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A third generation bilirubin sensor development by using gold nanomaterial as an immobilization matrix for signal amplification

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ABSTRACT

In present work, we employed gold nanorods for electrochemical sensing of bilirubin. A new method is developed by using covalently immobilized bilirubin oxidase (BOx) on gold nanorods and employed gold microelectrode. The sensing interface materials were characterized by dynamic light scattering (DLS), scanning electron microscopy (SEM), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The optimum response was observed at pH 7 and 35 ºC. The linear working range of the biosensor is 0.01 -500 µM. Fabricated sensing technology showed good evaluation parameters like precision (2.2 % and 3.2 %) and analytical recovery (98.2 % and 97.4 %). Bilirubin biosensor can be employed for early detection of bilirubin in blood serum to check jaundice, hyperbilirubinia and physiologic jaundice in infants. Copyright © 2015 VBRI Press.

Keywords: Bilirubin; gold nanorods; horse radish peroxidase; gold microelectrode.

Introduction

Nanostructured materials have ever increased attracting research interest among multidisciplinary researches in the last few years due to comparable characteristic length scale with the critical length scales of physical phenomena [**1**]. In comparison with bulk materials, nanostructured materials exhibit interesting and peculiar properties which it is well-known that such unique properties and improved performances are determined by their size, structure, and mutual interaction among the nanostructured species [**2**]. Nanomaterials have novel physical and chemical properties that can be incorporated into chemical and biological sensing [**3-7**]. Metal nanoparticles, metal oxide nanoparticles, semiconductor nanoparticles and nanostructural conducting polymers have been administered into biosensor applications [**8-10**]. Gold nanoparticles have many distinct properties and its different shapes which make them suitable for use in biological applications. Among them nanorods are considered exceptional candidates for biological electrochemical sensing [**11**]. Gold nanorods have prominent applications in drug delivery, photochemical therapy, as contrasting and therapeutic agents. Gold nanorods depict chemically inert nature and high biocompatibility in biological systems. These novel properties of gold nanorods make it suitable for biomedical applications. Chitosan is a biopolymer which possesses excellent thin film forming property; NH_2 groups of chitosan provide sites for attachment of biomolecules [**12-14**]. In the present report we used gold nanorods as a sensing material for binding of bilirubin oxidase (BOx) for ultrasensitive detection of bilirubin.

Bilirubin (BR) is a lipophilic and cytotoxic yellow orange pigment [**16**]. The clinical determination of serum BR concentration on a regular basis is important. High concentration of bilirubin in blood can cause brain damage or death in severe cases. BR metabolism disorder may result into hyperbilirubinemia. High BR concentration in infants leads to physiologic jaundice [**17**]. BR detection is done by direct spectroscopic techniques or colorimetric measurements followed by diazotization of analyte [**18, 19**]. The conventional techniques are slow and lack accuracy. The point of care diagnostic tools is more accurate, precise, portable, cheap and regular basis test can easily be performed as it can be available at patient bedside [**20**]. Amperometric biosensor displays a high sensitivity and low detection concentration of analyte [**21**]. Voltammetric methods have been employed for the current measurements with respect to applied potential waveforms. This technique is a culmination of electrochemical and biological recognition process. Various biosensors are fabricated for detection of bilirubin in serum such as zirconia coated silica nanoparticles/chitosan hybrid film [**16**], Fe_3O_4/hydroxyapatite/molecularly imprinted polypyrrole nanoparticles [**22**], conductive poly-terthiophene–Mn(II) complex for amperometric bilirubin detection [**23**], fiber optic sensor sensor [**24**], multiwalled carbon nanotubes [**25**].

We describe herein fabrication of sensing device with increased sensitivity, selectivity and stability and can provide excellent potential applicability in fast and sensitive electrochemical sensing of BR.

Experimental

Materials

Bilirubin, bilirubin oxidase and chloroauric acid trihydrate were procured from Sigma-Aldrich Co. Chitosan nanopowder from Sisco research laboratory (SRL). All other chemicals were of analytic reagent grade. Double distilled water (DW) was used throughout the experiments.

