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Immobilization of Bilirubin Oxidase Nanoparticles onto Gold Electrode for Amperometric Detection of Serum Bilirubin

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ABSTRACT

'Bilirubin oxidase nanoparticles' (BOxNPs) were prepared from commercial bilirubin oxidase by using the 'desolvation method'. An amperometric bilirubin biosensor was developed by immobilizing BOxNPs onto an Au electrode. The morphology in terms of size and shape of BOxNPs aggregates was studied by "transmission electron microscopy (TEM)", "dynamic light scattering (DLS)" and "Scanning electron microscopy (SEM)" which were utilized to characterize BOxNPs/Au electrode. BOxNPs/Au electrode demonstrated an optimum response time (1s), pH (8.5), and temperature (35°C) when polarized at a potential (+0.3 V). The lowest 'detection limit' (0.01 µM) was calculated by selecting a concentration range from 0.01 to 1000 µM for bilirubin. BOxNPs/Au electrode was employed to detect total serum bilirubin in healthy people and people suffering from jaundice. When the electrode was stored at a low temperature in sodium phosphate buffer (0.1 M, pH 7.0), it retained 50% of its original activity with storage stability of 150 days.

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Introduction

Bilirubin is one of the by-products of haemoglobin catabolism caused by the body's clearance of senescent red blood cells that contain heme. The elevated levels of bilirubin in the bile and urine could be a sign of certain diseases. It is responsible for the yellow discoloration in jaundice. Analysis of serum bilirubin in samples is an important test for liver function. A high concentration of bilirubin is associated with hepatocellular diseases like cirrhosis of the liver, hepatitis, brain impairment, or death because of kernicterus, particularly in newborns; whereas a lower concentration of bilirubin is related to a deficiency of iron and coronary heart disease [1-3]. Conventional techniques for measuring medical serum samples are direct qualitative analysis and diazo reaction [4,5]. However, the direct qualitative investigation of bilirubin has the drawback that its level is affected by "interference" by alternative "haem proteins" whereas the "diazo reaction" depends on pH value [6]. In addition, other methods of bilirubin determination are ""fluorometry", "enzymatic assay", "capillary cataphoresis", "High-Pressure Liquid Chromatography" (HPLC), "chemiluminescence" and "piezoelectric effect" [7-14]. These methods require expensive apparatus, time-bound sample preparation, and expert individuals to manage, and therefore aren't appropriate for routine. We have reviewed various methods for the determination of bilirubin with special emphasis on biosensors [15].

Nanoparticles are considered to be emerging, powerful, and vast applied materials in biosensing. Recently enzyme nanoparticles (ENPs) have come into the spotlight for their distinctive properties, including high electrical, thermal, optical, chemical, mechanical, and catalytic potential. Direct immobilization of biomolecules such as enzymes or proteins onto metal electrodes leads to a loss of enzyme activity due to modification in physical and chemical properties. This problem can be solved through the attachment of ENPs to metal electrodes. To solve these problems, enzymes were cross-linked in a well-organized manner to synthesize the aggregated enzyme molecules named enzyme nanoparticles (ENPs), before immobilization [16]. Liu et al in 2005 synthesized and characterized nanoparticles of horseradish peroxidase (HRP) for the first time using them for H_2O_2 biosensors [17]. Subsequently, the ENPs of various enzymes were characterized and employed for the construction of improved amperometric biosensors e.g. glucose oxidase for glucose, cholesterol esterase, and cholesterol oxidase for total cholesterol, uricase for uric acid, lipase, glycerol kinase, glycerol-3phosphate oxidase for triglyceride, creatininase, creatinase, sarcosine oxidase for creatinine, lactate dehydrogenase for lactate, urease for urea, pyruvate oxidase for pyruvic acid, L- lysine oxidase for L-lysine, xanthine oxidase for xanthine and β-galactosidase for lactose amperometric biosensors [18-28].

