reduction of hydrogen peroxide to water by two ferrous cytochrome *c*. Their common mechanistic feature is the formation of an oxoferryl intermediate upon binding of hydrogen peroxide to the ferric heme in the active site, and the intermediate storage of the second oxidation equivalent as a heme- or Trp-based radical, or as a second ferric heme group [10]. CCP can thus be applied for the electrocatalytic determination of hydrogen peroxide, which is of great importance in chemical, biological, clinical, environmental and many other fields.

Here we demonstrate the ability of gelatin to act as a matrix for the monoheme cytochrome *c* peroxidase (CCP) from yeast, mediated by

* Corresponding author at: Chemistry Department, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium.
E-mail address: karolien.dewael@ua.ac.be (K. De Wael).

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horse heart cytochrome *c* (HHC). Based on the positive charge of HHC and the relative small sizes of this protein, gelatin B (negative charge) was selected as a matrix. We anticipate that CCP is large enough to be trapped in the matrix by size alone. The electrocatalytic behavior of the enzymes towards hydrogen peroxide is investigated in detail.

2. Experimental

2.1. Chemicals and solutions

Horse heart cytochrome *c* (HHC), 2-[4-(2-hydroxyethyl)-piperazinyl] ethanesulfonic acid (HEPES), mercaptohexanol (MH) and sodium hydroxide were purchased from Sigma-Aldrich. *Saccharomyces cerevisae* cytochrome *c* peroxidase (C128A CCP) without the possibly interfering surface Cys residue was expressed in *Escherichia coli* and purified as previously described [11,12].

The HEPES buffer solution of 10 mM was set to pH 7.0 using a 0.15 M NaOH. Type B gelatin (Gel, IEP = 5, Bloom strength = 257), isolated from bovine skin by the alkaline process, was kindly supplied by SKW Biosystems (Ghent, Belgium).

2.2. Electrode preparation

The basic electrochemical setup consisted of a three-electrode cell using a saturated calomel reference electrode (SCE, Radiometer Analytical, France) and a platinum counter electrode. The gold working electrodes of 1.6 mm diameter (BASi, West Lafayette, USA, www.bioanalytical.com) were pretreated by mechanical polishing. Before its first use the electrode surface was briefly scoured by a silicon carbide emery paper of 1200 grit to obtain a fresh surface. To smoothen the resulting relatively rough surface it was further subjected to sequential polishing by polishing cloth covered with alumina powder of 1, 0.3 and 0.05 mm particle size (Buehler, USA) for respectively 5, 10 and 20 min. To remove any adherent Al_2O_3 particles the electrode surface was rinsed thoroughly with doubly deionized water and cleaned in an ultrasonic bath containing deionized water (Branson 3210, USA) for 2 min.

Before immobilizing gelatin onto the electrode, the gold surface was modified with a SAM. Therefore, the electrode was immersed in a water solution containing 1 mM 6-mercaptohexanol (MH) for 18 h at room temperature, which allowed the adsorption of a SAM onto the surface. Afterwards, the modified gold electrodes were rinsed with water to remove any physically adsorbed mercaptohexanol. The electrodes are denoted as MH|Au electrode.

To immobilize gelatin B onto a MH|Au electrode, 7 μL of a gelatin B solution (5 wt.%) was brought on the surface by using a syringe after which it was exposed to air for 2 h at 4 °C (drop drying). The gelatin B solution was prepared by mixing the gelatin B powder and the HEPES buffer solution at 40 °C. These electrodes are referred to in the text as GelB|MH|Au. When HHC and/or CCP were added to the gelatin-HEPES solution, the final electrodes are denoted as CCP|HHC|GelB|MH|Au, HHC|GelB|MH|Au or CCP|GelB|MH|Au. The concentration of HHC, CCP and gelatin is indicated between brackets. Finally, all electrodes were washed with the HEPES buffer solution and stored at 4 °C before use.