Construction of working electrode for voltammetric sensing of BR

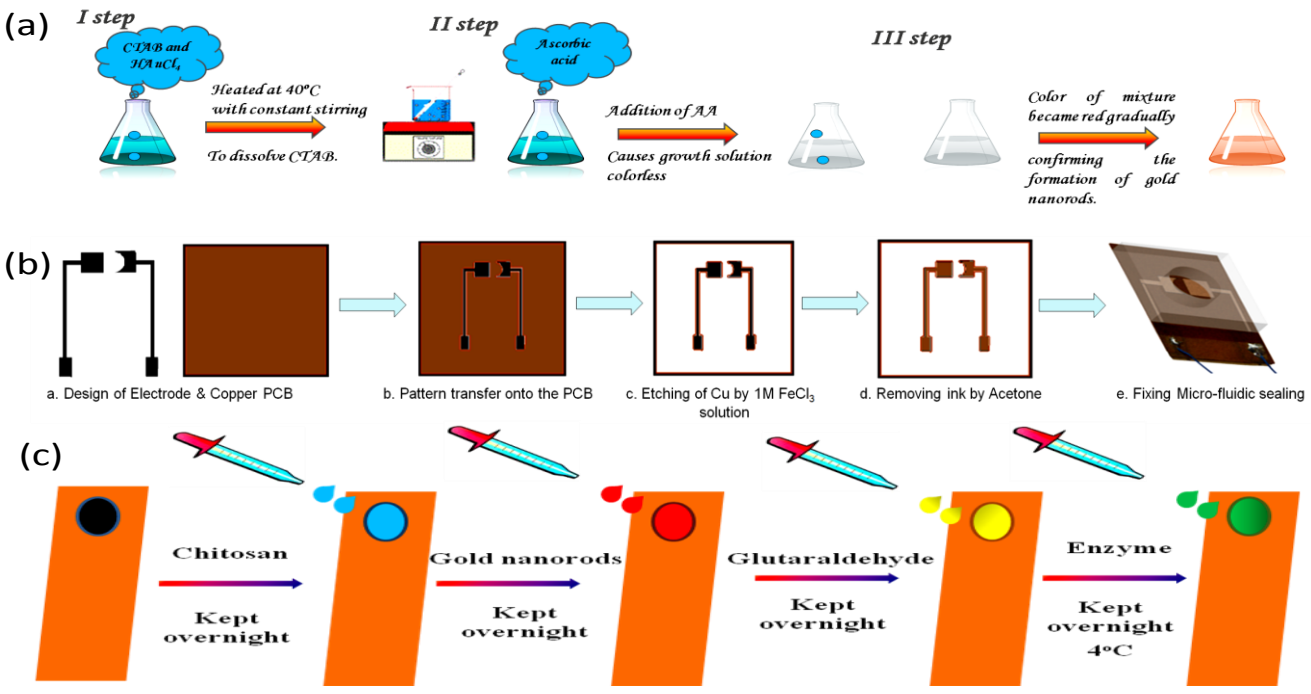
Au nanorods were synthesized by a seeding growth mechanism. Trisodium citrate capped seed gold nanoparticles of 3-4 nm were prepared [**26**]. It was followed by seed mediated growth of gold nanorods with cetyl trimethyl ammonium bromide (CTAB) as surfactant [**27**]. The preparation of growth solution was done by mixing CTAB (0.08 M) and $HAuCl_4$ (250 µM). The solution was then heated at 40ºC with constant stirring to dissolve CTAB. Then the prepared solution was treated with 10 mL of ascorbic acid preparation (AA, 10 mM) and 30 mL of $NaBH_4$ (3mM). The addition of AA made the growth solution colorless due to reduction of gold ions to gold atom. The colorless solution was added to the gold seed solution and then the solution was left undisturbed at 4ºC for 24 hrs (**Scheme.1a**).

