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Electrochemical studies on reconstituted horseradish peroxidase modified carbon paste electrodes

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Abstract

Horseradish peroxidase (HRP) is a heme protein that acts specifically on H_2O_2 as the electron acceptor. Hemin (Ferriprotoporhyrin-IX) is the prosthetic group of the enzyme. A direct molecular wire to the redox center of the enzyme is expected to enhance the electrochemical response of the enzyme. Native HRP was immobilized onto the surface of glassy carbon (GC) matrix using a 16-atom spacer arm. We have also immobilized the redox center of the enzyme (hemin) through one of the propionate groups onto the surface of glassy carbon matrix using an 11-atom spacer arm with amino terminus. Apoperoxidase was isolated according to the Teale's method and was allowed to reconstitute with the hemin-bound matrix for enzyme reconstitution. The HRP paste and reconstituted-HRP (rec-HRP) paste electrodes were used to study the electrochemical response to substrate H_2O_2 using electrochemical techniques like cyclic voltammetry (CV) and flow injection (FI) studies. Flow injection studies using HRP paste electrode showed a linearity from 25 to 200 μM H_2O_2. The rec-HRP paste showed ~100 times increase in the electron transfer rates compared to native HRP paste, and substrate linearity from 25 to 100 μM was observed.

Keywords: Enzyme electrodes; Horseradish peroxidase covalent coupling enzyme electrochemistry

1. Introduction

Determination of hydrogen peroxide is of practical importance (i) in biotechnology and food processing industries where H_2O_2 is used as a sterilizing and cleaning agent, (ii) in bioanalytical chemistry (oxidase enzyme), where H_2O_2 is one of the electroactive byproducts released [1,2] in the reaction and (iii) as an immunoenzyme marker, where horseradish peroxidase (HRP) is used as a marker enzyme. HRP (EC: 1.11.1.7) is an important enzyme catalyzing the oxidation of a number of electron donors through H_2O_2 as the electron acceptor. The electron donors can be a leuco-dye, which upon oxidation gives a colored product. The enzyme, when coupled with an oxidase system or in an immunoenzyme marker, can be used for various assays with high specificity. A number of electrochemical sensors for the determination of the H_2O_2 have been reported in the literature using either HRP in solution [3] or covalently immobilized [4] or crosslinked [5,6].

Amperometric determination of H_2O_2 using HRP relies on the principle that the enzyme reduces the peroxide molecule, thereby getting oxidized to form compound-I: the oxidized form of the enzyme. This compound-I is two oxidation state above that of the resting enzyme, the central metal ion iron gets oxidized to Ferryl (Fe (IV)) state and the second oxidation equivalent is stored as a porphyrin radical cation [7]. Compound-I can be represented as [(protein) (porphyrin) Fe (IV)=O]. A suitable mediator (electron donor) reduces the compound-I to the resting enzyme Fe (III) state. Most of the sensors recognize the oxidized form of the mediators at the electrode surface [8]. A number of peroxide sensors have been reported in the literature [9–14].

In the present paper, we have attempted to increase the electrochemical response of HRP paste electrode to the substrate H_2O_2 solution. This was achieved by attaching a molecular wire to the redox center of the enzyme directly followed by reconstitution of the enzyme. We have used cyclic voltammetry (CV) and flow injection (FI) studies without mediators in order to elucidate the increased response for the on-line monitoring of H_2O_2. If suitably modified, this can be used for immunological studies.

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2. Materials and methods

2.1. Materials

Glassy carbon (GC) Sigradur® G was procured from HTW (Hotchempatur Werkestoffe Geminewald, Germany). The GC matrix has highly disordered structure and fracture pattern kin to that of a glass. Horseradish peroxidase (HRP) and fumed silica were procured from Sigma (St. Louis, MO, USA). 1-Ethyl 3-(3-dimethyl aminopropyl) carbodiimide (EDC) and 1, 6 diaminohexane were obtained from Merck, Germany. Hemin was obtained from Himedia. All other chemicals were of AR grade from Qualigens, India.