2.3. Apparatus

A PGSTAT20 potentiostat controlled by GPES 4.9 005 software package running (ECO Chemie, The Netherlands) was used to record voltammetric curves. Prior to the start of each measurement, the pH of the solution was measured using an Orion Benchtop pH-meter model 420A (Thermo Fisher Scientific, USA). The solutions were thoroughly deoxygenated by bubbling nitrogen through the cell solution for 20 min and a nitrogen atmosphere was maintained over the solution during the experiment.

3. Results and discussion

3.1. Electrochemical behavior of HHC and CCP entrapped in a gelatin B matrix

Fig. 1 shows the electrochemical response of a GelB|MH|Au electrode (1), a CCP|GelB|MH|Au electrode (2) and a CCP|HHC|GelB|MH|Au electrode (3) in a 10 mM HEPES pH 7 buffer solution in a potential window from −0.4 to 0.4 V vs SCE with a scan rate of 50 mV/s. No oxidation or reduction process is observed for the GelB|MH|Au electrode and the CCP|GelB|MH|Au electrode (curves 1 and 2 respectively). Both the gelatine matrix and the CCP protein are not electrochemically active in this specific potential window. A complete electrochemical study of the behavior of gelatin at a gold electrode is described by the authors [13]. For CCP, probably the concentration is too low to be detectable by cyclic voltammetry. Nevertheless, the following results show that CCP is present and electrocatalytically active. When 111 μM HHC and 11.1 μM CCP are added to a GelB matrix, the corresponding current potential behavior is shown as curve 3 (only scan 20 is shown). The voltammogram shows a well defined oxidation and reduction peak. As CCP is not electrochemically active in this specific potential window, the redox couple could be explained as the oxidation and reduction of the heme group present in the HHC protein [14,15]. In a previous article [6], the electrochemical behavior of a HHC|GelB|MH|Au electrode was described.

The formal potential (E°′) for the redox reaction of HHC is 0.018 V, consistent with the value of cytochrome *c* in solution [16]. Fig. 1 (curve 3) shows a redox reaction at this potential, the presence of CCP does not influence the redox processes of HHC. The ratio of the cathodic and anodic peak currents is close to one for all scans. A stability study showed that from scan 1 a stable electrochemical signal is obtained with no leakage of the proteins into the HEPES cell solution. Gelatin is a protein, which provides an ideal microenvironment for HHC and CCP. The gelatin B matrix has an overall negative charge, while HHC is positively charged. Apart from the physical trapping in the hydrogel matrix, this additional interaction further stabilizes the entrapment. However, due to screening by the electrolyte, HHC is not permanently bound to the gelatine matrix, but remains mobile in the hydrogel. This can be seen from the concentration dependence of the catalytic activity, and the square-root dependence of the HHC current on scan rate [6]. This enables the application as membrane-free biosensor that can nevertheless immobilize multiple enzyme layers to enhance sensitivity.

3.2. Electrocatalysis

The electrocatalytic behavior of a CCP|HHC|GelB|MH|Au electrode towards hydrogen peroxide is studied in detail. Fig. 2 shows the cyclic

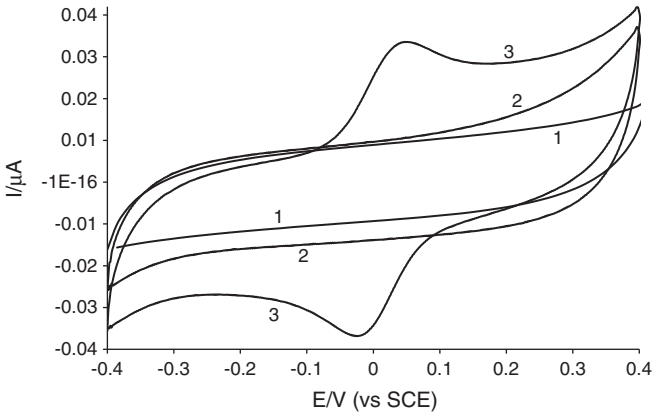

Fig. 1. The current potential behavior of a GelB|MH|Au (1), a CCP (14 μM)|GelB|MH|Au (2) and a CCP (11.1 μM)|HHC (111 μM)|GelB|MH|Au (3) electrode in a 10 mM HEPES pH 7 buffer solution with a scan rate of 50 mV/s.