Next step involves preparation of gold microelectrode printed circuit board (PCB) was cut by electrical cutting machine having dimensions (6 cm x 2 cm). The surface cleaning of cut PCB was done by distilled water. A sticker was placed on the PCB to get desired pattern. The piece was dipped in ferric chloride (1 M) in a petri dish and left for 35 mins at 40 ºC. Copper was etched and sticker was removed and gold plating was done on the copper. The contacts were made on the electrode by soldering wires to it. The operative reactions involved in ferric chloride based etching. The cupric ion gets oxidized to cuprous ion due to proportionation reaction.

Following reactions are involved in fabricating gold microelectrode (**Scheme.1b**).

$$2\ Fe^{3+} + Cu^0 \longrightarrow 2\ Fe^{2+} + Cu^{2+}$$
$$Cu^{2+} + Cu^0 \longrightarrow 2\ Cu^{+}$$

For surface modification of gold microelectrode, the gold nanorods, BOx, chitosan nanopowders and glutaraldehyde were decorated on electrode for electrochemical sensing of BR. Firstly, chitosan nanopowder solution (1 %) was prepared in ascorbic acid (AA) by constant stirring for 12 hours. The chitosan solution was drop dried on the electrode in the oven at 60 ºC. The electrode was left undisturbed for 12 hours. Then gold microelectrode was washed thoroughly with DW to remove unbound matter. After that, Gold nanorods (10 µl) solution was dip coated onto chitosan modified electrode and was kept overnight for physical adsorption. For cross-linking of enzyme, glutaraldehyde (2.5 %) was drop dried on the electrode at room temperature (RT). Then 10 µl of enzyme solution was drop dried on the modified electrode and kept for 24 h at 4 ºC. The electrode was finally washed with 0.1 M phosphate; buffer (pH 8.5) to remove unbound enzyme. This working electrode was characterized by SEM and CV at various stages of its fabrication (**Scheme.1c**).



Scheme. 1. Schematic diagram showing the stepwise fabrication of the (a) micro gold electrode (b) gold nanorods (c) modified electrode (BOx/nano Au / CHIT/ micro AuE).

Study of voltammetric and impediometric behavior of BR biosensor

To study the voltammetric and impediometric behavior, electrochemical cell composed of BOx/nano Au / CHIT/ micro AuE as working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode was recorded in PBS (50 mM, pH 6.5, 0.9 % NaCl) containing 5mM $[Fe(CN)_6]^{3-/-4}$ in presence of bilirubin. EIS and CV pattern of different phases of electrode fabrication was taken. Electrochemical impedance technique has been employed to investigate the charge transfer processes occurring at electrode/solution interface. CV was done for determination of the number of electrons transferred, whether a couple undergoes multiple oxidation/reduction steps, and whether there are other species in the solution that participate in the electrochemical process.

Different experimental conditions are also studied for maximum electrochemical response like pH, incubation temperature and time were optimized. For evaluation of functioning of biosensor, parameters like precision and accuracy were also studied.

Results and discussion

Evidence of preparation & characterization of working electrode

Fig. 1(**a**) shows TEM images, which was done to confirm the formation of gold nanorods. Microscopic illustration was reflecting the formation of nanorods with size 80 nm. **Fig. 1**(**b**) revealed the dynamic light scattering (DLS) pattern of gold nanorods which is also confirming the formation of nanorods. These results confirmed the formation of nanorods with size 80 nm.