2.2. Methodology

2.2.1. Immobilization

2.2.1.1. Activation of the matrix. Five hundred milligrams of GC matrix was heated at 120 °C for 6 h followed by washing with hot carbon tetrachloride in order to eliminate the volatile impurities present. The matrix was then heated with 30 ml of pirhana solution (a mixture of H_2SO_4/H_2O_2 in the ratio of 2:1) for 6 h in a hot water bath. This treatment introduces hydroxyl groups onto the surface of the GC matrix. The matrix was neutralized with alkali and washed in 0.1 M saline by spinning the matrix at 10,000 rpm for 10 min at room temperature. This matrix was also used as blank after drying (for comparison).

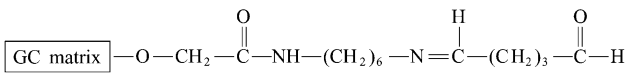
Attachment of 16-atom spacer arm. This was done in three steps:

(a) Carboxymethylation (CM): The activated matrix (250 mg) was incubated with 2% chloroacetic acid (5 ml) in alkaline medium (2 ml of 6 N NaOH) for 1 h. This step introduces carboxymethyl groups on the matrix.

(b) Addition of 1, 6 diaminohexane: The CM matrix was incubated with 5 ml of 1.5 M 1, 6 diaminohexane (pH 4.6): with EDC as the coupling agent for 4 h at room temperature. This step introduces 1, 6 diaminohexane to the free carboxyl terminus. The matrix at this step is called the amino matrix.

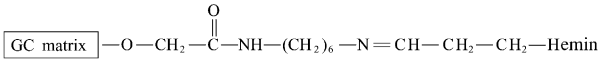
(c) Attachment of glutaraldehyde: The amino matrix obtained was treated with 2 ml of 3% glutaraldehyde for 3 h. This introduces glutaraldehyde to the free amino terminus matrix as a Schiff's base.

Coupling the HRP. The GC matrix with 16-atom spacer arm having aldehyde terminus was incubated with 0.5 mg of HRP (100 U) for 24 h.



16-atom spacer arm

Coupling of hemin. Hemin was attached to the amino matrix using one of the propionate groups present at 6/7 position of the heme. Five hundred micrograms of hemin was incubated with 100 mg of the amino matrix in the presence of EDC (40 mg) as the coupling agent at pH 4.6. Hemin of ~0.07 μg was bound per mg of GC matrix as determined by specrophotometric assay.



Hemin bound to amino terminus matrix (11-atom spacer arm)

Preparation of apoperoxidase. Apoperoxidase was prepared using Teale's method [15] with slight modification. Separation of the porphyrin from the apoperoxidase was done by giving the holoenzyme an acid shock followed by extracting the prosthetic group into a partially miscible organic solvent.

One milligram of the native enzyme was suspended in 5 ml of 1 mM Tris adjusted to pH 2 in order to split the porphyrin from the apoenzyme. This was followed by the extraction of hemin in 5 ml of cold 2-butanone. These two phases of liquids are partially miscible with each other, and hence, two layers could be seen. The upper organic layer was carefully separated from the lower aqueous layer. The extraction was repeated twice. The lower protein layer was suspended in 10 mM Tris (pH 8) followed by dialysis against 0.1% saline for 10 h at 4 °C. The saline was changed at least twice.

Reconstitution of the enzyme. 100 mg of hemin-bound matrix was incubated for 20 h at 4 °C with 400 μg of apoperoxidase. The excess protein was washed with saline. The activity of the reconstituted enzyme was checked using standard activity assay. Approximately 74% of apoperoxidase could retain its capacity to reconstitute to active holoenzyme in 1:1 stoichiometric proportions in solution as seen from the activity assay.

2.2.2. Protein estimation

Lowry's method of protein estimation was used. A 0.2 mg/ml of standard protein BSA was used to obtain the standard graph. Alkaline hydrolysis was done in order to cleave the protein from the matrix and the supernatant obtained after spinning the matrix was used for estimation.

2.2.3. Activity assay

Activity assay was done using *o*-dianisidine. It is a leuco dye capable of existing in two states, reduced (colorless) and oxidized (colored). In the presence of substrate H_2O_2, the enzyme HRP undergoes oxidation to form compound-I, which is subsequently reduced by the donor back to the resting enzyme in two steps. The oxidized form of the donor is colored with maximum absorbance at 500 nm. A calibration curve was obtained using 0.2 U HRP as the standard. A final concentration of 0.4 mM *o*-dianisidine and 0.084 mM H_2O_2 was used in the assay reaction mixture.

