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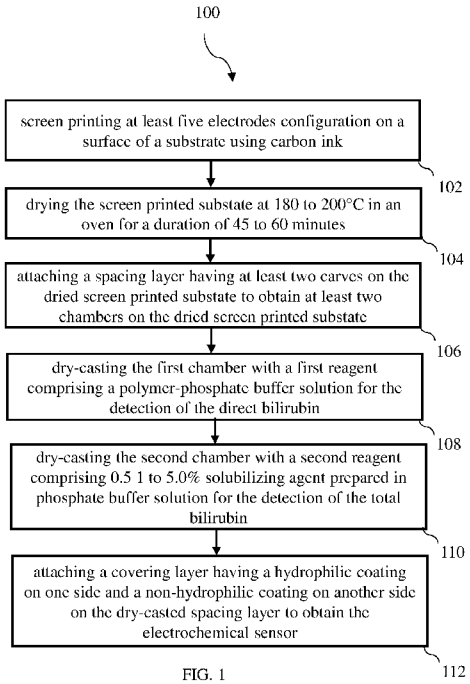


FIG. 1

(57) **Abstract:** The present patent invention introduces an electrochemical sensor specifically designed for accurate detection of bilirubin levels in biological samples The sensor incorporates a unique electrode configuration consisting of two working electrodes (201), two counter electrodes (202), and a common reference electrode (203), all fabricated with gold-based trimetallic nanocomposites. The sensor further employs isolated chambers, enabling separate detection of direct and total bilirubin. The chambers are dry-casted with specific reagents to optimize the detection process. A covering layer (208), featuring a hydrophilic coating and non-hydrophilic outer layer, facilitates sample introduction via capillary action. A small vent opening ensures efficient air escape during sample entry. The fabrication process involves screen printing, drying, and layer attachment. The electrochemical sensor of the present invention provides improved accuracy and reliability, making it highly suitable for precise diagnosis and monitoring of medical conditions related to liver health.

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AN ELECTROCHEMICAL SENSOR FOR DETECTION OF BILIRUBIN WITH GOLD-BASED TRIMETALLIC NANOCOMPOSITES

EARLIEST PRIORITY DATE:

This Application claims priority from a patent application filed in India having Patent Application No. 202311040216, filed on June 13, 2023 and titled "AN ELECTROCHEMICAL SENSOR FOR DETECTION OF BILIRUBIN WITH GOLD-BASED TRIMETALLIC NANOCOMPOSITES"

FIELD OF INVENTION

Embodiments of the present invention relates to electrochemical sensors used for the detection of bilirubin. More specifically, the invention pertains to an electrochemical sensor that employs a specific electrode configuration, gold-based nanocomposite materials, and isolated chambers for the detection of direct and total bilirubin.

BACKGROUND

Bilirubin is a yellow-colored catabolic product of heme released from senescent red blood cells. It exists in blood in two different forms: unconjugated and conjugated. Unconjugated bilirubin is highly toxic and insoluble, and it must be converted to its soluble (conjugated) form through conjugation with glucuronic acid for excretion. This conjugation process takes place in the liver, catalyzed by the enzyme UDP-glucuronosyltransferase. The measurement of bilirubin levels in biological samples, such as blood or serum, is important in clinical diagnosis and monitoring of various medical conditions, such as jaundice, cirrhosis, thalassemia, Gilbert syndrome, and hepatitis. Various techniques have been developed for bilirubin detection, including spectrophotometric methods and electrochemical sensors.

Conventional methods of bilirubin analysis, such as spectroscopic techniques and diazo reactions, have limitations in terms of susceptibility to interferences from other serum components, pH-dependence, lack of selectivity, high cost, and time-consuming sample preparation. Recent advancements have focused on the use of metallic nanoparticles, such as gold, silver, copper, and platinum, to enhance the analytical performance of electrodes in terms of sensitivity, stability, and detection limit.

Electrochemical sensors offer several advantages over other detection methods, such as simplicity, sensitivity, and rapid response. These sensors utilize specific electrode configurations and materials to facilitate the electrochemical reactions involved in the detection process. However, there is a need for improved electrochemical sensors that provides accurate and reliable detection of bilirubin.

In recent times, metallic nanoparticles like gold, silver, copper, and platinum have gained widespread usage in enhancing the performance of electrodes for improved sensitivity, stability, and detection limit in analytical applications. Tri-metallic nanoparticles are becoming increasingly significant due to their distinct and enhanced physical and chemical properties when compared to their mono-metallic and bimetallic counterparts.

The incorporation of a third metal into the system has demonstrated the ability to enhance electrocatalytic activity and stability, making it particularly advantageous for fabricating electrochemical sensors with improved linearity and detection limit. Non-enzymatic sensor fabrication methods have garnered attention as an alternative to enzymatic biosensors due to their cost-effectiveness, rapid response, and exceptional sensitivity in certain cases. Moreover, non-enzymatic sensors are highly desirable as they are not reliant on various factors such as temperature, pH, and ionic strength, which greatly affect the catalytic activity of enzymatic sensors.

It is estimated that 6 out of every 10 babies develop Jaundice due to the presence of excess of bilirubin in the blood. Its monitoring in new-born is of utmost importance as untreated jaundice for too long may cause brain damage, a condition called

kernicterus. In conventional testing, sample (blood/serum) is processed to collect plasma/serum and then treated with various harsh chemical reagents for different time intervals to form a colored product, and bilirubin concentration is then determined from its color intensity. This method has several disadvantages such as complex sample processing, time-consuming, high sample volume, interferences from other blood components, and high toxicity of chemicals used. For effective treatment, all forms of bilirubin need to be quantitatively detected and regularly monitored.

Jaundice is a substantial public health issue in India, according to studies from the National Institute of Communicable Diseases (NICD) with number of deaths increased in the last 20 years. In comparison to adults, infants are more susceptible to jaundice with high mortality rate. Therefore, continuous monitoring of all forms of blood bilirubin level is always necessary for its efficient therapy. However, it is very difficult to achieve the same through conventional methods, which is also very time-consuming and needs trained personnel.

While numerous studies have been published on the non-enzymatic detection of bilirubin using nanomaterial-modified disc electrodes within the physiological range, the literature remains limited in comparison to physiologically relevant analytes like glucose, urea, uric acid, and hydrogen peroxide. Although the analytical performance of these disc-based electrodes was satisfactory, they suffer from several drawbacks such as high cost, lack of portability, limitations in point-of-care testing, and the need for high sample volumes.

Reference is also been made to Patent documents such as **US6030827**, **US20100224488A1**, **US6326208**, **US5563072**, **US4338095A**, **JP3868379B2**, **US20170192021A1**, **US2007281321**, **US6882873**, etc. Most of these patent documents discloses enzymatic sensors. Enzyme based sensor has several limitations such as complicated immobilization step, high cost of the enzyme, delayed response, and low shelf-life. Further, an optical-based sensor has been disclosed in US4338095A. However, this sensor has this limitation that other

interferants present in the blood sample may cause absorbance and the false result may be produced.

The non-patent documents referenced include "*Non-enzymatic detection of bilirubin based on a graphene-polystyrene sulfonate composite*," 2015, RSC Advances; "*An amperometric bilirubin biosensor based on a conductive polyterthiophene-Mn(II) complex*," 2008, Biosensors and Bioelectronics; "*Superior sensing performance of multi-walled carbon nanotube-based electrodes to detect unconjugated bilirubin*," 2013, Thin Solid Films; "*Synthesis of the multi-walled carbon nanotubes-COOH/graphene/gold nanoparticles nanocomposite for simple determination of Bilirubin in human blood serum*," 2013, Sensors and Actuators; "*Electrocatalytic oxidation of bilirubin at ferrocenecarboxamide modified MWCNT-gold nanocomposite electrodes*," 2009, Microchimica Acta; and "*Selective nonenzymatic bilirubin detection in blood samples using a Nafion/Mn-Cu sensor*," 2014, Biosensors and Bioelectronics. These non-patent documents disclose sensors which are based on conventional disc electrodes which are modified with nanomaterials to detect bilirubin in the physiological range. Though the analytical features are quite good, there are several disadvantages of using disc-based electrodes such as high cost, portability issue, POC testing, and high sample volume requirement.

There is a growing need for detection of bilirubin on account of higher incidences of neo-natal jaundice and for some medicines whose side effect includes increased bilirubin levels and there is an increased demand for portable devices for detection of bilirubin.

There remains a need for a reliable, simple, and realistic platform that overcomes the limitations of previous bilirubin analysis methods, particularly in quality control laboratories and in-house assays. Also, there is need for introducing a highly sensitive non-enzymatic screen-printed sensor modified with a nanostack of tri-metallic nanoparticles for the direct analysis of bilirubin in undiluted human serum samples.

Therefore, there is a need for an innovative non-enzymatic sensor for direct bilirubin analysis in undiluted human serum samples, thus improving the accuracy, efficiency, and practicality of bilirubin detection.