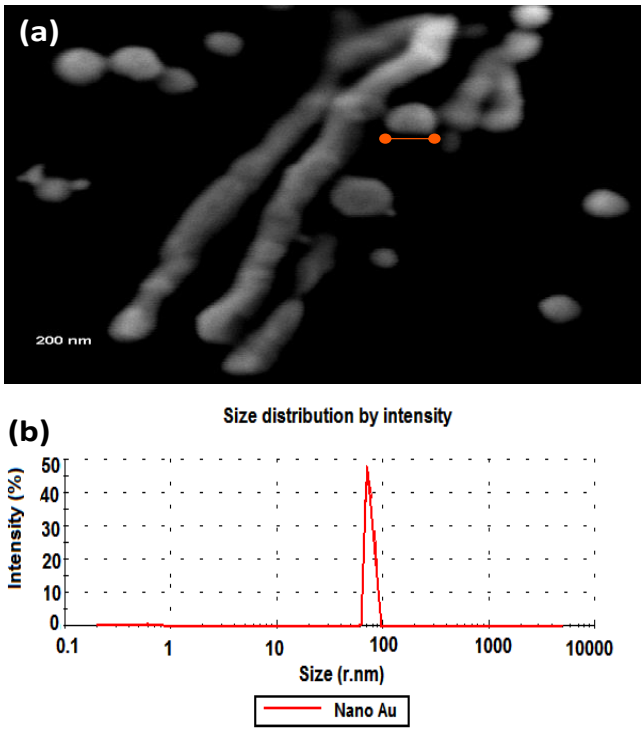


Fig. 1. (a) Transmission electron microscope (TEM) image of gold nanorods and (b) DLS spectra of gold nanorods.

SEM images illustrate various stages of electrode by microscopic examination of formation of sensing biomaterials. Surface topography of various phases of electrode (a) bare gold microelectrode (b) nano Au / CHIT/ micro AuE (c) and BOx/nano Au / CHIT/ micro AuE were also observed. Microscopic illustration of the bare electrode shows uncovered but rough surface. While gold nanorods and chitosan nanopowder decorated gold microelectrode reveals rods and spherical like structure on the surface. After enzyme incorporation, surface topography revealed presence of some globular structure which confirmed the immobilization of biomolecule (**Fig. 2(c)**).

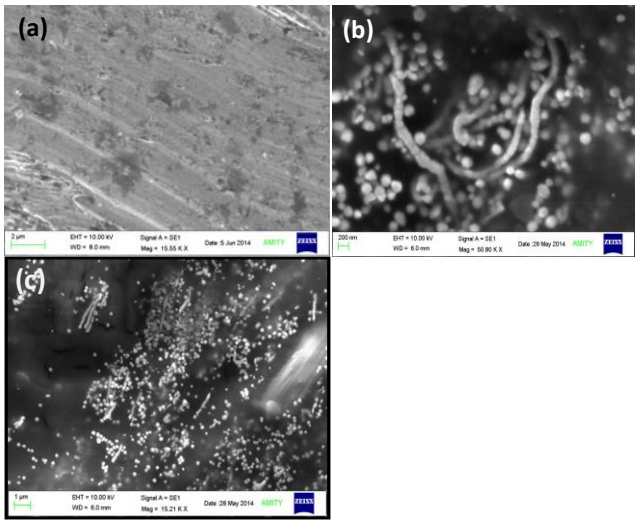


Fig. 2. SEM image of (a) bare micro AuE (a), nano Au /CHIT/micro AuE (b) and (c) BOx/nano Au/CHIT/micro AuE.

Electro-chemicals techniques are done to validate the presence of various sensing materials. First, CV technique was done with the modified gold microelectrode as the sensing electrode in the scanning potential range of -0.1 to +0.1 V s^{-1} at the scan rate 20 mV s^{-1} in 0.1 M phosphate buffer pH 7.5 in the presence of bilirubin (**Fig. 3**(**A**)). Insignificant sensing signal was observed in case of unmodified electrode and upon nanorods and CHIT decoration on the gold microelectrode sensing signal was significantly increased. It can be due to conducting properties of gold nanorods which decreases the resistance between reference and working electrode. Synergistic effect of both CHIT and gold nanorods provides more electron flow to the surface of electrode. When enzyme (BOx) integrated with sensing surface there is much increase in the sensing signal (BOx/nano Au / CHIT/ micro AuE). It might be due to more production of electrons due to interaction between enzyme and substrate. As this immobilization matrix might provide favorable environment and close proximity for biomolecule.