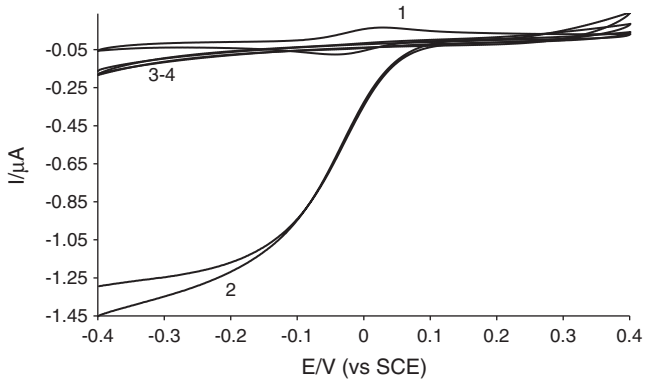

Fig. 2. The current potential behavior of a CCP (11.1 μM)|HHC (111 μM)|GelB|MH|Au electrode in the absence (1) and presence of 4.6 mM H_2O_2 (2) in a 10 mM HEPES pH 7 buffer solution with a scan rate of 50 mV/s. The current–potential curves recorded at a HHC|GelB|MH|Au and a CCP|GelB|MH|Au electrode in the presence of 4.6 mM H_2O_2 are indicated as 3 and 4 respectively.

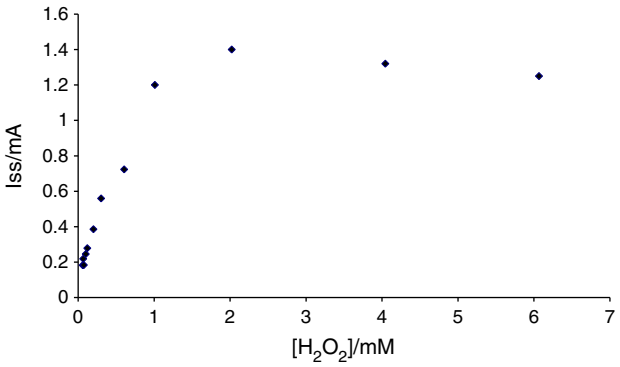

Fig. 4. Current as a function of H_2O_2-concentration for a CCP (11.1 μM)|HHC (111 μM)|GelB|MH|Au electrode in a 10 mM HEPES pH 7 buffer solution with a scan rate of 50 mV/s.

voltammograms of the enzyme electrode in the absence (1) and presence (2) of 4.6 mM hydrogen peroxide at a scan rate of 50 mV/s. These electrodes yield catalytic electrochemical signals observed as reversible, sigmoidal waves with a limiting current corresponding to an enzymatic velocity. The value of E_{cat} is −12 mV, given as Fig. 3, the value is close to that of free HHC in solution and slightly shifted towards a more negative potential when compared with the free HCC/diheme CCP experiment [17]. The shift can be attributed to the interaction amongst neighbor redox centers. The oxidation and reduction waves are substantially symmetric with a peak separation of $\Delta E_p = 3$ mV and a full width at half maximum of ca. $\Delta E_{FWHM} = 120$ mV. The value for ΔE_{FWHM} is somewhat greater than the theoretical value of 90.6 mV [18,19]. This nonideality of the peak indicates that some enzyme molecules experience a different microenvironment than do the majority of the enzymes. The voltammetric behavior of a CCP|HHC|GelB|MH|Au electrode in the presence of hydrogen peroxide changes obviously, the reduction current is increased and the oxidation peak disappeared. The fact that the oxidation peak is absent, indicates a very fast oxidation rate.

The necessity of the presence of both HHC and CCP in the gelatin matrix for the electrocatalytic reduction of hydrogen peroxide was proven by the fact that no catalytic wave was observed when one of these compounds was excluded from the matrix. The voltammetric behavior of a HHC|GelB|MH|Au electrode and a CCP|GelB|MH|Au electrode in the presence of 4.6 mM hydrogen peroxide is presented as curves 3 and 4 respectively in Fig. 2. Only when HHC and CCP are both present, a reversible, sigmoidal wave is obtained.