Recently, we prepared nanoparticles (NPs) of bilirubin oxidase (BOx), characterized and immobilized them onto polyethylene (PE) film. These PE film-bound BOxNPs were used for discrete enzymic colorimetric analysis of serum bilirubin [29]. BOxNPs bound to PE film through absorption had a leakage problem when reused. To overcome this problem, the covalent immobilization of BOxNPs onto Au wire/ electrode was done, which resulted in

the development of an amperometric biosensor. The developed amperometric biosensor was then used for the detection of serum bilirubin. The advantage of using BOxNPs in biosensors is that these can be immobilized directly onto metal electrodes without undergoing denaturation and do not require any other nanoparticles to promote electron transfer. These also allow fast electron transfer.

Materials and Methods

Reagents

'Bilirubin oxidase' (BOx),15 IU/mg (EC 1.4.3.14), and various reagents such as '4-aminophenazone', 'Tris HCl', 'glutaraldehyde', (GA) and 'bilirubin' were purchased from Merck. Oher required reagents like 'Potassium ferricyanide', and 'Potassium Ferrocyanide' purchased from the 'Sisco research laboratory' in Mumbai, India. Gold wire (1.5 cm×0.05 cm) (23 carat) had been bought from the local market.

Assay of Bilirubin Oxidase

In our reported paper, we had already described the assay of Box [29].

Preparation of Bilirubin Oxidase Nanoparticles (BOxNPs)

The synthesis of BOxNPs was done by using ethanol and its further cross-linking with glutaraldehyde (desolvation method) [16].

Initially, a desolvating agent (ethanol 4-5 ml) was added to a solution of BOx (2 mg/ml) for preparation of BOxNPs at a plummeting rate (0.5 ml/min), centrifuged at 500 rpm. Later, glutaraldehyde (2ml) was added to the earlier synthesized solution by stirring for a day to make sure that cross-linking of BOxNPs resulted in the formation of ENPs. The synthesized cross-linked BOxNPs were washed and isolated by centrifugation (14000xg, 25 min) from the native BOx solution. This was followed by redispersion of the prepared pellet in distilled water (DW) and sonication for a further 5 min. The functionalized groups (–SH) were supposed to be introduced onto BOxNPs by continuously mixing for 7-8 hours with the addition of cysteine. Then, the functionalized BOxNPs are finally stored at 4°C until the next use.

Immobilization of BOxNPs onto Au electrode/Preparation of enzyme/working electrode (BOxNPs/Au)

Prior to the modification on the surface, a standard piranha solution (conc. H_2SO_4 + H_2O_2 in the molar ratio of 3:1) was used to wash gold (Au) wire/electrode. This was done for half an hour and then this Au electrode was rinsed with DW. Alumina slurry was used to polish the Au electrode. The polycrystalline Au electrode was dipped into a 2 ml suspension of functionalized BOxNPs by magnetic stirring (4°C, 12 hours), which provided a covalent coupling of BOxNPs onto the Au electrode through the thiolate-Au bond. The BOxNPs/Au electrode was further rinsed with reaction buffer (0.1M, pH 7.0) very cautiously [16].

The following reaction occurred for the covalent coupling between "thiol-functionalized cross-linked BOxNPs" and polycrystallized Au electrode to form the Au-thiolate bond for BOxNPs"

$$Au_n\text{-}AuO + 2\ (BOxNPs)\text{-}SH \longrightarrow (A_u n\ (n\text{-}S\text{-}BOxNPs)_2 + H_2O$$

Polycrystalline-AuE Functionalized BOxNPs BOx -E (Bilirubin oxidase -Electrode)

To measure the response of BOxNPs, cyclic voltammetric measurements were done using three electrode systems including 'BOxNPs/Au (working)', 'Ag/AgCl (reference)', and 'Pt wire (auxiliary)'. The prepared BOxNPs/Au electrode was immersed in 20 ml of 0.1M Tris–HCl buffer (pH 8.5). The reaction was further initiated by the addition of bilirubin (0.1mM), which was oxidized to biliverdin by BOxNPs producing an electroactive H_2O_2. The electro-oxidation of H_2O_2 occurs under high potential for the generation of electrons or current flow (in milliampere, mA) at the BOxNPs/Au electrode to detect H_2O_2. Figure 1 shows the occurrence of possible electrochemical reactions during the response measurement:

