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Short communication

# Electrochemical studies on horseradish peroxidase covalently coupled with redox dyes

B.S.B. Salomi, Chanchal K. Mitra *

*School of Life Sciences, University of Hyderabad, Hyderabad 500046, India*

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**Abstract**

The present study aims at investigating the use of redox dyes as non-diffusional electron mediators in hydrogen peroxide biosensors using horseradish peroxidase (HRP). We observe that the two redox dyes Safranine O and Neutral Red covalently bound to HRP, efficiently mediate electron transfer from the active site of the enzyme to the electrode surface. Dyes bound to the enzyme using a spacer arm diaminohexane further enhance the electron transfer. The enzyme electrodes show a linear response to the concentration of $H_2O_2$ up to 500 μM concentration and with a detection limit of around 50 μM. The dyes can be used as coupled mediators to develop a successful electro-optical biosensor.


*Keywords:* Horseradish peroxidase; Redox mediators; Safranine O; Neutral Red; Spacer arm

## 1. Introduction

Determination of hydrogen peroxide is of practical importance in food processing industries where $H_2O_2$ is used as a sterilizing and cleaning agent, in bioanalytical chemistry where $H_2O_2$ is one of the electroactive byproducts released in the reaction, and where horseradish peroxidase (HRP) is used as an immunoenzyme marker (Turner et al., 1987). In view of its simplicity, low-cost and real time detection electrochemical techniques have been considered ideal for the determination of $H_2O_2$. Although $H_2O_2$ is electroactive, the potentials needed are prone to interference and therefore an enzymatic route is often preferred as this is highly selective. HRP is an important enzyme catalyzing the oxidation of a number of electron donors through $H_2O_2$ as the electron acceptor. It is a hemin containing monomeric enzyme with a single polypeptide chain and a ferriprotoporphyrin IX prosthetic group (Shannon et al., 1966). It shows a characteristic Soret band at 402 nm due to the presence of heme. The enzyme is oxidized by $H_2O_2$, and the enzyme in turn is reduced by a suitable electron donor via the formation of intermediate compounds. A number of electrochemical sensors for $H_2O_2$ using either HRP in solution or covalently immobilized or cross-linked have been reported (Green and Hill, 1986; Tatsuma et al., 1989; Yao et al., 1985; Shul'ga and Gibson, 1994). However, the redox center or active center of HRP is electrically insulated by a relatively thick protein shell, hindering its direct electrical communication with the electrodes (Wollenberger et al., 1991; Ho et al., 1993; Ruzgas et al., 1995). Therefore, mediators have been employed to shuttle electrons between the active site of HRP and the electrodes (Korell and Spichiger, 1994). The mediators can replace the natural electron donors. $HRP_{[ox]}$ produced by $H_2O_2$ is brought back to its native state, i.e., $HRP_{[red]}$, by the oxidation of the mediator, which in turn is reduced at the electrode surface. The chemical modification of HRP with the electron relay groups enables a non-diffusion mediated electron-transfer and yields short inter-relay electron-transfer distances (Heller, 1992; Willner and Katz, 2000; Degani and Heller, 1988; Schuhmann et al., 1991).

A vast number of redox dyes remains untapped as mediators. These dyes have a dual advantage of spectroscopic and electrochemical detection. We have studied the use of coupling the enzyme HRP with redox dyes such as Safranine O and Neutral Red (Fig. 1) to enhance the electroactivity of the enzyme near the electrode surface. These dyes belong to the class of phenazines and contain a reactive amino group, which is used for coupling them to the enzyme. Use of a linker bridging the dye and the enzyme to study its role in the electron transfer process is also investigated.

* Corresponding author. Tel.: +91 40 23134668; fax: +91 40 23010120.
*E-mail address:* c_mitra@yahoo.com (C.K. Mitra).

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## 2. Materials and methods

### 2.1. Materials

Horseradish peroxidase (EC: 1.11.1.7) type II (Catalog no.: P 8250), and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC) are procured from Sigma. 1,6-Diaminohexane is obtained from Merck, hydrogen peroxide from Qualigens, Neutral Red from Acros and Safranine O from Aldrich.

Electrochemical studies are carried out on a CH Instruments (model 660A) electrochemical workstation using a three-electrode setup with a glassy carbon working electrode, an Ag/AgCl reference electrode and a platinum counter electrode. The total cell volume is ~5 ml.

### 2.2. Methodology

#### 2.2.1. Covalent coupling of the dyes to HRP

The amino groups of the dyes (Fig. 1) are covalently attached to the carboxyl groups present in horseradish peroxidase using EDC (carbodiimide derivative) as the coupling agent (Bauminger and Wilchek, 1980). The dyes are initially reduced using sodium dithionite before the coupling reaction. 200 μl of 5 mM EDC (1 μmol) prepared freshly in distilled water is added to a mixture containing 200 μl of 0.25 mM HRP (50 nmol) in 0.1 M phosphate buffer of pH 7 and 200 μl of 5 mM reduced dye (1 μmol) solution, and incubated overnight at room temperature. The enzyme coupled with the dye is purified on a gel column (vide infra).