The electrocatalytic reduction peak of hydrogen peroxide by these type of electrodes is used to quantitatively determine the concentration of hydrogen peroxide. Fig. 4 shows the calibration curve. The linear response is from 0 to 0.3 mM. The detection limit for H_2O_2 is found to be 1×10^{-5} M. When exceeding this concentration, the calibration curve tends to deviate from its linear path and finally reaching a maximum value, $I_{ss,max}$ of 1.4 mA. This behavior is typical for a Michaelis–Menten kinetic mechanism. The electrochemical approach of the Lineweaver–Burk equation [20] can be used to calculate the apparent Michaelis–Menten constant (K_M) where $1/I_{ss}$ is plotted against 1/c where I_{ss} is the steady-state current after the addition of the substrate and c the bulk concentration of the substrate (Fig. 5). By analysis of the slope and the intercept, the value of K_M is determined to be 0.3689 mM, which is in good correlation with other peroxidases in hydrogel structures [21]. As is well known, the smaller the K_M is, the higher the analyte affinity, and thus the catalytic ability at given concentration. Therefore, the peroxidase activity of CCP entrapped in the gelatin matrix is greatly enhanced. Gelatin creates a biocompatible environment and assists in the electron transfer between proteins and the bulk electrode.

The HCC/CCP system may offer potentials for selective peroxide detection because of the low overpotentials required, the decreased activity of the gold electrode by modification, and the intrinsic substrate specificity of the enzyme. It was also investigated whether hydrogen peroxide has any effect on the stability of any of the reaction partners. Fig. 6 shows that upon recording subsequent voltammograms in the presence of 4.6 mM H_2O_2, the limiting current gradually decreases. Scans 20 and 30 are presented by curves 2 and 3 respectively. The decrease in limiting current indicates that one of the compounds is chemically attacked by hydrogen peroxide. Based on former experiments, probably HHC is attacked by hydrogen peroxide, a similar effect of hydrogen peroxide as observed for yeast cytochrome *c* [22]. However,

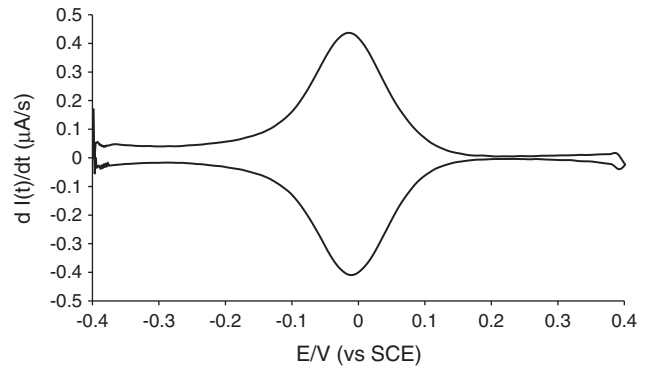

Fig. 3. Derivate of the catalytic current as a function of the potential, based on the data obtained in Fig. 2.

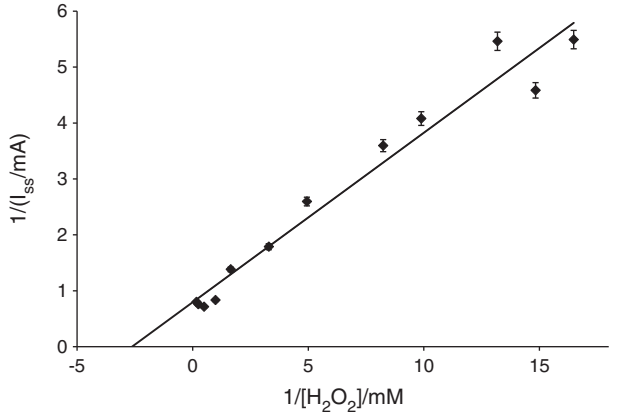

Fig. 5. Lineweaver–Burk plot based on the data of Fig. 3.