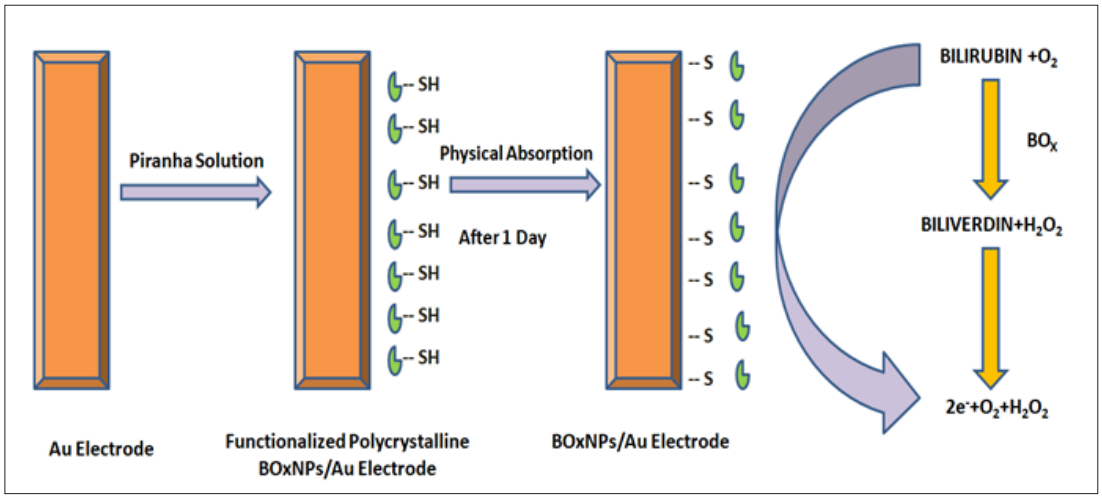


Figure 1: Schematic Illustration of the Construction of BOxNPs/Au (BOxNPs- Bilirubin Oxidase Nanoparticles; Au- Gold Electrode) and Chemical Reactions Involved in the Immobilization of Bilirubin Oxidase Nanoparticles on the Modified Electrode

In amperometric bilirubin biosensors, the following electrochemical reactions are involved:

$$\text{Bilirubin} + O_2 + H_2O \xrightarrow{BO_x} \text{Biliverdin} + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{High Potential}} O_2 + 2H+ + 2e^-$$

$$2e^- \xrightarrow{\text{BOxNPs}} \text{Au electrode}$$

Optimization and Evaluation of BOxNPs/Au Electrode

To optimize the operating conditions like pH, temp., substrate (bilirubin) concentrations, and response time, the immobilized BOxNPs/Au electrode was tested under various assay conditions. To measure optimal pH, the pH of the reaction buffer was varied from 7.0 to 10.0 at an interval of 0.5 using the subsequent buffers (0.1Msodium phosphate in pH range7.0-7.5) and 0.1M Tris -HCl for pH range 8.0-10.0). The reaction mixture was incubated at various temperatures (25-45ºC) and response time was varied between 0 to10 seconds. The influence of different bilirubin concentrations was examined in the range of 0.01-1000 µM. Various parameters like analytical recovery, precision, and interference were assessed to evaluate BOxNPs/Au electrode.

Characterizations of BOxNPs and BOxNPs/Au Electrode

Characterizations of BOxNPs and BOxNPs/Au electrode was conducted using various methods such as 'Dynamic Light Scattering (DLS)', 'Transmission Electron Microscopy (TEM)', and 'Scanning Electron Microscopy (SEM).' The morphology of BOxNPs was carried out by 'TEM' and 'DLS' in terms of size and shape. SEM was used to characterize the BOxNPs/Au electrode.

Determination of Total Bilirubin in Sera by BOxNPs/Au Electrode

Samples of blood were taken from the hospital of 'Pandit Bhagwat Dayal Sharma, Postgraduate Institute of Medical Sciences' Rohtak, Haryana, India. The samples of blood were kept at 25ºC for 35 min with resulting clots without any interruption. The clots were removed in a refrigerated centrifuge (1,500 – 2,500 x g for 5 min.) and the supernatant (serum) obtained was transferred into a fresh tube through a pipet. The serum samples were stored at low temperatures (2-8 °C) in half ml of aliquots until used. The measurement of total serum bilirubin was done by the present BOxNPs/Au electrode. The method used to evaluate the BOxNPs/Au electrode under its optimal assay conditions was similar to that described above, using serum in place of bilirubin solution. The concentration of serum bilirubin was calculated from the standard curve between bilirubin concentrations and current in mA.