Electrochemical impedance spectroscopy (EIS) was also done in order to validate the results. This technique is an effective method for probing the surface properties of sensing electrode. EIS of (a) bare gold microelectrode (b) nano Au / CHIT/ micro AuE (c) and BOx/nano Au / CHIT/ micro AuE) in a solution containing 1 mM $Fe(CN)_6^{3-/-4}$ with 0.1 M KCl at 0.20 mV s^{-1} (frequency range of 0.01 Hz –10 kHz) and in the presence of bilirubin. Sensing

electrode (BOx/nano Au / CHIT/ micro AuE) showed small Rct value which means that sensing electrode demonstrated small resistance for the transfer of electrons and thus produces amplified signal. When nanorods and CHIT get decorated on the unmodified electrode then there is also decrease in the Rct value as compared to unmodified electrode. This means that immobilization matrix involved in this sensor provides biocompatible environment which facilitates the transfer of electrons. EIS studies are corresponding with the CV pattern of modified electrode.

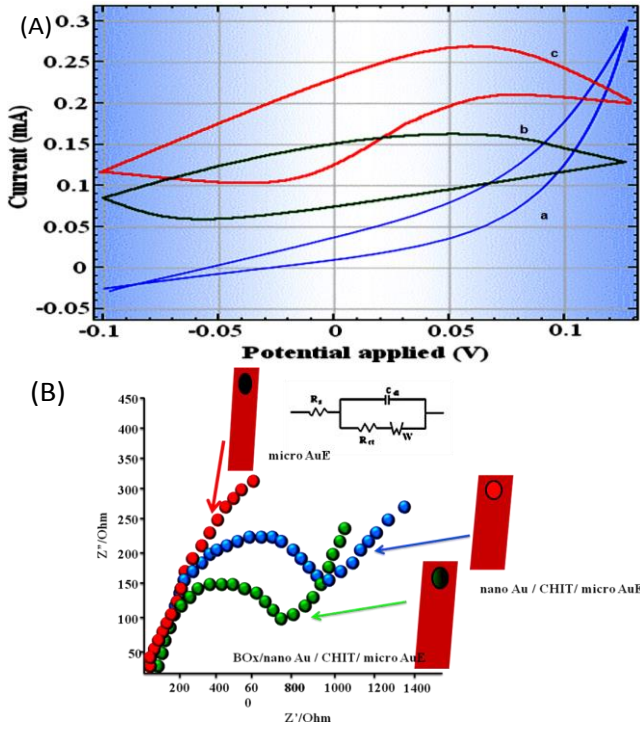


Fig. 3. (A) CV pattern of (a) bare micro AuE (a), nano Au / CHIT/ micro AuE (b) and BOx/nano Au / CHIT/ micro AuE in the scanning potential range of -0.1 to +0.1 V s−1 at the scan rate 20 mV s−1 in 0.1 M phosphate buffer pH 7.5 in the presence of bilirubin. (B) EIS of (a) bare micro AuE (a), nano Au / CHIT/ micro AuE (b) and BOx/nano Au / CHIT/ micro AuE containing 1 mM Fe(CN)6 3−/4− with 0.1 M KCl at 0.20 mV s−1 in the presence of bilirubin. (frequency range of 0.01 Hz – 10 kHz).

Impedimetric detection of bilirubin

Correlation between the electron transfer resistance (Rct) and the bilirubin concentrations was varied in the range of 500 to 1000 μM was studied in a solution containing 1 mM $Fe(CN)_6^{3-/4}$ with 0.1 M KCl at 0.20 mV s^{-1} (frequency range of 0.01 Hz –10 kHz). Time of incubation was kept 20 s, after 20 s electrochemical signals was produced. Quick response of nano Au sensor might be due to the sensing interface as it may be due to favorable orientation, biocompatibility and conductive pathway to transfer electrons. Value of Rct decreased with increase in bilirubin concentration (**Fig. 4(a)**). Because bilirubin oxidase acts on the bilirubin and after interaction hydrogen peroxide is generated. Increase in concentration of bilirubin produces more hydrogen peroxide and more hydrogen peroxide means more electrons. The results of experiments carried out in triplicate sets reveal reproducibility of the system within 3%. Calibration was also performed using CV and square-wave voltammetry (SWVs) with increasing concentration of bilirubin. CV response was observed for high concentrations of bilirubin (100- 600 μM) (**Fig. 4**(**b**)) while SWV response was observed for low concentrations of bilirubin (10-100 μM). The peak current is linearly related to the various concentrations of bilirubin (**Fig. 5**). Limit of detection was found to be 0.005 μM.

