# Tuning of Oxidation Reduction Potential of Enzyme Glucose Oxidase with Amperometry for the Estimation of Copper in Food Samples

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**Abstract**

A new amperometric biosensor for copper determination in food samples was developed through the immobilization of glucose oxidase (GOx) in polyacrylamide gel. The response time for the reaction was studied by observing the percentage inhibition with time at 0.0125 ppm concentration of $Cu^{2+}$. 2.30 minutes was the optimized response time for the estimation of $Cu^{2+}$ ion in the solution. Linear range for the detection of $Cu^{2+}$ by the biosensor was between 0.0125-0.1 ppm. Bioprobe was operationally stable for five days followed by a fall in activity.

*Keywords*: biosensor, copper, amperometric, stabilization, glucose oxidase.

## Introduction

Copper is present in cereals, meat, liver, oysters, nuts and green vegetables. It is necessary for the iron absorption, tyrisinase activity and cofactor for vitamin C requiring hydroxylation. It also protects the heart by increasing high density lipids (HDL) [1]. Various methods are available for the determination of copper in solution [2, 3, 4]. The majority of these methods is tedious and time consuming, and may require expensive equipments in addition to considerable technical skills. Therefore, it is of great importance to get a rapid and reliable method of quantifying copper in food. Biosensors are promising tools in that respect since they combine the specificity of the biological reconnaissance of relevant analytes with the versatility of the transducers. Moreover, a good biosensor will require fewer operations than conventional methods. In the present work, a biosensor with the glucose oxidase (GOx) immobilized in

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polyacrylamide gel is developed [5] for the determination of $Cu^{2+}$ in food samples. Sensitivity, reproducibility and lifetime of the biosensor were studied.

**Experimental**
*Materials and methods*
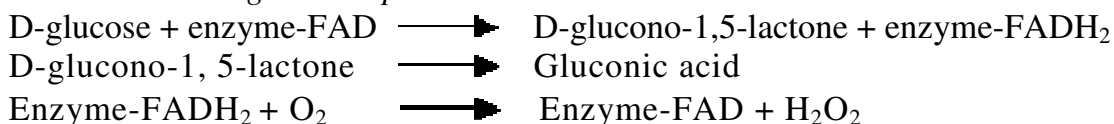*Preparation of the biosensor*
*Polyacrylamide gel immobilization*
Gox immobilization was carried out by Skrylabin and Koshcheenko [5] procedure with slight modification. 5 mL of 30% acrylamide and bis solution (28 g acrylamide and 2 g bis-acrylamide in 100 mL phosphate buffer 0.1 M, pH 6.0) were mixed with 5 mL of enzyme solution (15 U/mL). Mixture was gently agitated and left for 10 minutes at room temperature. 0.5 mL (0.5%) of ammonium persulphate were added to initiate the polymerization which was further catalysed by the addition of 100 µL (50%) of N,N,N,N- tertramethylethylenediamine (TEMED). Solution was mixed gently to avoid froth formation and allowing to polymerize for half an hour. Polymerize gel slab was cut into square blocks (1.0 x 1.0 cm) for further study.
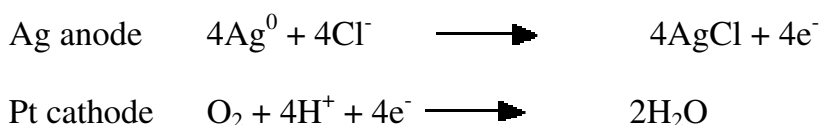
*Biosensor*
Assembly of biocomponent consists of purified GOx immobilized with polyacrylamide gel confined to nylon membrane to form the biocomponent of the biosensor. Nylon membrane does not impart any resistance to the transport of substrate to the catalytic sites or diffusion of product back into the solution. The membrane side containing the immobilized enzymes was applied on the outer membrane of the electrode jacket in contact with the platinum electrode and secured with an O-ring. The reference electrode was an helix of Ag/AgCl which was housed in the same stem as the Pt electrode (Fig. 1). The biosensor, thus prepared, was then dipped in 5 mL of sodium acetate buffer pH 6.0 (50 mM) containing 100 ppm as substrate and dissolved oxygen (DO) was measured with a DO meter. In the process the following reactions take place [6,7]:

*Reaction at biological components*

D-glucose + enzyme-FAD $\longrightarrow$ D-glucono-1,5-lactone + enzyme-$FADH_2$
D-glucono-1, 5-lactone $\longrightarrow$ Gluconic acid
Enzyme-$FADH_2$ + $O_2$ $\longrightarrow$ Enzyme-FAD + $H_2O_2$