The enzyme bound matrix was suspended in 10 mM phosphate buffer (pH 7) followed by the addition of *o*-dianisidine and H_2O_2. After incubation for 5 min, the matrix was spun at 6000 rpm for 2 min. The supernatant obtained was used for optical density measurement.

2.2.4. Electrode paste preparation

Modified paste electrodes were prepared using fumed silica as the binder. A combination of 66% matrix (blank/modified GC matrix), 33% fumed silica and 1% NaCl was used. Blank paste electrode (BPE), HRP paste, hemin paste electrode (HPE) and rec-HRP paste electrodes were used in our study.

2.2.5. Instrumentation

Standard three-electrode configuration was used for all the electrochemical studies. For FI studies, we have setup a simple device using a PC-controlled two-way solenoid pinch valve to control the solution flow, a home-made potentiostat was used in order to apply the voltage as seen in Fig. 1. Working electrode was a micropipette tip with enzyme modified GC paste packed tightly in it. Counter electrode (Pt wire) and reference electrode (Ag wire) were used. A flow rate of 2 ml/min under gravity was maintained. H_2O_2 solution was allowed to flow through an inlet tube (leading into the electrochemical cell) under the control of a two-way solenoid pinch valve. Supporting electrolyte used in the present study was 100 mM KCl. The working electrode was kept at a bias of +50 mV vs. Ag/AgCl. For both CV and Fl studies, software in Borland C++ (V 3.1) was written.

3. Results and discussions

From the protein estimation experiments, 2.25 μg and 0.57 μg per mg of matrix were observed for the HRP and rec-HRP, respectively. From the activity assay 0.0046 U and 0.002 U per mg of matrix were obtained for HRP and rec-HRP, respectively. The original activity of the enzyme was 200 U/mg protein. Therefore, 2.25 μg of enzyme is expected to have ~0.2 U of activity. The observed activity is approximately 100 times lower. For the reconstituted enzyme, the activity is 200 times lower. The reduced activity may be attributed to (1) different kinetics of the immobilized enzyme and (2) loss of activity due to immobilization.

The CV of modified electrodes showed variations in the peak positions as seen in Figs. 2 and 3. HRP paste electrode showed quasi-reversible behavior with the oxidation peak at −50 mV and a reduction peak at −150 mV (figure not shown). BPE shows no peaks, only the capacitive currents