SUMMARY

Embodiments of the present invention relates to an electrochemical sensor for detection of bilirubin. The invention mainly focuses on an electrochemical sensor that employs a specific electrode configuration, gold-based nanocomposite materials, and isolated chambers for the detection of direct and total bilirubin.

In accordance with another embodiment of the present invention, an electrochemical sensor for detection of bilirubin comprising a substrate having at least five electrodes configuration screen printed on a surface of the substrate. The at least five electrodes configuration comprises two working electrodes, two counter electrodes and a common reference electrode. The two working electrodes fabricated with gold-based trimetallic nanocomposites. The gold-based trimetallic nanocomposites are selected from a group consisting of Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni. A spacing layer having at least two carves affixed on the at least five electrodes configuration on the surface of the substrate. The spacing layer is configured to create at least two isolated chambers on the surface of the substrate. The at least two isolated chambers comprise a first isolated chamber configured to carry out detection of direct bilirubin and a second isolated chamber configured to carry out detection of total bilirubin. A covering layer affixed onto a top surface of the spacing layer. The covering layer comprises a hydrophilic coating and a non-hydrophilic coating. The hydrophilic coating is affixed onto the top surface of spacing layer and the non-hydrophilic coating is configured to function as an outer layer.

The present invention introduces the use of novel trimetallic nanocomposites synthesized using gold-based (Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni) materials. These nanocomposites exhibit superior electrochemical properties, enhancing the

sensitivity and accuracy of the sensor compared to conventional sensors. The reagent formulation used in the chambers of the sensor includes specific compositions tailored for the analysis of direct and total bilirubin. The presence of solubilizing agents and hydrophilic polymers ensures efficient detection and quantification of both forms of bilirubin.

To further clarify the advantages and features of the present invention, a more particular description of the invention will follow by reference to specific embodiments thereof, which are illustrated in the appended figures. It is to be appreciated that these figures depict only typical embodiments of the invention and are therefore not to be considered limiting in scope. The invention will be described and explained with additional specificity and detail in the appended figures.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described and explained with additional specificity and detail with the accompanying figures in which:

FIG. 1 is a flow diagram representing steps of a process for fabrication of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present invention;

FIG. 2 depicts a side view of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present disclosure;

FIG. 3 demonstrates a fabrication process of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present disclosure;

FIG. 4 depicts the electrochemical response of fabricated Au-Ag-Cu nanocomposite modified sensor investigated towards bilirubin oxidation using linear sweep voltammetry (LSV) technique: **FIG. 4a** depicts Au-Ag-Cu nanocomposite synthesized using glucose (R1) as reducing agent in comparison to bare and Au-Ag nanocomposite modified electrode, and **FIG. 4b** depicts Au-Ag-

Cu nanocomposite synthesized using sodium borohydride (R2) as reducing agent, in accordance with an embodiment of the present disclosure;

FIG. 5 depicts the electrochemical response of fabricated Au-Ag-Co nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure;

FIG. 6 depicts the electrochemical response of fabricated Au-Ag-Ni nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure;

FIG. 7 depicts comparison studies of the electrochemical response among all the three fabricated trimetallic gold-based nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure;

FIG. 8 depicts analytical performance of all the all the three fabricated trimetallic gold-based nanocomposite modified sensor modified electrodes assessed using chronoamperometry for the detection of total and direct bilirubin on a single strip, in accordance with an embodiment of the present disclosure; and

FIG. 9 depicts an interference study performed on new miniaturized designs to check the effect of some common interfering compounds such as glucose (500 μM), ascorbic acid (50 μM), dopamine (0.5 μM), and uric acid (50 μM) in the presence of 20 mg/dl bilirubin, in accordance with an embodiment of the present disclosure.

Further, those skilled in the art will appreciate that elements in the figures are illustrated for simplicity and may not have necessarily been drawn to scale. Furthermore, in terms of the construction of the device, one or more components of the device may have been represented in the figures by conventional symbols, and the figures may show only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the figures with details that will be readily apparent to those skilled in the art having the benefit of the description herein.

DETAILED DESCRIPTION

For the purpose of promoting an understanding of the principles of the disclosure, reference will now be made to the embodiments illustrated in the figures, and specific language will be used to describe them. It will nevertheless be understood that no limitation of the scope of the disclosure is thereby intended. Such alterations and further modifications in the illustrated system, and such further applications of the principles of the disclosure as would normally occur to those skilled in the art are to be construed as being within the scope of the present disclosure.

The terms "comprises", "comprising", or any other variations thereof, are intended to cover a non-exclusive inclusion, such that a process or method that comprises a list of steps does not include only those steps but may include other steps not expressly listed or inherent to such a process or method. Similarly, one or more components, compounds, and ingredients preceded by "comprises... a" does not, without more constraints, preclude the existence of other components or compounds or ingredients or additional components. Appearances of the phrases "in an embodiment", "in another embodiment" and similar language throughout this specification may, but not necessarily do, all refer to the same embodiment.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by those skilled in the art to which this disclosure belongs. The system, methods, and examples provided herein are only illustrative and not intended to be limiting.

In the following specification and the claims, reference will be made to a number of terms, which shall be defined to have the following meanings. The singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise.

FIG. 1 is a flow diagram representing steps of a process for fabrication of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present invention.

FIG. 2 depicts a side view of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present disclosure.

FIG. 3 demonstrates a fabrication process of an electrochemical sensor for detection of bilirubin, in accordance with an embodiment of the present disclosure.

Embodiments of the present invention relate to an electrochemical sensor for detection of bilirubin. The invention mainly focuses on an electrochemical sensor that employs a specific electrode configuration, gold-based nanocomposite materials, and isolated chambers for simultaneously detection of direct and total bilirubin in a given sample.

In accordance with another embodiment of the present invention, an electrochemical sensor for detection of bilirubin comprising a substrate having at least five electrodes configuration screen printed on a surface of the substrate. The at least five electrodes configuration comprises two working electrodes (201), two counter electrodes (202) and a common reference electrode (203) as depicted in **FIG. 2**. The two working electrodes (201) are fabricated with gold-based trimetallic nanocomposites. The gold-based trimetallic nanocomposites are selected from a group consisting of Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni.

A spacing layer (206) having at least two carves affixed on the at least five electrodes configuration on the surface of the substrate. The spacing layer (206) is configured to create at least two isolated chambers on the surface of the substrate. The at least two isolated chambers comprise a first isolated chamber configured to carry out detection of direct bilirubin and a second isolated chamber configured to carry out detection of total bilirubin as depicted in **FIG. 3**.

A covering layer (208) affixed onto a top surface of the spacing layer. The covering layer (208) comprises a hydrophilic coating and a non-hydrophilic coating. The

hydrophilic coating is affixed onto the top surface of spacing layer (206) and the non-hydrophilic coating is configured to function as an outer layer. The substrate comprises a polyethylene terephthalate (PET) sheet.

In another embodiment of the present invention, the electrochemical sensor comprises the first isolated chamber is dry-casted with a first reagent comprising a hydrophilic polymer, and a phosphate buffer solution for the detection of the direct bilirubin. The second isolated chamber is dry-casted with a second reagent comprising the hydrophilic polymer, 0.1 to 5.0% solubilizing agent prepared in one of deionized (DI) water and phosphate buffer solution for the detection of the total bilirubin. The outer layer of the covering layer (208) is configured to provide a wicking mechanism to the electrochemical sensor wherein a drop of sample applied is drawn into the first isolated chamber and the second isolated chamber through a capillary action. The covering layer (208) comprises a small vent opening configured to ensure escaping of air from the first isolated chamber and the second isolated chamber as sample enters. The hydrophilic polymer is selected from one of carboxy methyl cellulose, PVP, hydroxy ethyl cellulose, and combination thereof. The solubilizing agent is selected from one of Tergitol, Pluronic F-127, and combination thereof.

In another embodiment of the present invention, a process for fabrication of an electrochemical sensor for detection of bilirubin comprising the steps of screen printing at least five electrodes configuration on a surface of a substrate using carbon ink, at step 102. The at least five electrodes configuration comprises two working electrodes (201), two counter electrodes (202) and a common reference electrode (203). The two working electrodes (201) are modified with gold-based trimetallic nanocomposites. The screen-printed substrate is dried at 180 to 200 °C in an oven for a duration of 45 to 60 minutes, at step 104.

A spacing layer (206) having at least two carves is attached on the dried screen printed substate to obtain at least two chambers on the dried screen printed substate, at step 106. The least two chambers comprise a first chamber configured to carry

out detection of direct bilirubin and a second isolated chamber configured to carry out detection of total bilirubin. At step 108, the first chamber is dry-casted with a first reagent comprising a polymer-phosphate buffer solution for the detection of the direct bilirubin. At step 110, the second chamber is dry-casted by adding 3.0-5.0 µL of a second reagent at 45-55 °C for 5-10 min. The second reagent comprising 0.1 to 5.0% solubilizing agent prepared in one of the DI water and phosphate buffer solution for the detection of the total bilirubin. At step 112, a covering layer (208) is having a hydrophilic coating attached on one side and a non-hydrophilic coating on another side on the dry-casted spacing layer (206) to obtain the electrochemical sensor.