*Reaction at transducer*

Ag anode $\quad$ $4Ag^0 + 4Cl^-$ $\longrightarrow$ $4AgCl + 4e^-$

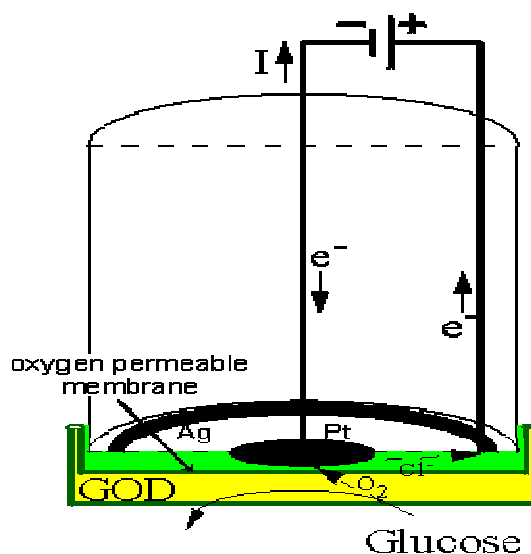Pt cathode $\quad$ $O_2 + 4H^+ + 4e^-$ $\longrightarrow$ $2H_2O$

**Figure 1.** Schematic diagram of an amperometric biosensor.

### Sensitivity and linear range studies

The activity of GOx was inhibited in the presence of $Cu^{2+}$ showing increased inhibition with increased concentration of $Cu^{2+}$. Fall in DO was directly proportional to the enzyme activity. $Cu^{2+}$ ions inhibit the GOx activity so there was less DO fall. To find the response time, % inhibition was observed in the presence of 0.025 ppm $Cu^{2+}$ after time intervals (30 seconds) for five minutes. Introducing different concentrations of $Cu^{2+}$ ions (0.0125-0.1 ppm) in the reaction mixture the linear range was optimized. In all the experiments fall in DO with the GOx activity was determined with respect to reference, i.e., polymerize gel slab square blocks (1.0 x 1.0 cm) without the enzyme. Michalis constant (Km) and inhibitor constant were determined by line weaver Burk plot at various concentrations of the inhibitors.

### Stability, reproducibility and regeneration of bioprobe

The operational stability of the bioprobe was studied by using the probe for oxidizing glucose over intervals of time (days) and its activity in terms of DO fall was observed. The probe was stored at 4 ºC in sodium acetate buffer pH 6.0 (50 mM). This experiment was repeated 36 times to analyze the reproducibility of the biosensor. The regeneration characteristics of the enzyme probe (ability to reactivate) were studied by using the sodium acetate buffer and ethylenediaminetetraacetic acid (EDTA).

## Results and discussion
### *Optimization of response time and linear range for detection of Cu$^{2+}$*

The study is based on the inhibition bioassay principle in which activity of glucose oxidase was inhibited in the presence of Cu$^{2+}$. D-glucose is oxidized to gluconic acid with the enzyme GOx in the presence of molecular oxygen. The activity of the enzyme is inhibited by the Cu$^{2+}$, hence reduced the rate of oxidation of glucose. It leads to less utilization of O$_2$ and as a result less DO fall in the presence of Cu$^{2+}$. This mechanism of inhibition was used for the estimation of Cu$^{2+}$ in food samples. The response time for the reaction was studied by observing the percentage inhibition with time at 0.0125-ppm concentration of Cu$^{2+}$. From the results (Fig. 2) it was observed that in the first 2-3 minutes there was increase in the percentage inhibition and after that it was relatively stable. Thus a time of 2.30 minutes was optimized to study the response of Cu$^{2+}$ ion in the solution. There was increase in percentage inhibition as the concentration of Cu$^{2+}$ was increased up to 0.1 ppm, but after that it was constant (Fig. 3). Linear range for the detection of Cu$^{2+}$ by the biosensor was in the range 0.0125-0.1 ppm. The immobilized enzyme showed an
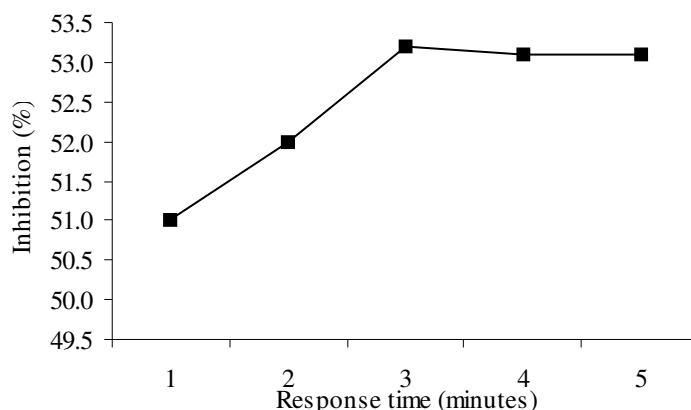