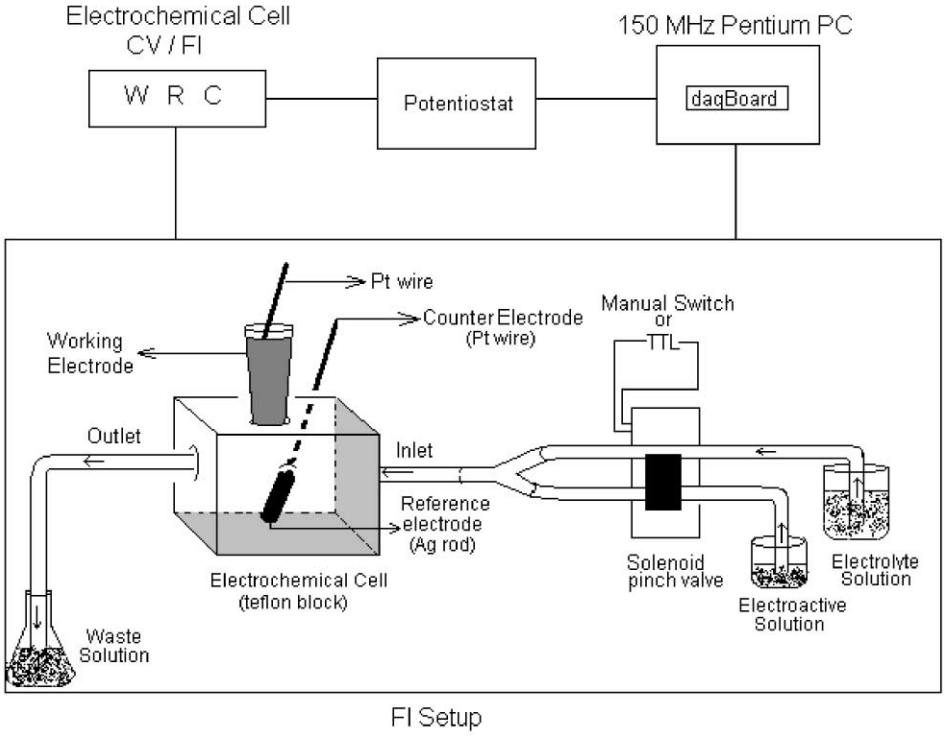


Fig. 1. Block diagram of the instrumentation. Schematic representation of a home-made setup for carrying out flow injection (FI) studies. The principle of working of FI setup is that upon the opening of the solenoid pinch valve (TTL pulses or manually) for a small interval of 10 s or so, a small plug of the electroactive solution ~300 μl flows into the electrochemical chamber. The working electrode at an optimum bias subsequently detects this plug flowing over the surface causing an increase in the current. The current decays as the plug moves out of the electrochemical cell into the outlet tube and waste solution flask. The width of the peak approximately corresponds to the time the H_2O_2 solution was allowed to flow. In the presence of the supporting electrolyte alone, there are no peaks observed.

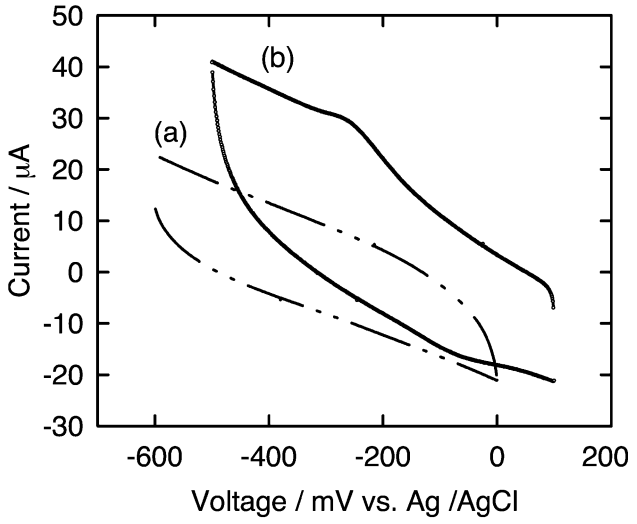


Fig. 2. CV of (a) blank paste electrode. (b) Hemin paste electrode showing an oxidation peak at −50 and −275 mV.

are seen (Fig. 2(a)). HPE showed an oxidation peak at −50 mV and a reduction peak at −275 mV as seen in Fig. 2(b). Upon reconstitution with apoperoxidase, an oxidation peak at +50 mV and a reduction peak at −100 mV were observed as seen in Fig. 3(a). In the presence of 0.1 mM H_2O_2 , there is an increase in the reduction peak observed as shown in Fig. 3(b), indicating that there occurs some electron transfer processes.

The FI response of HRP paste electrode shows a linearity response from 25 to 200 μM (figure not shown). Fig. 4 shows the FI response of rec-HRP paste. Each peak corresponds to a single injection of ~300 μl of sample. Peaks are sharp and clear. An enhanced current response (~100 times) compared to native HRP paste and the response time

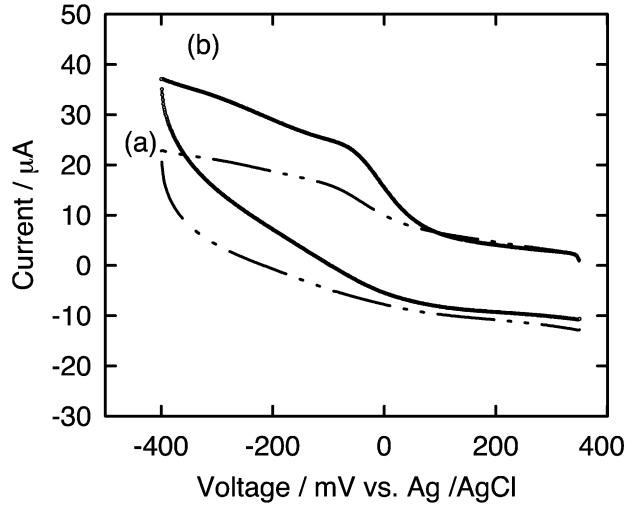


Fig. 3. CV of rec-HRP paste electrode (a) in supporting electrolyte alone and (b) with 0.1 mM H_2O_2, an increase in the reduction peak at −50 mV is observed clearly.