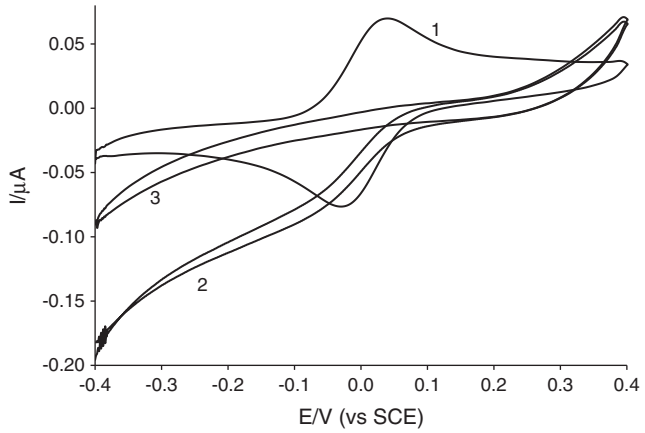

Fig. 6. The current potential behavior of a CCP (11.1 μM)|HHC (111 μM)|GelB|MH|Au electrode in the absence (1) and presence of 4.6 mM H_2O_2 (2–3) in a 10 mM HEPES pH 7 buffer solution with a scan rate of 50 mV/s. Curve 2 is scan 20, curve 3 is scan 30.

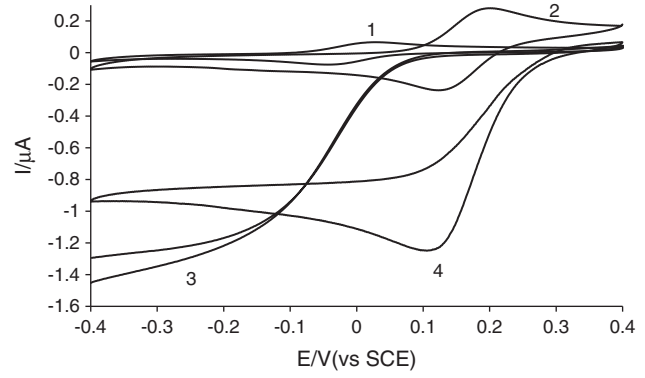

Fig. 7. The current potential behavior of a CCP (11.1 μM)|HHC (111 μM)|GelB|MH|Au (curves 1 and 3) and a CCP|ferro|GelB|MH|Au (curves 2 and 4) electrode in the absence (1,2) and presence of 4.6 mM H_2O_2 (3,4) in a 10 mM HEPES pH 7 buffer solution with a scan rate of 50 mV/s.

it should be noted that the concentration of hydrogen peroxide used for this stability test is an order of magnitude higher than the linear response range, and thus represents a greatly accelerated durability test. Moreover, H_2O_2 is an atypically aggressive analyte in the context of biosensors.

To prove the role of HHC as a mediator, HHC was replaced by ferrocene methanol (ferro) in the matrix. The electrochemical behavior of a CCP|HHC|GelB|MH|Au electrode (curve 1) and a CCP|ferro|GelB|MH|Au electrode (curve 2) is shown in Fig. 7. When 4.6 mM hydrogen peroxide is added to the cell solution, curves 3 (CCP|HHC|GelB|MH|Au) and 4 (CCP|ferro|GelB|MH|Au) are obtained. As can be observed, the electrocatalytic wave is shifted towards more positive potentials when HHC is replaced by ferro. However, although this mediator provides a good electrocatalytic system, it is not as stably trapped in the matrix as HHC due to its small dimensions and absence of charge.

4. Conclusion

This article describes the use of gelatin B as a biocompatible matrix for HHC and CCP. Hydrogen peroxide could be detected electrocatalytically at CCP/HHC/GelB modified gold electrodes. The necessity of the presence of HHC as well as CCP for the detection of hydrogen peroxide was proven. When one of the compounds is absent, no catalytic wave was observed. The fact that HHC acts as a mediator was proven by substitution of HHC by ferrocene in the enzyme matrix. These results open up new perspectives for developing biosensors.

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