The substrate comprises a polyethylene terephthalate (PET) sheet. The gold-based trimetallic nanocomposites are selected from a group consisting of Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni. The covering layer (208) comprises a small vent opening configured to ensure escaping of air from the first isolated chamber and the second isolated chamber as sample enters.

The embodiments and examples described above are provided for illustrative purposes and are not intended to limit the scope of the invention. Various modifications and alternative embodiments may be implemented without departing from the spirit and scope of the present invention. Therefore, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given.

EXAMPLES

Materials and Methods

Materials

Chloroauric acid tetrahydrate ($HAuCl_4.3H_2O$), Silver nitrate ($AgNO_3$), and Bilirubin was procured from Sigma-Aldrich, while copper sulfate, nickel sulfate, soluble starch, sodium borohydride and cobalt chloride were purchased from Loba-chemie. BioRad level 1 and 2 were purchased from local distributor. Carbon ink

was procured from Dycotec Materials (UK) while opaque white PET sheets (Melinex, 339, DTF) were used as a substrate for screen-printing.

Synthesis of Trimetallic nanocomposites

I. Gold-based nanocomposite

(i) Au-Ag-Cu

For the modification of electrode material, a novel trimetallic nanocomposite consisting of gold, silver and copper was synthesized using wet chemistry. The process involves reduction of salts of silver (0.5-2.0 mM), gold (0.5-2.0 mM) and copper (1.0-10.0 mM) in DI water in the presence of starch (0.5-2%) as stabilizing agent and glucose (0.2-1.0 M) as reductant at 80-100 °C. Sodium hydroxide (0.5-1.0 M) was added to the above mixture and again incubating for 10-15 minutes at same temperature to obtain a dark-coloured colloidal solution of nanocomposite. In another approach, the nanocomposite was prepared by using sodium borohydride (10-100 mM) as reductant in non-alkaline solution. After nanocomposite synthesis, ethanol was added to the dispersion and the particles were separated by centrifugation at 5000-10000 rpm for 10-15 min.

(ii) Au-Ag-Co

For the synthesis of Au-Ag-Co trimetallic nanoparticles, a chemical reduction process was used wherein starch and sodium borohydride were used as stabilizing agent and reducing agent, respectively. Without stabilizing agent, instant aggregation of the nanoparticles was observed. The process involves reduction of salts of silver (0.5-2.0 mM), gold (0.5-2.0 mM) and cobalt (1.0-10.0 mM) in DI water in the presence of starch (0.5-2%) as stabilizing agent and sodium borohydride (10-100 mM) as reductant. On addition of sodium borohydride, an instant change in colour of solution was observed which confirms the formation of nanoparticles. The reaction was allowed to proceed for another 10-15 minutes. After nanocomposite synthesis, ethanol was added to the dispersion and the particles were separated by centrifugation at 5000-10000 rpm for 10-15 min.

(iii) Au-Ag-Ni

The process involves reduction of salts of silver (0.5-2.0 mM), gold (0.5-2.0 mM) and nickel (1.0-10.0 mM) in DI water in the presence of starch (0.5-2%) as stabilizing agent and sodium borohydride (10-100 mM) as reductant. Again, on addition of sodium borohydride, an instant change in color of solution was observed which confirms the reduction of ions. The reaction was allowed to proceed for another 10-15 minutes. After nanocomposite synthesis, ethanol was added to the dispersion and the particles were separated by centrifugation at 5000-10000 rpm for 10-15 min.

Sensor fabrication

FIG. 3 demonstrates the fabrication process of electrochemical sensor for bilirubin. A Unitech tabletop screen printer was used for the fabrication of carbon electrodes in a five-electrode configuration: two working (WE), two counter (CE), and a common reference electrode (RE). The PET sheets were used as received without any pre-treatment. The carbon ink was used to print the electrodes and were dried at 180-200°C in an oven for 45-60 min. A middle double-sided adhesive layer having two cut-out is fixed to the base PET substrate in a way to create two isolated chambers (chamber 1 and 2) with one chamber specific for direct bilirubin and other for total bilirubin. Finally, a covering layer (208) consists of the hydrophilic coating on one side is affixed to the spacing layer. The hydrophilic side of covering layer (208) was toward the sample-contact zone while non-hydrophilic side is present on the outer side. This outer covering provides a wicking mechanism to the fabricated sensor wherein a drop of sample applied at one end (sample inlet) is drawn into the sample-receiving chamber through capillary action. This covering layer (208) also includes a small vent opening to ensure escaping of air from the chamber as sample enters it. With this assembly, the sample volume required to cover the entire working zone lowered to 2-3 µl. This five-electrodes modified strip is designated as dual-chamber (DC) strips.

Dried Reagent chemistry

The fabricated sensor can be used to analyze both, direct bilirubin or total bilirubin depending on the chamber to which sample is introduced. Each chamber analyzes bilirubin fraction as a function of the composition of the reagent pre-dried in the chamber. Chamber 1 is intended toward the analysis of direct bilirubin while chamber 2 is for total bilirubin analysis.

The two novel surfactants including Tergitol and Pluronic F-127 as solubilizing agents which have not been reported earlier for total bilirubin analysis are used in the present invention. A 0.1-5.0% stock solution of the solubilizing agent was prepared in DI water and dissolved slowly to prevent froth formation. The specific reagent is then casted into the chamber by adding 3.0-5.0 µL of reagent solution, and dried at 45-55 °C for 5-10 min. These solubilizing agents have an advantage over previously reported sodium dodecyl sulfate (SDS) in that they are able to solubilize unconjugated fraction of bilirubin at a very low concentration of solubilizing agent and are therefore highly efficient for total bilirubin determination.

The fabricated sensor can be used to analyze both, direct bilirubin or total bilirubin depending on the chamber to which sample is introducing. Each chamber analyzes bilirubin fraction as a function of the composition of the reagent pre-dried in the chamber. Chamber 1 is intended toward the analysis of direct bilirubin while chamber 2 is for total bilirubin analysis as depicted in **Table 1**. The composition of chamber may be:

Table 1. The composition of chamber.

Sr. No.	Chamber	Reagent	Composition	Analyte
1	Chamber-1 (C-1)	R-1	Nanomaterial, Solvent (Water/buffer), hydrophilic polymer	Direct bilirubin
2	Chamber-2 (C-2)	R-2	Nanomaterial, Solvent (Water/buffer), hydrophilic polymer, solubilizing agent	Total bilirubin

For chamber-1 and chamber-2, the reagent can either be casted directly on the working electrode or may be deposited in the entire chamber. The presence of solubilizing agent ensures the solubilization of the unconjugated fraction of bilirubin to provide total bilirubin estimation. Preferred examples for hydrophilic polymers for the above composition are carboxy methyl cellulose, PVP or hydroxy ethyl cellulose.

FIG. 4 depicts the electrochemical response of fabricated Au-Ag-Cu nanocomposite modified sensor investigated towards bilirubin oxidation using linear sweep voltammetry (LSV) technique: **FIG. 4a** depicts Au-Ag-Cu nanocomposite synthesized using glucose (R1) as reducing agent in comparison to bare and Au-Ag nanocomposite modified electrode, and **FIG. 4b** depicts Au-Ag-Cu nanocomposite synthesized using sodium borohydride (R2) as reducing agent, in accordance with an embodiment of the present disclosure.

FIG. 5 depicts the electrochemical response of fabricated Au-Ag-Co nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure.

FIG. 6 depicts the electrochemical response of fabricated Au-Ag-Ni nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure.

FIG. 7 depicts comparison studies of the electrochemical response among all the three fabricated trimetallic gold-based nanocomposite modified sensor investigated towards bilirubin oxidation using LSV technique, in accordance with an embodiment of the present disclosure.

Results and Discussion

Electrochemical activities of various nanocomposite-modified electrodes toward bilirubin oxidation were evaluated by chronoamperometric and LSV (linear sweep voltammetry). The LSV was carried out at 5.0 mg/dl bilirubin prepared in 0.1 M phosphate buffer (pH 8.0) at a step potential and scan rate of 5.0 mV and 50 mV s^{-1} respectively.

I. Gold-based nanocomposite

(i) Au-Ag-Cu nanocomposite

Initially LSV technique was used to analyze the response of fabricated Au-Ag-Cu nanocomposite modified sensor. As depicted in **FIG. 4a**, in comparison to bare and Au-Ag nanocomposite modified electrode, Au-Ag-Cu nanocomposite modified electrode shown a higher current output toward bilirubin oxidation. The oxidation potential for bilirubin was obtained at around 0.35 V with a slight shift in comparison to the bare electrode. Since Au-Ag-Cu nanocomposite was synthesized using two different reducing agents, a comparison was also made between the electrochemical response of both electrodes. As depicted in **FIG. 4b**, Au-Ag-Cu nanocomposite synthesized using sodium borohydride (R2) as reducing agent shown a lower current output in comparison to Au-Ag-Cu nanocomposite synthesized using glucose as a reducing agent (R1). It implies that the synthetic process involved significantly affects the electrochemical response of the fabricated sensor.