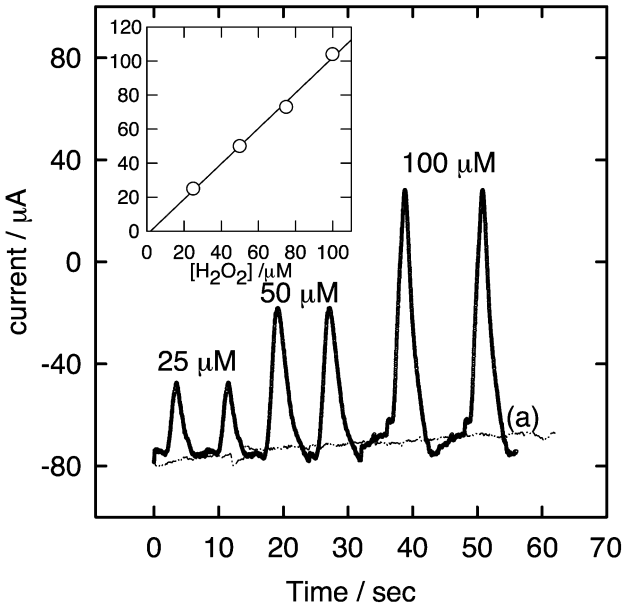


Fig. 4. Main graph shows the flow injection response of rec-HRP paste electrode at a bias of +50 mV. Each peak corresponds to a sample volume of ~300 μl. The peaks are very clear and are sharper than the native enzyme electrode. The current values are ~100 times higher than the native electrode and the response time as calculated from the width of the peak is ~10 s. Linearity from 25 to 100 μM in this case was observed as seen in the inset. (a) Represents the baseline current without the substrate.

of 10 s was observed. Linearity from 25 to 100 μM was observed as seen in Fig. 4 inset.

HRP (molecular weight ~40 kDa) is an enzyme where the depth of the redox center is 4.1 Å, which is relatively low when compared to other oxidoreductases such as glucose oxidase (8.7 Å) and diaphorase (6.3 Å). In the resting enzyme, ferriprotoporphyrin-IX is present, which is usually found in the periphery of the protein shell. By nature, the enzyme is built for an efficient transfer/acceptance of electrons on contact with the substrate, and hence, might also directly communicate with the electrode. This may be the reason for a good response by the native enzyme itself (graphs not shown) when compared to the other systems. In spite of this, their rate of electrochemical reduction/oxidation may vary with their orientation on the surface of the electrode [16]. The electrochemical response can thus be increased by either binding to the electrode surface functions that can interact with the specific region of the protein so as to facilitate a proper orientation or by binding the prosthetic group directly to the electrode surface followed by reconstitution with the apoprotein [8]. There have been reports on ways in which the sensitivity of the molecule to H_2O_2 can be improved, for instance, use of recombinant types (carbohydrate-free) and with six histidine tags on the C terminus has been reported [17,18].

In order to improve the electron transfer between the electrode and the enzyme, the redox center of the enzyme was directly attached to the electrode. Our results from FI studies support this.

On-line monitoring of H_2O_2 is very important in several areas like biotechnology. Thus, the rec-HRP modified pastes can be used for efficient electron transfer in a suitable biosensor. The paste electrodes can be easily screen printed onto a plastic surface and can be used in disposable biosensor strips.

4. Conclusions

The molecular wiring to the active site of the enzyme is effective as seen from the electrochemical studies. Higher currents suggest that lower electrochemical activation energy is required when compared to the native enzyme as the redox center is directly bound to the electrode surface and a direct electron transfer is possible. CV studies clearly indicate that the redox center is better accessible and there are lower capacitance currents seen in the case of rec-HRP electrodes. This is a preliminary study, and better response of the electrode system can be obtained by either changing the site where the porphyrin moiety is attached (for instance, instead of binding through one of the propionate groups, other groups like vinyl groups on the molecule can be used after certain modification as both the propionate groups are very essential for the enzyme activity) or by modulating the spacer arm, for instance, introducing conjugate double-bond systems, so that the electron transfer within the chain is resonance stabilized and hence faster.

We notice here that the effective activity (equivalent to the soluble enzyme) is approximately 100–200 times less, when the immobilized enzymes are considered. However, we note that the current values (in FI experiments) are approximately 100 times larger for the rec-HRP system. This suggests that the electron transfer rates may be 10,000 times higher (compared to the enzyme in solution) which is very significant. The immobilization process and the site are to be optimized for full realization of the power of this technique. In this preliminary work, we have conclusively demonstrated the possibility of the electron transfer through the spacer arm.