Re-Usability and Shelf life of BOxNPs/Au Electrode

The shelf life of the BOxNPs/Au electrode was measured by calculating its activity per week while storing it at 4°C in a Na_3PO_4 buffer (0.1M, pH- 7.0). BOxNPs/Au electrode was washed 4-5 times with a similar buffer before using it in the next test.

Results and Discussion

Characterizations of Bilirubin Oxidase Nanoparticles (BOxNPs)

The characterization of BOxNPs was done by DLS and TEM. DLS results confirm the formation of BOxNPs nanoparticles. As shown in Figure 2(A), the size of BOxNPs depends upon the original level of the enzyme. The single peak appeared with the average size (70-90 nm). Figure 2(B) showed the TEM of BOxNPs, which revealed the spherical shape of nanoparticles with varying diameters (25-100nm).

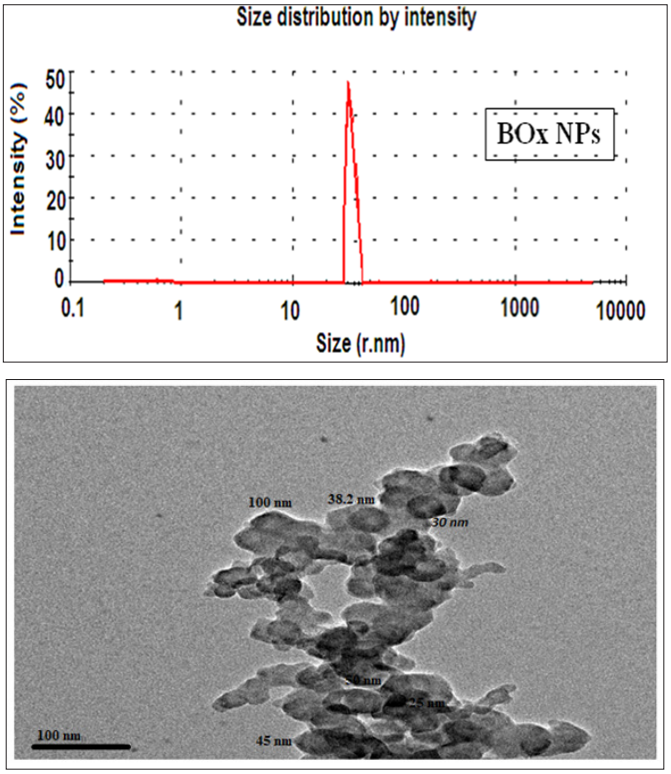
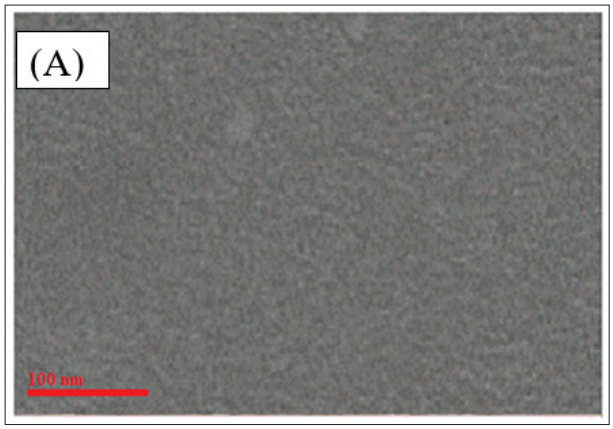


Figure 2: (A) Dynamic Light Scattering (DLS) of Bilirubin Oxidase Nanoparticles (BOxNPs) Confirms "Single Peak" with Mean Sizes of 70 nm and 90 nm, (B) Transmission Electron Microscopic (TEM) Image of Bilirubin Oxidase Nanoparticles (BOxNPs) Synthesized by the Desolvation Method Observing its Spherical Shape, in the Range (25-100 nm)

SEM Characterization of BOxNPs/Au Electrode

SEM was used to find the surface morphology of the Au electrode and a modified Au electrode i.e. BOxNPs/Au electrode. In Figure 3(A) a homogeneous surface could be seen on the bare Au electrode while in Figure, 3(B) a cloudy structural morphology could be seen with some pearl-shaped structures, which confirmed the immobilization of the BOxNPs aggregates onto Au electrode.