(ii) Au-Ag-Co nanocomposite

Next, the electrochemical response of Au-Ag-Co modified electrode was investigated toward bilirubin oxidation using LSV technique. As depicted in **FIG.**

5, modification with Au-Ag-Co nanocomposite enhanced the current output for bilirubin oxidation in comparison to bare and bimetallic nanocomposite modified electrode. A slight shift in the oxidation potential was also observed around 0.4V.

(iii) Au-Ag-Ni nanocomposite

The electrochemical response of Au-Ag-Ni modified electrode was also recorded toward bilirubin oxidation. As depicted in **FIG. 6**, Au-Ag-Ni nanocomposite also improvises the sensor response by enhancing the electro-oxidation of bilirubin in comparison to the bare electrode. There was a significant improvement in the current intensity around 0.35 V specific for bilirubin oxidation compared to bare and Au-Ag nanocomposite modified electrode.

A comparison was also done among all the three trimetallic gold-based nanocomposite synthesized. It is depicted in **FIG. 7** that in comparison to the bare electrode, all three trimetallic nanocomposite materials could enhance bilirubin electrooxidation with different intensities.

FIG. 8 depicts analytical performance of all the all the three fabricated trimetallic gold-based nanocomposite modified sensor modified electrodes assessed using chronoamperometry for the detection of total and direct bilirubin on a single strip, in accordance with an embodiment of the present disclosure.

Analytical performance of bilirubin sensor

Next, the analytical performance of all the nanocomposites (gold-based) modified electrodes were assessed using chronoamperometry for the detection of total and direct bilirubin on a single strip. For this, Lyphochek assayed chemistry controls level 1 and level 2 with pre-determined values of direct and total bilirubin were procured from a local distributor of BioRad Laboratories, and further analyzed using as nanocomposite modified dual chamber strip sensor. For all gold-based-nanocomposites, a significant current difference was obtained between the direct (C-1) and total bilirubin (C-2) of BioRad 1 indicating the ability of sensor to discriminate between the two fractions of bilirubin as depicted in **FIG. 8**.

FIG. 9 depicts an interference study performed on new miniaturized designs to check the effect of some common interfering compounds such as glucose (500 μM), ascorbic acid (50 μM), dopamine (0.5 μM), and uric acid (50 μM) in the presence of 20 mg/dl bilirubin, in accordance with an embodiment of the present disclosure.

Interference study was also performed on new miniaturized designs to check the effect of some common interfering compounds such as glucose (500 μM), ascorbic acid (50 μM), dopamine (0.5 μM), and uric acid (50 μM) in the presence of 20 mg/dl bilirubin. For comparison, two different matrixes, buffer and serum, were used and the interferants were added prior to bilirubin addition to analyze bilirubin oxidation in the presence of all the interferants in the solution. It has been observed that the presence of interferants in either matrix did not significantly affect the sensor response toward bilirubin, and a sharp peak was obtained following bilirubin addition in comparison to other interferants as depicted in **FIG. 9**. Since the average signal deviation was found to be less than 5.0% (tolerance limit) in all interfering species, thus, it confirms the highly selective nature of the fabricated device.

Therefore, the present invention provides an electrochemical sensor for detection of bilirubin that enables the simultaneous detection of both forms of bilirubin, namely direct and total, on a single strip. This is a significant advancement as it eliminates the need for separate tests or devices for analyzing different forms of bilirubin, streamlining the diagnostic process.

The sensing platform of the present invention incorporates a novel nanostack, which consists of various nanocomposites synthesized using gold-based based materials. These nanocomposites enhance the sensitivity of the fabricated sensor, improving its ability to detect and measure bilirubin accurately. The fabrication of the electrochemical sensor involves a five-electrode configuration, including two working electrodes, two counter electrodes, and a common reference electrode. This configuration allows for precise and reliable measurements of bilirubin levels, contributing to the accuracy and efficiency of the sensor.

The electrochemical sensor for detection of bilirubin of the present invention is designed with two isolated chambers, with each chamber specifically dedicated to the analysis of a particular form of bilirubin. This dual-chamber design enables the simultaneous measurement of direct and total bilirubin in real-time, offering convenience and time-saving benefits in clinical settings.

The present invention introduces the use of novel trimetallic nanocomposites synthesized using gold-based (Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni) materials. These nanocomposites exhibit superior electrochemical properties, enhancing the sensitivity and accuracy of the sensor compared to conventional sensors. The reagent formulation used in the chambers of the sensor includes specific compositions tailored for the analysis of direct and total bilirubin. The presence of solubilizing agents and hydrophilic polymers ensures efficient detection and quantification of both forms of bilirubin.

The fabrication process involves the use of a miniaturized design, allowing for a compact and portable sensor. The use of screen-printed carbon electrodes and PET sheets as substrates contributes to the cost-effectiveness and ease of manufacturing the sensor.

The integration of both direct and total bilirubin detection capabilities into a single sensor of the present invention eliminates the need for separate devices or tests for each form of bilirubin. This consolidation reduces the overall cost of diagnostic procedures, as healthcare providers and laboratories can achieve accurate results using a single, cost-effective sensor instead of multiple devices.

The present invention provides the simultaneous detection of direct and total bilirubin on a single strip, coupled with the real-time analysis capability, improves the efficiency of the diagnostic process. Healthcare professionals can obtain comprehensive bilirubin measurements quickly, leading to reduced waiting times for patients and increased patient throughput. This time-saving aspect can translate into cost savings for healthcare facilities and improve overall operational efficiency.

The present invention provides the miniaturized and portable design of the sensor that allows for point-of-care testing, enabling bilirubin analysis at the patient's bedside or in non-laboratory settings. This eliminates the need to send samples to centralized labs for testing, reducing transportation costs and turnaround time. Point-of-care testing can be particularly beneficial in resource-limited or remote areas, where access to laboratory facilities may be limited.

The technical advancements in the present invention, such as the enhanced sensitivity of the nanostack and the precise five-electrode configuration, contribute to improved accuracy and reliability of bilirubin measurements. Accurate and reliable diagnostic results help in avoiding misdiagnosis or unnecessary treatments, leading to cost savings in terms of healthcare expenditures.

The availability of a rapid and reliable bilirubin detection method of the present invention can facilitate better patient management and treatment decisions. Physicians can monitor bilirubin levels more efficiently, enabling timely interventions and adjustments in treatment plans. This can potentially reduce the duration of hospital stays, minimize the risk of complications, and optimize healthcare resource utilization.

The present invention provides a high level of selectivity for bilirubin detection, as indicated by the minimal interference from common interfering compounds such as glucose, ascorbic acid, dopamine, and uric acid. This characteristic ensures accurate and reliable measurements even in the presence of potential interfering substances.

In another aspect of this invention, the fabricated device can be directly used for the simultaneous analysis of both forms of bilirubin in un-diluted human blood, plasma and serum samples. The conventional spectrophotometric approach cannot measure bilirubin directly in the blood, and always requires serum as the sample for bilirubin determination. In laboratory environment, initially serum is extracted from the blood samples, and which is then mixed/diluted with various chemical reagents and incubated for different time intervals that cause a spectrophotometric color change which is monitored and corrected for the dilution factor to determine the bilirubin

concentration. The average analysis time is 30 min starting from the serum separation from blood samples. Further the analyzer may require up to 3 ml of blood per assay. However, the proposed device provides a reagent-less analytical approach for quantitative analysis of bilirubin in blood/plasma/serum samples. Moreover, a response time of less than one minute and sample requirement of 3-5 µl offers many advantages.

Therefore, the present invention provides the development of a user-friendly portable sensor for measuring direct and total bilirubin levels in blood/serum samples. The developed sensor needs only a drop of around 3-5 microliter of sample and provides the concentration of each fraction of bilirubin within a minute. This device would be highly applicable in the hospitals, nursing centers, and pathology labs by providing a safe and convenient strip-based system for rapid analysis of bilirubin. The present invention discloses the fabrication of a single strip electrochemical sensor which can detect all forms of bilirubin in a single drop of blood and provides its concentration within a minute.

While specific language has been used to describe the invention, any limitations arising on account of the same are not intended. As would be apparent to a person skilled in the art, various working modifications may be made to the method in order to implement the inventive concept as taught herein.

The figures and the foregoing description give examples of embodiments. Those skilled in the art will appreciate that one or more of the described elements may well be combined into a single functional element. Alternatively, certain elements may be split into multiple functional elements. Elements from one embodiment may be added to another embodiment. For example, order of processes described herein may be changed and are not limited to the manner described herein. Moreover, the actions of any flow diagram need not be implemented in the order shown; nor do all of the acts need to be necessarily performed. Also, those acts that are not dependent on other acts may be performed in parallel with the other acts. The scope of embodiments is by no means limited by these specific examples.