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References

[1] A.P.F. Turner, I. Karube, G.S. Gibson (Eds.), Biosensors Fundamentals and Applications, Oxford Univ. Press, New York, 1987.
[2] B.R. Horrocks, D. Schmidtke, A. Heller, A.J. Bard, Scanning electrochemical microscopy 24. Enzyme ultramicroelectrodes for the measurement of hydrogen peroxide at the surfaces, Anal. Chem. 65 (1993) 3605–3614.
[3] M.J. Green, H.A.O. Hill, Amperometric enzyme electrodes, J. Chem. Soc., Faraday Trans. 1 82 (1986) 1237–1243.
[4] T. Tatsuma, Y. Okawa, T. Watanabe, Enzyme monolayer- and bilayer-modified tin oxide electrodes for the determination of hydrogen peroxide and glucose, Anal. Chem. 61 (1989) 2352–2355.
[5] T. Yao, M. Sato, Y. Kobayashi, T. Wasa, Amperometric assay of total and free cholesterols in serum by combined use of immobilized cholesterol esterase and cholesterol oxidase reactors and peroxidase electrodes in flow injection system, Anal. Biochem. 149 (1985) 387–391.
[6] A.A. Shul'ga, T.D. Gibson, An alternative microbiosensor for hydrogen peroxide based on an enzyme field effect transistor with a fast response, Anal. Chim. Acta 296 (1994) 163–170.
[7] H. Yamada, T. Yamazaki, Proton balance in conversion between five oxidation states of horseradish peroxidase, Arch. Biochem. Biophys. 165 (1974) 728–738.
[8] D. Savitri, C.K. Mitra, Electrochemistry of reconstituted glucose oxidase paste electrodes, Bioelectrochem. Bioenerg. 4 (1998) 67–73.
[9] T. Tatsuma, T. Watanabe, S. Tatsuma, T. Watanabe, Substrate purging enzyme electrodes. Peroxidase/catalase electrodes for H_2O_2 with an improved upper sensing limit, Anal. Chem. 66 (1994) 290–294.
[10] J.P. Lowry, R.D.O. Neill, Homogenous mechanism of ascorbic acid interference in hydrogen peroxide detection at the enzyme-modified electrodes, Anal. Chem. 64 (1992) 453–456.
[11] U. Korell, U.E. Spichiger, Novel membraneless amperometric peroxide biosensor based on tetrathiafuvaline, *p*-tetracyanoquino dimethane electrode, Anal. Chem. 66 (1994) 510–515.
[12] M.G. Garguilo, N. Huynh, A. Proctor, A.C. Michael, Amperometric sensors for peroxide, choline and acetylcholine based on electron transfer between horseradish peroxidase and a redox polymer, Anal. Chem. 65 (1993) 523–528.
[13] T. Tatsuma, T. Watanabe, Peroxidase model electrodes: heme peptide modified electrodes as a regentless sensor for H_2O_2, Anal. Chem. 63 (1991) 1580–1585.
[14] J.A. Cox, R.K. Jaworski, Voltammetric reduction and determination of hydrogen peroxide at an electrode modified with a film containing palladium and iridium, Anal. Chem. 61 (1989) 2176–2178.
[15] F.W.J. Teale, Cleavage of the haem-protein link by acid methylethylketone, Biochim. Biophys. Acta 35 (1959) 543.
[16] A. Heller, Electrical connection of enzyme redox centers to electrodes, J. Phys. Chem. 96 (1992) 3579–3587.
[17] G. Presnova, V. Grigorenko, A. Egorov, T. Ruzgas, A. Lindgren, L. Gorton, T. Borchers, Direct heterogeneous electron transfer of recombinant horseradish peroxidase on gold, Faraday Discuss., 116 (2000) 289–9, discussion 335–351.
[18] E.E. Ferapontova, V.G. Grigorenko, A.M. Egorov, T. Borchers, T. Ruzgas, L. Gorton, Mediatorless biosensor for H(2)O(2) based on recombinant forms of horseradish peroxidase directly adsorbed on polycrystalline gold, Biosens. Bioelectron. 16 (3) (2001) 147–157.