#### 2.2.2. Coupling of Neutral Red to HRP using a linker

The amino group in the dye is initially modified with glutaraldehyde and coupled to one of the amino group of diaminohexane. The other amino group of the linker is coupled to the carboxyl groups present in the acidic residues of HRP using carbodiimide (EDC). This is carried out in three steps as follows:

(i) 200 μl of 500 mM glutaraldehyde (0.1 mmol) is added to 200 μl of 5 mM dye (1 μmol) solution prepared in 0.1 M phosphate buffer of pH 7 and incubated at room temperature for 3 h before removing the excess reagents by gel filtration on a 10 ml Sephadex G25 column.

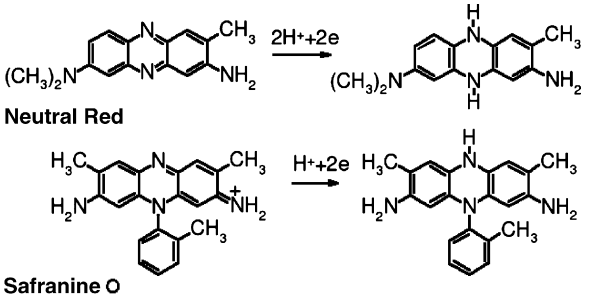


Fig. 1. Structures of the two dyes used in the present study—Safranine O and Neutral Red. Both dyes belong to the phenazine class of dyes and contain a reactive amino group suitable for covalent attachment.

(ii) To 500 μl of 0.25 mM of the glutaraldehyde modified dye (125 nmol), 500 μl of 25 mM diaminohexane (12 μmol) is added and incubated for 3 h and purified on the Sephadex column.

(iii) Finally 100 μmol of EDC is added to a mixture containing 500 μl of 25 μM HRP (12.5 nmol) dissolved in 0.1 M phosphate buffer pH 7 and 500 μl of 25 μM of the dye covalently bound to diaminohexane (12.5 nmol). The reaction mixture is purified and analyzed as given in Section 2.2.3.

This process couples the dye to the enzyme with a minimum of 12-atom long spacer arm giving a greater degree of conformational freedom to the dye. The possibility of glutaraldehyde polymerization in presence of oxygen might increase the length of the spacer arm.

#### 2.2.3. Separation of the coupled sample from the reaction mixture

Sephadex G25 beads are allowed to swell overnight in 0.1 M phosphate buffer before packing it into a 15 ml column. The reaction mixture is loaded onto the Sephadex column equilibrated with the reaction buffer. Initial 3 ml of eluate (void volume) is discarded. One milliliter fractions of the eluate are collected and their absorbance at the $\lambda_{max}$ of the dye and $\lambda_{max}$ of HRP, i.e., 403 nm is monitored. The degree of coupling (DOC), i.e., number of dye molecules per protein molecule is calculated using the formula,

$$\mathrm{DOC} = \frac{A_{max} \times M_W}{[\mathrm{protein}] \times \varepsilon_{dye}} \quad (1)$$

where $A_{max}$ is the absorbance at $\lambda_{max}$ of the dye; $M_W$ the molecular weight of the protein; [protein] the protein concentration in mg/ml and $\varepsilon_{dye}$ is the molar extinction coefficient of the dye at its absorbance maximum. The degree of coupling is a useful number but one must remember that this is an approximation as we have ignored possible changes in the absorption upon coupling.

#### 2.2.4. Activity assay

HRP activity assay is carried out using a leucodye *o*-dianisidine, which is colorless in reduced form and colored in oxidized form with maximum absorbance at 500 nm. HRP is oxidized in presence of $H_2O_2$ and in turn oxidizes the dye. The color obtained is directly proportional to the amount of oxidized HRP (enzyme activity). 2 ml of 1 mM $H_2O_2$ and 2 ml of 0.21 mM dye is added to 1 ml of the enzyme samples of varying concentration (enzyme units) and the absorbance at 500 nm is monitored after 5 min of incubation at room temperature. A standard plot is used for the activity assays of the enzyme coupled with the dyes.

#### 2.2.5. Electrochemical studies on labeled HRP

The labeled enzyme is immobilized (by adsorption) on a polished glassy carbon electrode. Five microliter of the enzyme sample (enzyme at a concentration of ~10 μM) is placed on the electrode surface and allowed to dry at room temperature. The unadsorbed protein is removed by gentle washing of the electrode surface with distilled water before carrying out the