WE CLAIM:

1. An electrochemical sensor (200) for detection of bilirubin, comprising:

a substrate having at least five electrodes configuration screen printed on a surface of the substrate,

wherein the at least five electrodes configuration comprises two working electrodes (201), two counter electrodes (202) and a common reference electrode (203),

wherein the two working electrodes (201) fabricated with gold-based trimetallic nanocomposites,

wherein the gold-based trimetallic nanocomposites are selected from a group consisting of Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni;

a spacing layer (206) having at least two carves affixed on the at least five electrodes configuration on the surface of the substrate, wherein the spacing layer (206) is configured to create at least two isolated chambers on the surface of the substrate,

wherein the at least two isolated chambers comprise a first isolated chamber configured to carry out detection of direct bilirubin and a second isolated chamber configured to carry out detection of total bilirubin;

a covering layer (208) affixed onto a top surface of the spacing layer, wherein the covering layer (208) comprises a hydrophilic coating and a non-hydrophilic coating,

wherein the hydrophilic coating is affixed onto the top surface of spacing layer (206) and the non-hydrophilic coating is configured to function as an outer layer, and

wherein the electrochemical sensor (200) is configured to simultaneously detect the direct bilirubin and the total bilirubin in a given sample.

2. The electrochemical sensor as claimed in claim 1, wherein the substrate comprises a polyethylene terephthalate (PET) sheet.

3. The electrochemical sensor as claimed in claim 1, wherein the first isolated chamber is dry-casted with a first reagent comprising a hydrophilic polymer, and a phosphate buffer solution for the detection of the direct bilirubin.

4. The electrochemical sensor as claimed in claim 1, wherein the second isolated chamber is dry-casted with a second reagent comprising the hydrophilic polymer, 0.1 to 5.0% solubilizing agent prepared in one of deionized (DI) water and phosphate buffer solution for the detection of the total bilirubin.

5. The electrochemical sensor as claimed in claim 3 and 4, the hydrophilic polymer is selected from one of carboxy methyl cellulose, PVP, hydroxy ethyl cellulose, and combination thereof.

6. The electrochemical sensor as claimed in claim 4, wherein the solubilizing agent is selected from one of Tergitol, Pluronic F-127, and combination thereof.

7. The electrochemical sensor as claimed in claim 1, wherein the outer layer of the covering layer (208) is configured to provide a wicking mechanism to the electrochemical sensor wherein a drop of sample applied is drawn into the first isolated chamber and the second isolated chamber through a capillary action.

8. The electrochemical sensor as claimed in claim 1, wherein the covering layer (208) comprises a small vent opening configured to ensure escaping of air from the first isolated chamber and the second isolated chamber as sample enters.

9. A process for fabrication of an electrochemical sensor for detection of bilirubin, comprising:

screen printing at least five electrodes configuration on a surface of a substrate using carbon ink,

wherein the at least five electrodes configuration comprises two working electrodes (201), two counter electrodes (202) and a common reference electrode (203),

wherein the two working electrodes (201) are modified with gold-based trimetallic nanocomposites;

drying the screen printed substate at 180 to 200° C in an oven for a duration of 45 to 60 minutes;

attaching a spacing layer (206) having at least two carves on the dried screen printed substate to obtain at least two chambers on the dried screen-printed substrate,

wherein the least two chambers comprise a first chamber configured to carry out detection of direct bilirubin and a second isolated chamber configured to carry out detection of total bilirubin;

dry-casting the first chamber with a first reagent comprising a polymer-phosphate buffer solution for the detection of the direct bilirubin;

dry-casting the second chamber by adding 3.0-5.0 µL of a second reagent at 45-55 °C for 5-10 min,

wherein the second reagent comprising 0.1 to 5.0% solubilizing agent prepared in one of deionized (DI) water and phosphate buffer solution for the detection of the total bilirubin; and

attaching a covering layer (208) having a hydrophilic coating on one side and a non-hydrophilic coating on another side on the dry-casted spacing layer (206) to obtain the electrochemical sensor.

10. The process as claimed in claim 9, wherein the substrate comprises a polyethylene terephthalate (PET) sheet.

11. The process as claimed in claim 9, wherein the gold-based trimetallic nanocomposites are selected from a group consisting of Au-Ag-Cu, Au-Ag-Co, and Au-Ag-Ni.

12. The process as claimed in claim 9, wherein the covering layer (208) comprises a small vent opening configured to ensure escaping of air from the first isolated chamber and the second isolated chamber as sample enters.

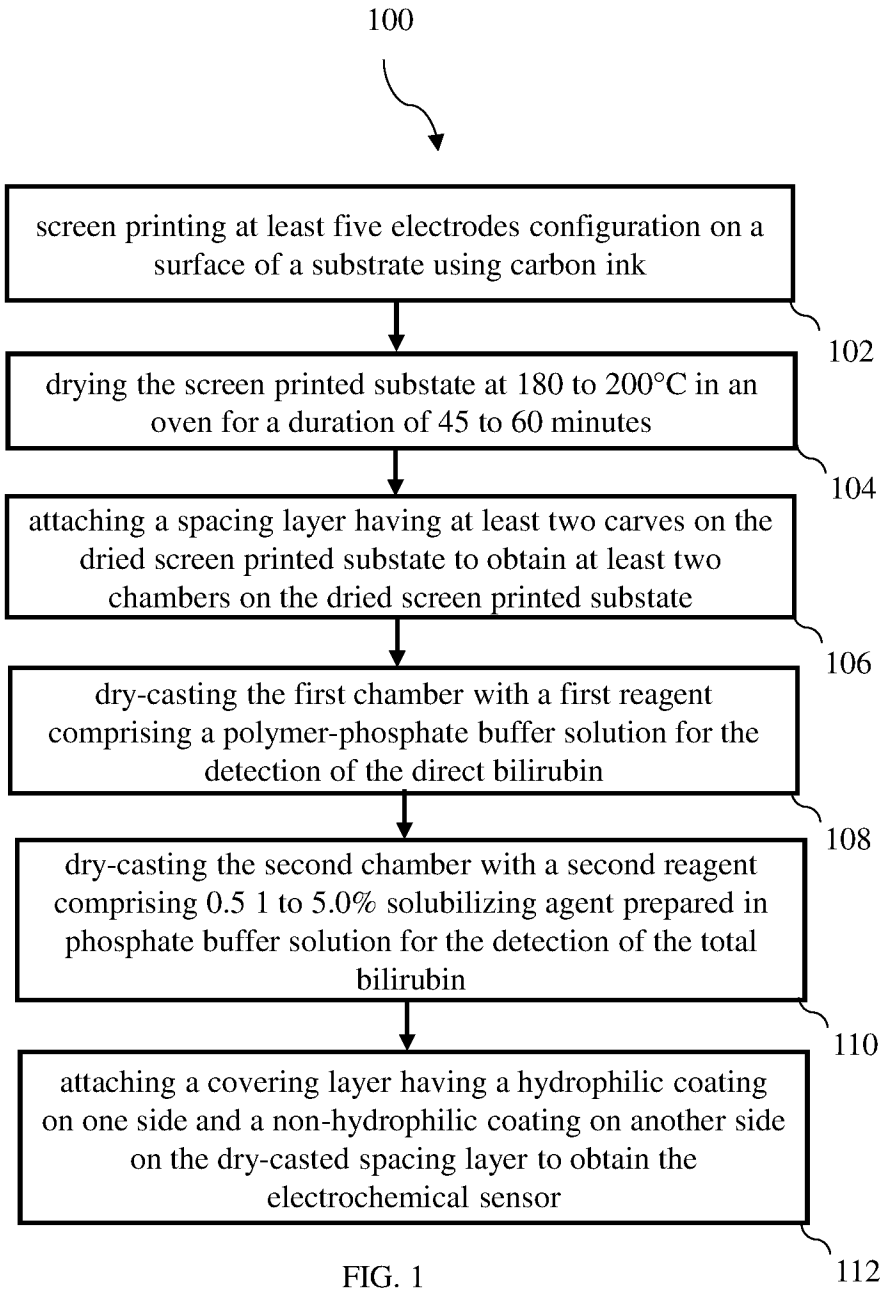

100
screen printing at least five electrodes configuration on a surface of a substrate using carbon ink
102
drying the screen printed substate at 180 to 200°C in an oven for a duration of 45 to 60 minutes
104
attaching a spacing layer having at least two carves on the dried screen printed substate to obtain at least two chambers on the dried screen printed substate
106
dry-casting the first chamber with a first reagent comprising a polymer-phosphate buffer solution for the detection of the direct bilirubin
108
dry-casting the second chamber with a second reagent comprising 0.5 1 to 5.0% solubilizing agent prepared in phosphate buffer solution for the detection of the total bilirubin
110
attaching a covering layer having a hydrophilic coating on one side and a non-hydrophilic coating on another side on the dry-casted spacing layer to obtain the electrochemical sensor
112