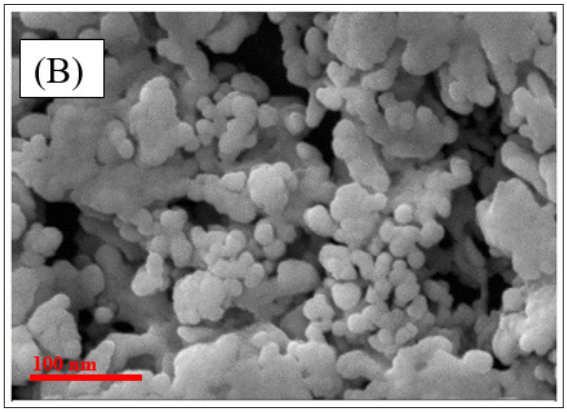


Figure 3: Scanning Electron Microscopic (SEM) Images A) Gold (Au) Electrode Showing a Homogeneous Surface and B) BOxNPs Immobilized on the Gold (Au) Electrode with a Cloudy Structural Morphology with a Pearl-Shaped Structure

Optimization of BOxNPs/Au Electrode

The effects of various factors such as pH-range, temperature, and time on the biosensor response were observed. The optimum current was observed at pH 8.5 which is nearly equal to the 'dissolved oxygen metric (DO) BOx electrode' and slightly higher than that of BOxNPs-PE film (pH 8.0), BOx/GrONPs/NiNPs/ITO (pH 8.0) [29-31]. The optimum temperature of the biosensor was found at 35^{o}C. The decrease in response of the BOxNPs/Au electrode was observed at high temperatures due to enzyme thermal denaturation. The response time of the biosensor was 1s, which is lower than earlier biosensors [29,32-34].

Evaluation of BOxNPs/Au Electrode

A wider linear relation was observed between biosensor response and bilirubin concentration (0.01–1000 µM) (Figure 4), as compared to the previously reported biosensors. (0.0–80 mM) [31-36]. The current biosensor showed a low limit of detection 0.1 µM (S/N¼3), as compared to the biosensors reported earlier. [31,32,35]. The analytical recovery of added bilirubin in sera (15 mM and 20 mM) was 94.5% and 96.0% respectively, demonstrating the reliability of BOxNPs/Au electrode. The within and between coefficients of variation were 5.45%) and 6.25% respectively for the serum bilirubin on the same day as well as after one week of storage at -20 degrees. This precision study revealed the high repeatability of the method.