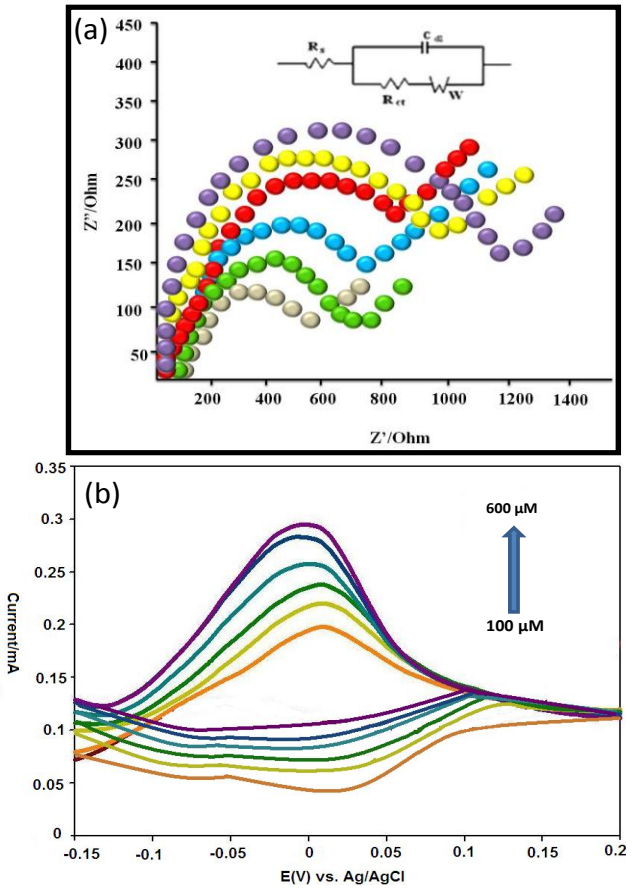


Fig. 4. (a) Impedimetric response of nano Au sensor for bilirubin detection and (b) CVs of modified electrode using different concentrations of bilirubin.

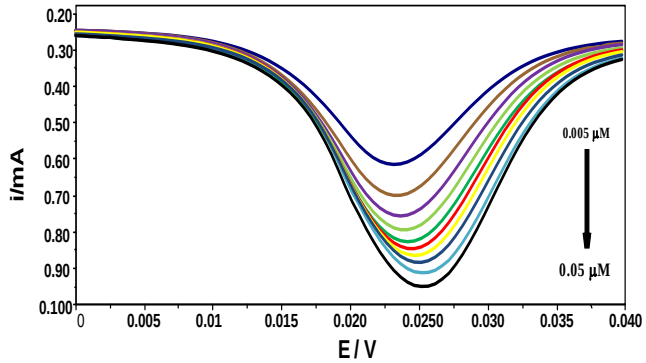


Fig. 5. SWVs of modified electrode using different lower concentrations of substrate.

Optimization & analytical performances of nano Au sensor

The response of nano Au sensor has been studied at different pH and temperatures. The result of the pH value on the response current of the BOx/nano Au / CHIT/ micro AuE was varied between 4.0 and 9.0 in 0.05 M PBS. The

optimum pH with the maximum activity of the immobilized BOx was at pH 7.0. Effect of temperature on biosensor was also studied in order to ensure the optimization. The current response reaches a maximum at approximately 50 ºC, and then goes down as the temperature turn higher. In order to maintain steady with the temperature of human body, 35 ºC is selected for this work. The effects of serum interferents on the bilirubin measurement have been studied by taking physiological concentration of interferents such as glucose, uric acid, urea and cholesterol. None had any significant interference. So nano Au sensor is anti-interferant (**Table 1**).