FIG. 1

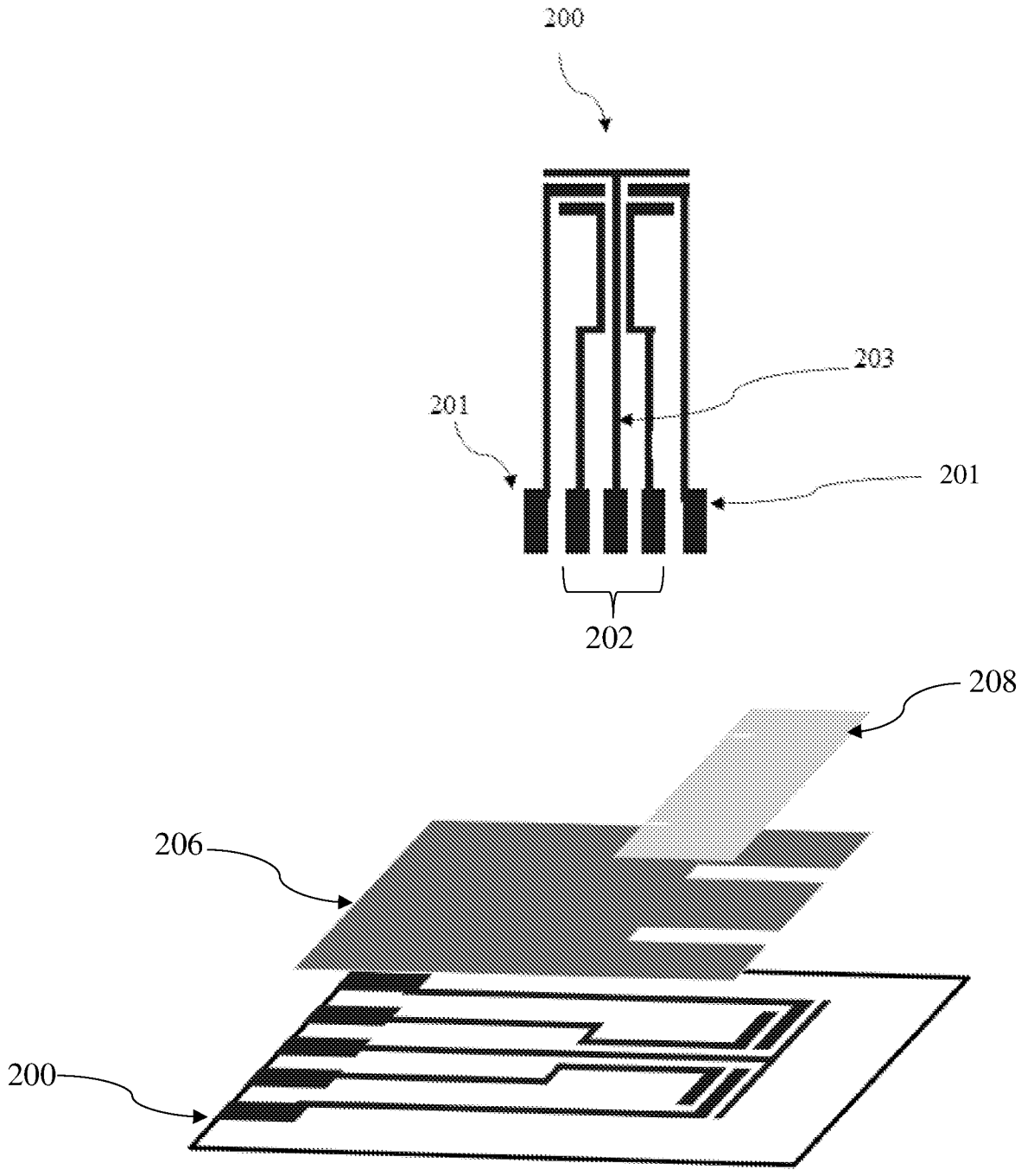


FIG. 2

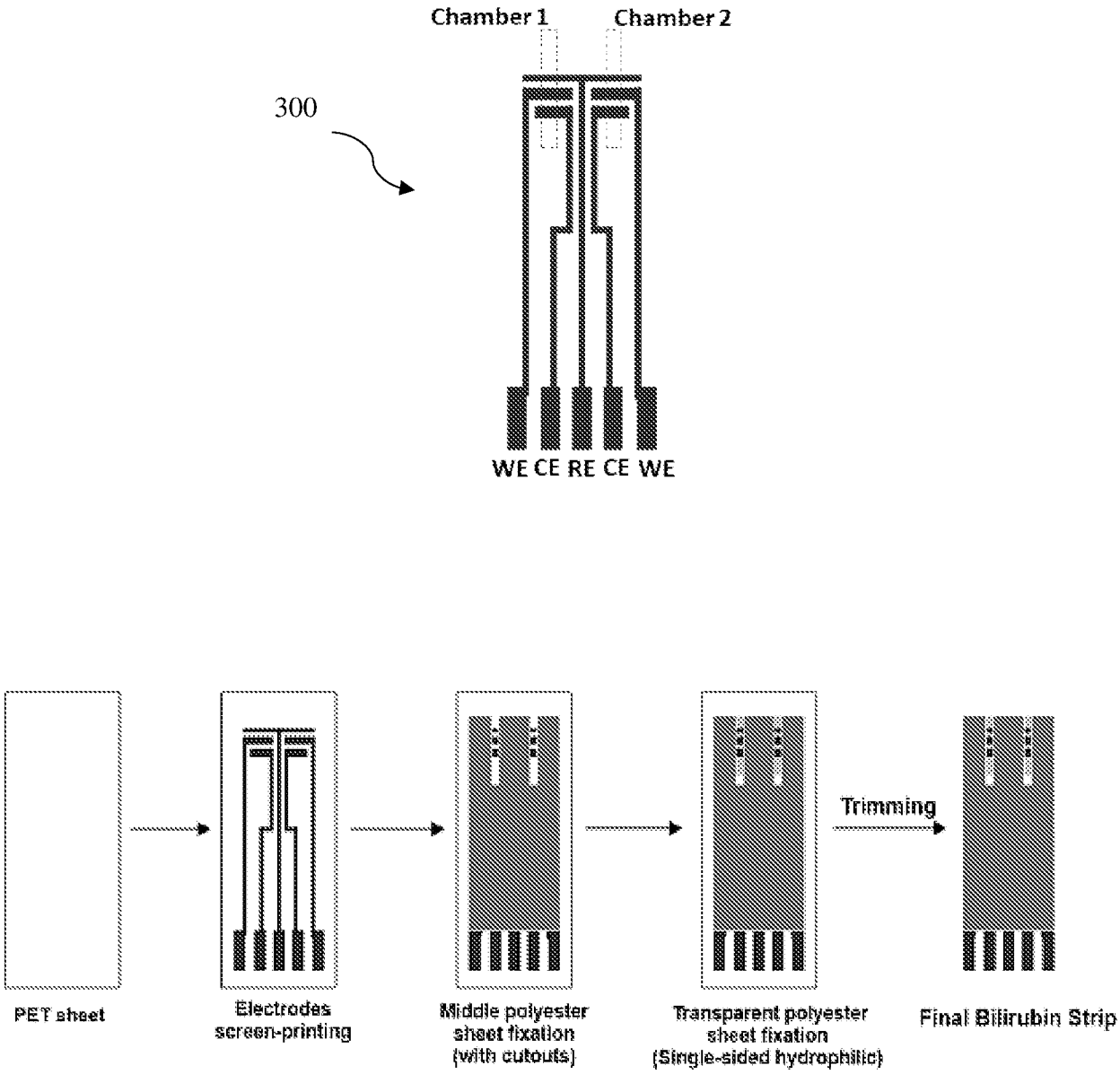
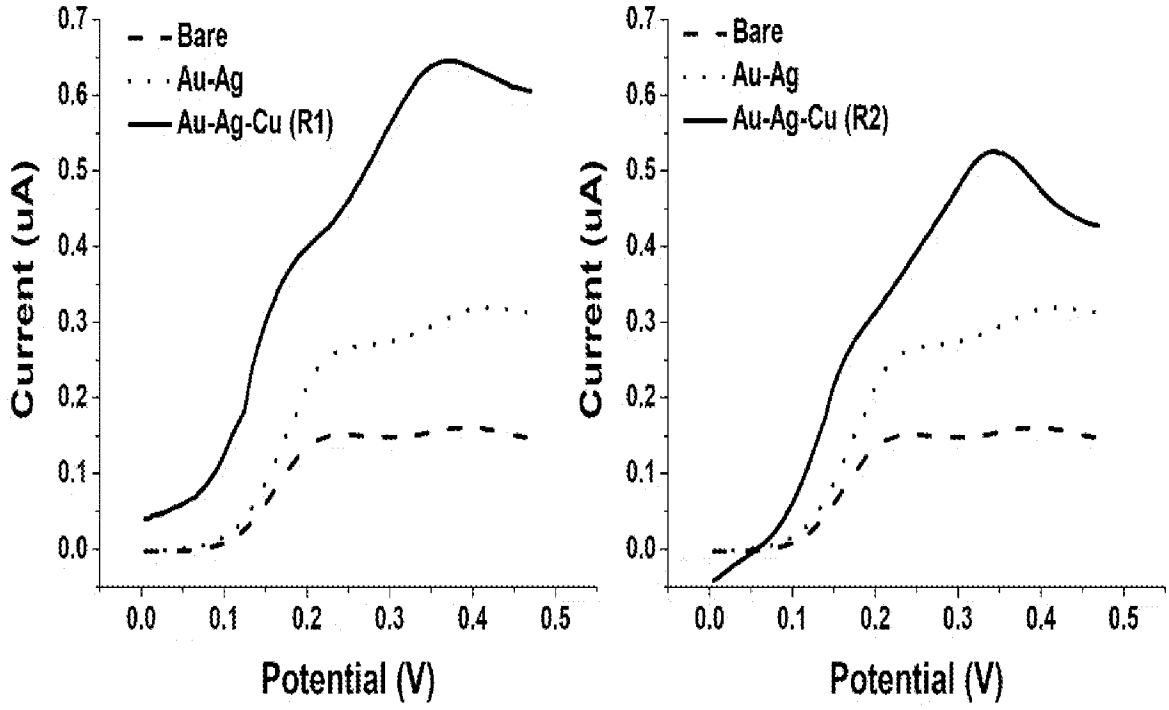