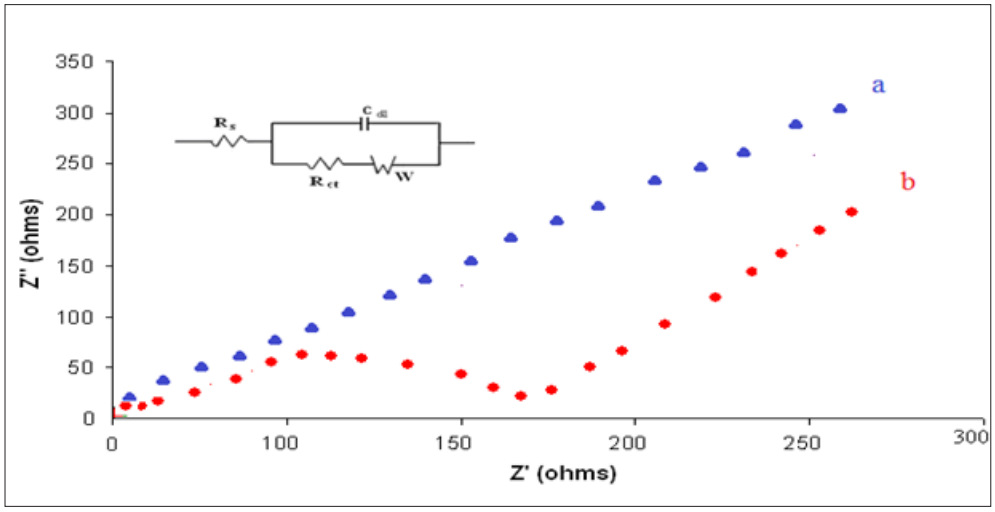


Figure 4: Electro Impedance Spectra of (a) Gold (Au) Electrode (b) Bilirubin Oxidase Nanoparticles/ Gold (BOxNPs/Au) Electrode

Application of BOxNPs/Au Electrode

The present biosensor was used to measure the bilirubin levels in sera of individuals who were apparently healthy and those suffering from jaundice. The bilirubin level ranged from 0.3–19 µM in healthy individuals and 25–69 µM in jaundice patients. (Table 1).

Correlation Between Bilirubin 1: Serum Bilirubin Ranges in Apparently Healthy Individuals and Jaundice Patients, as determined by Bilirubin Biosensor based on Bilirubin Oxidase Nanoparticles (BOxNPs) Modified Au Electrode Mean ± SD* (n = 4)

Sex	Age (Years)	Healthy Individuals(µM)	Sex	Age (Years)	Jaundice patients (µM)
M	34	05± 0.07	F	38	30 ± 0.04
F	29	16 ± 0.05	M	67	44 ± 0.02
M	45	18 ± 0.04	F	58	26 ± 0.03
F	82	12 ± 0.02	M	47	36 ± 0.02
M	46	17 ± 0.04	M	39	42 ± 0.03
F	55	12 ± 0.05	F	59	47 ± 0.02
M	32	19 ± 0.07	F	77	25 ± 0.01
F	37	17 ± 0.04	M	44	47 ± 0.05
M	16	07 ± 0.05	M	54	41 ± 0.03
F	24	03 ± 0.03	F	48	56 ± 0.04

M	10	18 ± 0.04	F	46	47 ± 0.01
F	64	16 ± 0.04	F	62	44 ± 0.03
M	75	04 ± 0.04	M	74	48 ± 0.04
F	77	06 ± 0.06	F	48	32 ± 0.02
M	89	12 ± 0.03	F	35	47 ± 0.03
F	19	14 ± 0.05	M	45	56 ± 0.02
M	29	05 ± 0.05	F	66	44 ± 0.04
F	47	05 ± 0.04	F	24	38 ± 0.05
M	62	08 ± 0.05	M	84	44 ± 0.03
F	77	14 ± 0.02	F	66	55 ± 0.02
M	46	05 ± 0.04	M	44	58 ± 0.03
F	79	06 ± 0.04	F	72	56 ± 0.02
M	19	07 ± 0.06	F	77	49 ± 0.02
F	26	04 ± 0.02	M	68	69± 0.04
M	27	08 ± 0.04	F	84	68 ± 0.02
F	25	06± 0.05	]M	77	41 ± 0.03

SD = Standard Deviation

The bilirubin values for 20 serum samples measured by the present biosensor (y) were correlated with those obtained by standard colorimetric method (x), using the "regression equation" The experiment was repeated three times. The results obtained showed a good correlation (r = 0.99) with the 'standard method', while the 'regression equation' y =0.9807x + 0.7075 (Figure 5). These outcomes showed the accuracy of the method.