Evaluating parameters like analytic recovery, precision and accuracy were studied for sensitive detection of analyte. The mean analytic recoveries of added bilirubin 20 and 40 mmol L^{-1} (final conc. in reaction mixture) were 98.2 ± 1.5 and 97.4 ± 1.5, respectively.

Table 1. Interference effect of various compounds on nano Au Sensor.

Interferents	Relative response (%)
Glucose	100
Fructose	100
Ethanol	100
Ascorbic acid	140
Citric acid	100
Lactic acid	100
Malic acid	100
Tartaric acid	100
Alanine	100
Leucine	100
Urea	90
Uric acid	90
Cholesterol	90
Pyruate	100

To test the reproducibility and reliability of the present biosensor bilirubin level in ten serum samples was estimated on single day (within batch) and five times again after storage at -20 ºC (between batch). Precision value was found to be 2.2 % (with in batch) and 3.2 % (between batch). Accuracy of proposed method was 99 %. The bilirubin level in apparently healthy persons and jaundice patients, as measured by the present biosensor was in the range 3– 16 and 25–68 µM, respectively (**Table 2**).

In order to determine the accuracy of the present method, bilirubin values in 10 serum samples were determined by the present enzyme electrode method (y) and compared with those obtained by the colorimetric method (x), the values obtained by both the methods were co-related.

The long-term stability of sensor was also tested after a month. It is revealed that the increase in the value of Rct has been found to be about 20 % after 1 week while Rct increases sharply resulting in about 60 % loss in about 10 weeks. A comparison of analytic parameters of various nanoparticles based biosensors for detection of bilirubin with the present biosensor is summarized in **Table 3**.

Table 2. Determination of bilirubin by present nanosensor and colorimetric method.

S.No.	Present Method (µM)		Colorimetric Method (µM)	
	Healthy Persons	Diseased Persons	Healthy Persons	Diseased Persons
1.	10	25	8	23
2.	10	30	10	28
3.	20	25	18	25
4.	15	40	15	40
5.	10	25	10	25
6.	15	60	18	63
7.	15	65	14	65
8.	15	30	15	29
9.	5	30	5	30
10.	5	30	5	30

Table 3. Comparison of the present method with other biosensing methods.

Matrix/method	Enzyme	Response time (s)	Detection limit (µM)	Linearity (µM)	Stability months	Reference
Amperometric array based biosensor MWCNT	BOx		8 ± 0.2	0 - 150		[17]
Photoelectrochemical method		<1	0.007	0.1-17	3	[19]
Fe_3O_4/hydroxyapatite/molecularly imprinted polypyrrole nanoparticles						
Amperometric conductive poly-terthiophene-Mn(II) complex	BOx	<5	40 ± 3.8	0.1-50	2	[20]
Fibre optic sensor		10	0.1	0.09-300		[21]
Potentiostat prepared from molecular imprinting				-		[24]
Determination of Bilirubin using Oxygen electrode	BOx	<180	10	200		[25]
Ferrocene carboxamide modified MWCNT-gold nanocomposites		<5	1.2	100		[26]
Electrochemical impediometric detection of bilirubin taking gold nanorods as sensing interface	BOx	2	0.005	0.01-500	1	Present

Conclusion

Nano Au sensor was fabricated by using gold nanorods as sensing interface and its applicability is for detection of bilirubin via EIS technique. The Nano Au sensor exhibits improved biosensing characteristics like linearity as 0.005 to 1000 µM, fast response time of 15 s, specific & anti-interferants. The response of Nano Au sensor with serum samples shows its implications towards the development of biosensor for commercial disease (jaundice) monitoring device.

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