Chamber 1
Chamber 2
300
WE CE RE CE WE
PET sheet
Electrodes screen-printing
Middle polyester sheet fixation (with cutouts)
Transparent polyester sheet fixation (Single-sided hydrophilic)
Trimming
Final Bilirubin Strip

FIG. 3

400



4a 4b

FIG. 4

500

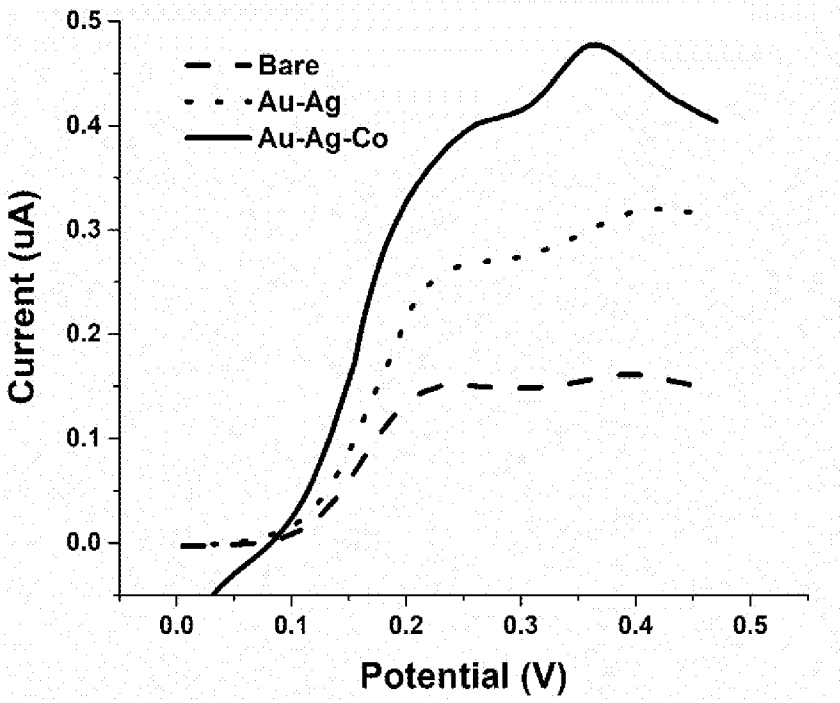


FIG. 5

600

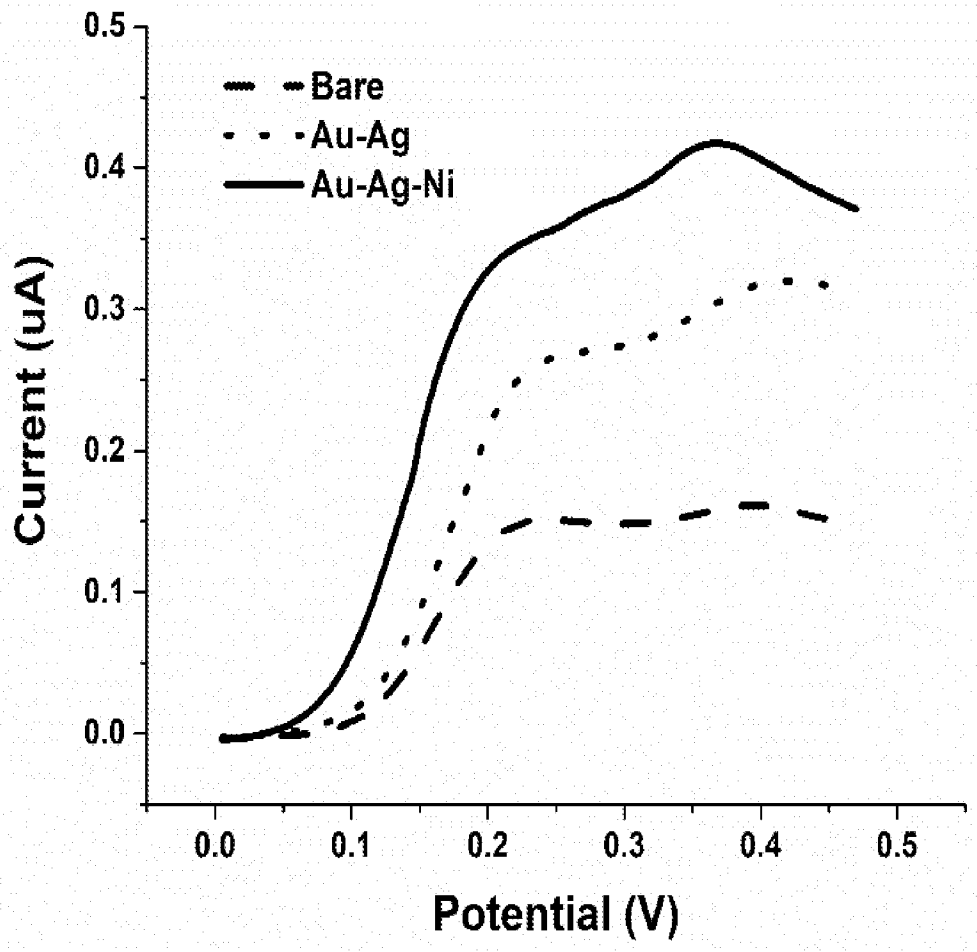


FIG. 6

700

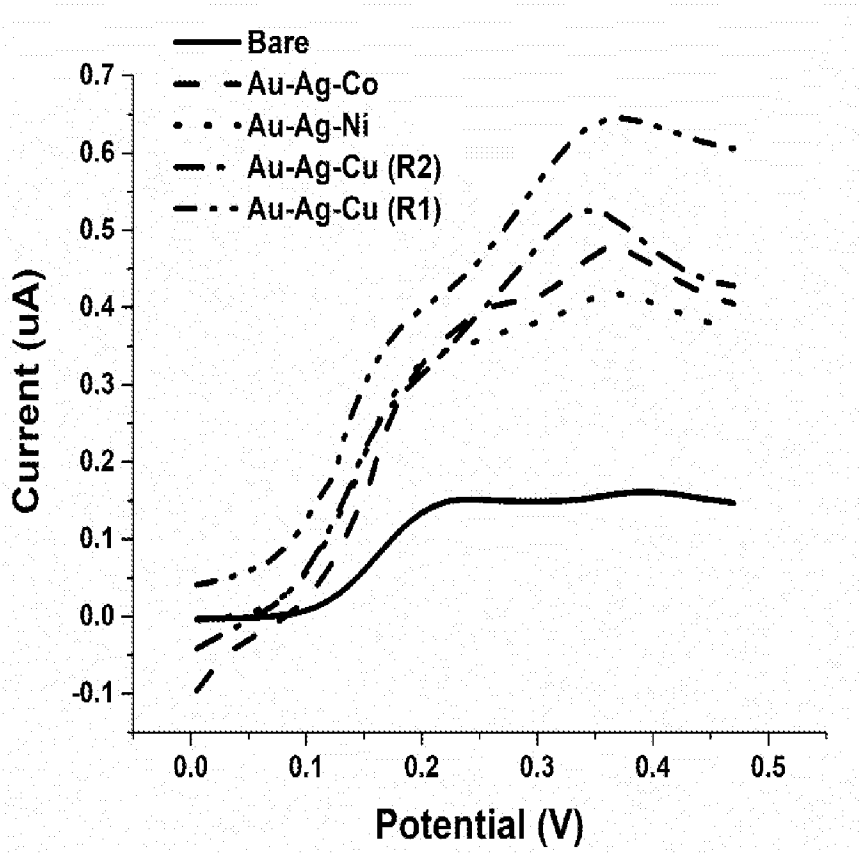

Bare
Au-Ag-Co
Au-Ag-Ni
Au-Ag-Cu (R2)
Au-Ag-Cu (R1)
Current (uA)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
Potential (V)