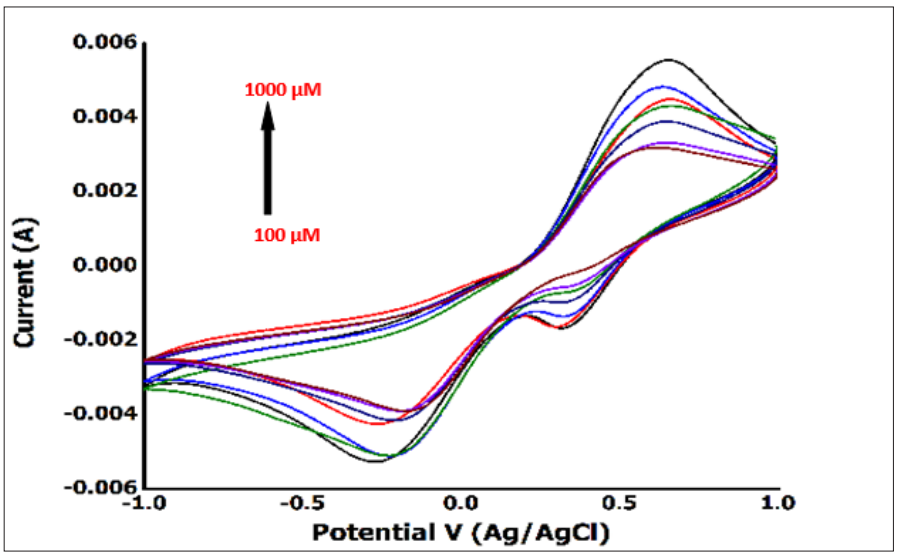


Figure 5: The Effect of Different Concentrations of Bilirubin varying from (0.01 - 1000 µM) Carried out in Tris HCl Buffer (pH 8.5) on Bilirubin Oxidase Nanoparticles/Gold (BOxNPs/Au) Electrode

Effect of Interference on BOxNPs/Au Electrode

The effect of interference on the present bilirubin biosensor was studied by comparing the amperometric response before and after adding some interferents in the reaction mixture at their physiological concentrations like "uric acid" (0.28 mM), "creatinine" (0.14 mM), "glycine" (0.45 mM), "glucose" (4.2 mM), and "ascorbic acid" (0.035 mM), along with bilirubin (100 µM) in Tris-HCl buffer (0.1 M, pH- 7.5). The results revealed that those above interferents had no effect on the analytic performance of the present biosensor.

Shelf life and Re-Usability of BOxNPs/Au Electrode

The BOxNPs/Au electrode was stored at a lower temperature (4°C) and the electrode response was measured at regular intervals to calculate its shelf life. The BOxNPs/Au electrode still had 50% of its original activity after 150 days of daily use for 100 times, which is higher/equal to earlier biosensors [29,32,35]. The enhanced storage stability of BOxNPs/Au than earlier enzyme electrodes was due to the covalent coupling of BOx onto the Au electrode.

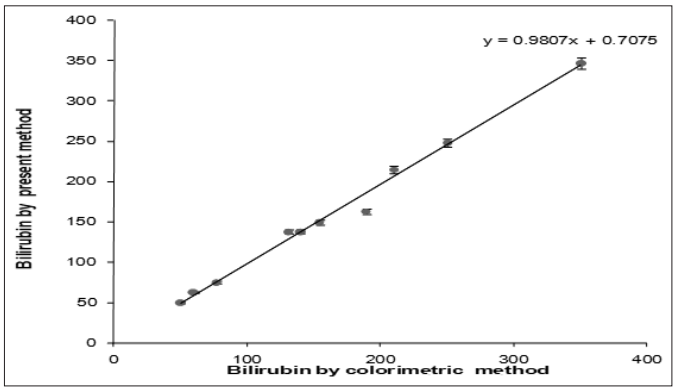


Figure 6: Correlation Between Bilirubin Values as Measured byStandard Colorimetric Method (x-axis) and Present Biosensor Method using Bilirubin Oxidase Nanoparticles/Gold (BOxNPs/Au) Electrode (y-axis)

Conclusion

BOxNPs/Au electrode was developed based on the covalent coupling of BOxNPs onto polycrystalline Au electrode. This electrode was found to have improved/better analytic performance, as it showed a lower limit of detection (0.1 μM), a lower response time (1s), and longer shelf life (150 days). The further advantage of this current technology is that it does not require any other nanoparticles to facilitate the transfer of electrons. Future research can be focused to improve other amperometric biosensors using enzyme nanoparticles (ENPs).

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CRediT Authorship Contribution Statement

Rachna Rawal: Methodology, Conceptualization, Visualization, Writing - original draft, Investigation. Poonam R. Kharangarh: Visualization, Formal analysis. C.S. Pundir: Supervision, writing, review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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