**Figure 2.** Specific response time of biosensor for Cu$^{2+}$ (Cu$^{2+}$, 0.05 ppm, pH 6.0 and Temp. 30 ºC).

apparent inhibition by Cu$^{2+}$ at concentrations higher than 0.0125 mg/L dm$^{-3}$ with inhibition constant of 0.95 ± 0.12 mol dm$^{-3}$. The enzyme showed an apparent $K_m$ value higher than that of the free enzyme. The apparent $V_{max}$ of enzyme decreased by a factor of 0.35 with respect to that of the native enzyme. The optimum temperature of the free and immobilized enzymes remained similar.
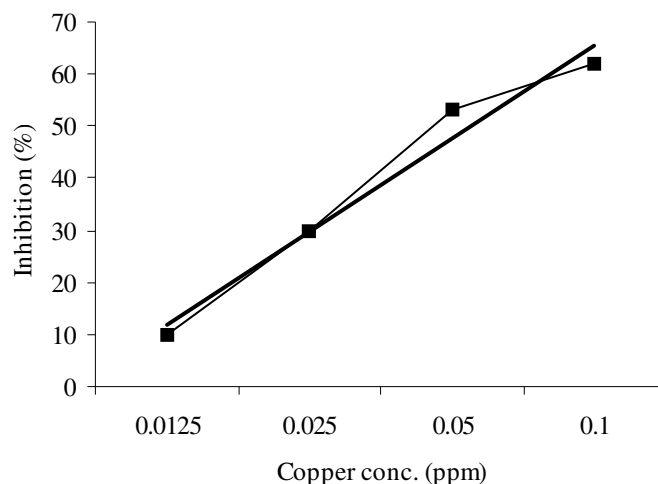
**Figure 3.** Linear range of $Cu^{2+}$ (pH 6.0, Temp. 30 ℃).

### *Stability, reproducibility and regeneration of bioprobe*

Operational stability of enzyme based bioprobe was studied over a few days. It was found that probe remains fully active for five days (Fig. 4), followed by a fall in activity. The probe was used for 36 cycles and it has 97% reproducibility. The regeneration characteristic of enzyme bioprobe was studied with 0.2 M sodium acetate buffer and 0.01 M EDTA. When regeneration was carried out with the sodium acetate buffer (0.2 M), we achieved 40-45% and 82.3% regeneration with one hour and overnight treatment, respectively. In the second treatment the probe was kept in 0.01 M EDTA for 4 hrs and 97.8% reactivation was achieved.
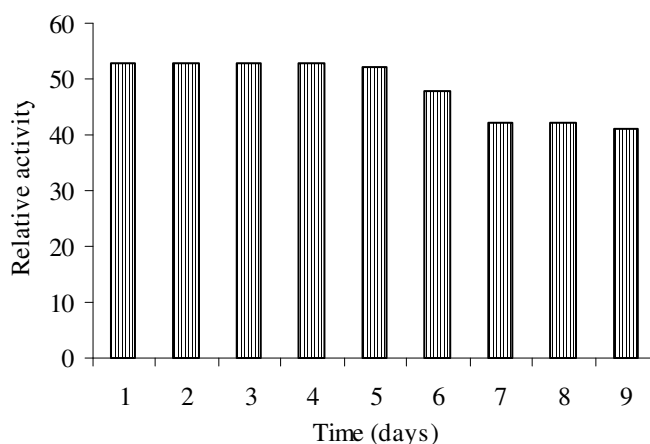


**Figure 4.** Operational stability of the bioprobe.

Developed biosensor was applied to monitor the presence of $Cu^{2+}$ ions in food samples, i.e. almond and meat liver. Concentration of $Cu^{2+}$ was 0.025 and 0.0185 ppm in almond and meat liver, respectively. To measure the

reliability of the biosensor, 5 mL digested food sample were mixed with 0.5 mL of $Cu^{2+}$ solution. Concentration detected from the linear graph was 0.0295 ppm, but theoretically it had 0.0375 ppm $Cu^{2+}$. So, from the results it was concluded that the developed biosensor has 78.6% reliability.

## Conclusions

The response time for the reaction was studied by observing the percentage inhibition with time at 0.0125 ppm concentration of $Cu^{2+}$. In the first 2-3 minutes there was increase in the percentage inhibition and after that inhibition was relatively stable. Thus, a time of 2.30 minutes was optimized as response time for the estimation of $Cu^{2+}$ ion in the solution. Linear range for the detection of $Cu^{2+}$ by the biosensor was between 0.0125-0.1 ppm. The immobilized enzyme showed an apparent inhibition by $Cu^{2+}$ at concentrations higher than $0.0125$ mg $dm^{-3}$ with inhibition constant of $0.0126$ mg $dm^{-3}$. Bioprobe was operationally stable for five days followed by a fall in activity. The probe has 97 % reproducibility after 36 cycles. The developed biosensor was applied to monitor the presence of $Cu^{2+}$ ions in food samples, i.e. meat liver and almond, and it has showed 78.6% reliability.

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