FIG. 7

800

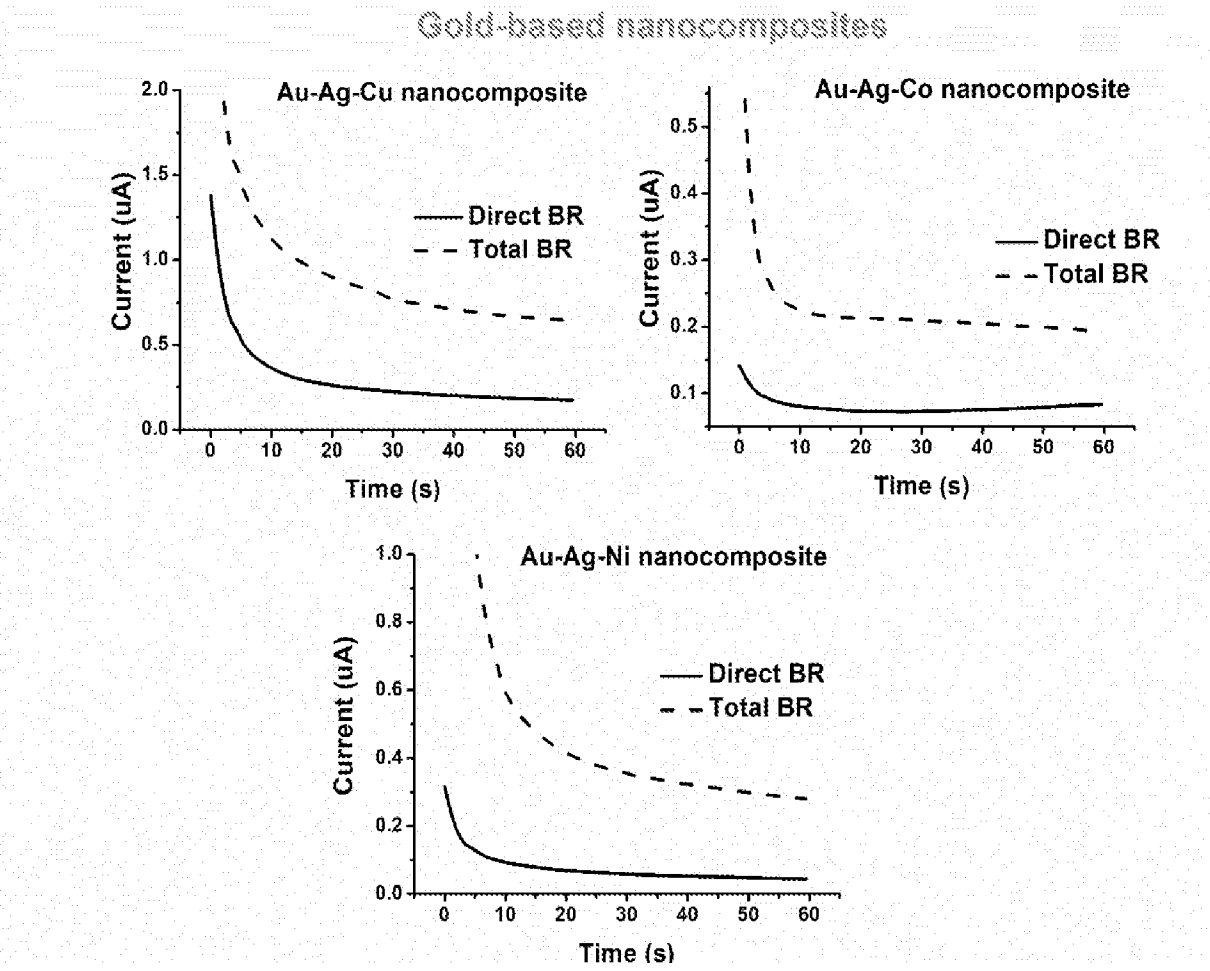

Gold-based nanocomposites
Au-Ag-Cu nanocomposite
Current (uA)
Time (s)
Direct BR
Total BR
Au-Ag-Co nanocomposite
Current (uA)
Time (s)
Direct BR
Total BR
Au-Ag-Ni nanocomposite
Current (uA)
Time (s)
Direct BR
Total BR

FIG. 8

900

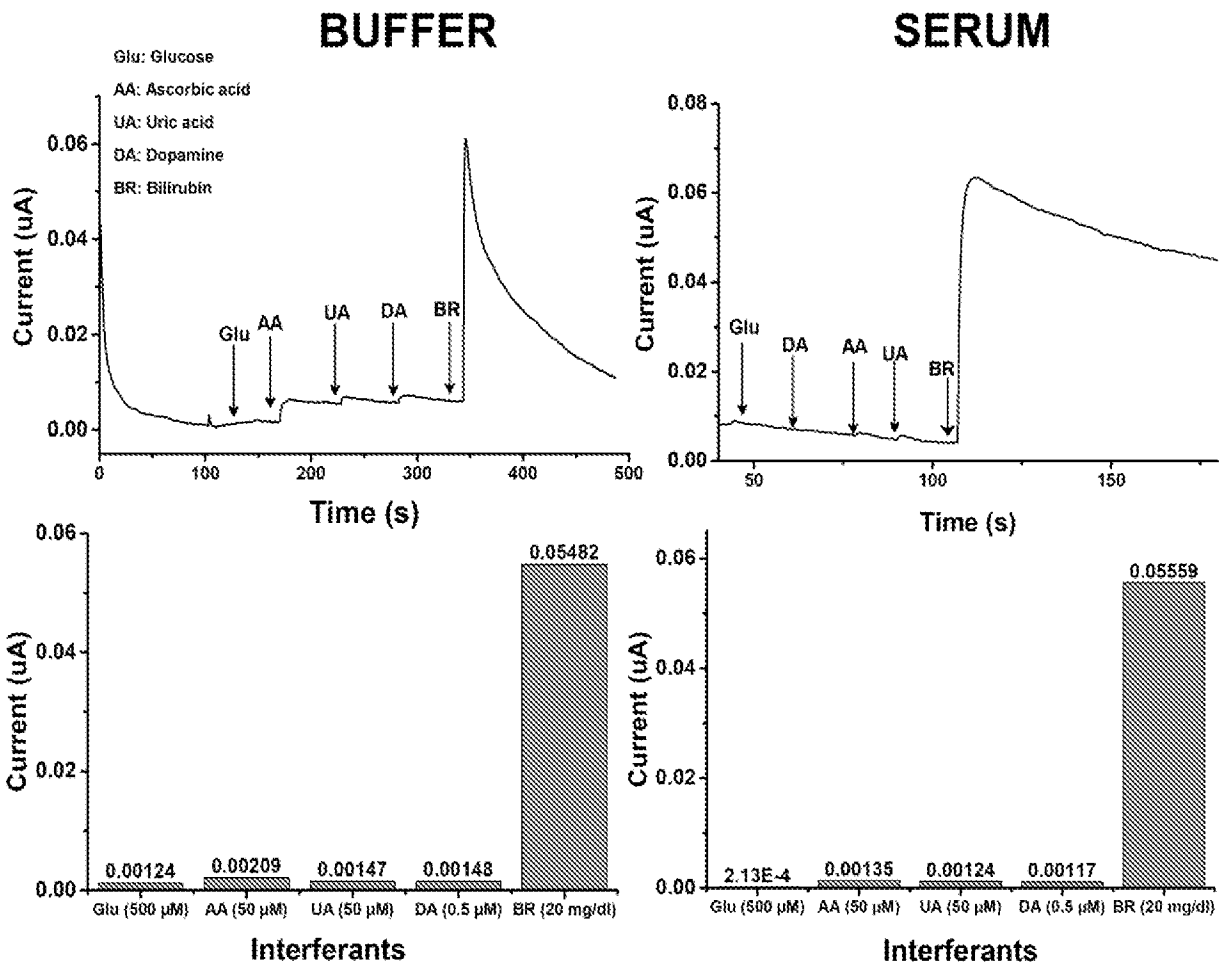

BUFFER
SERUM
Glu: Glucose
AA: Ascorbic acid
UA: Uric acid
DA: Dopamine
BR: Bilirubin
Current (uA)
Time (s)
Glu
AA
UA
DA
BR
0.00124
0.00209
0.00147
0.00148
0.05482
2.13E-4
0.00135
0.00124
0.00117
0.05559
Glu (500 µM)
AA (50 µM)
UA (50 µM)
DA (0.5 µM)
BR (20 mg/dl)
Interferants

FIG. 9