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(54) **Title:** AN ELECTROCHEMICAL BIOSENSOR

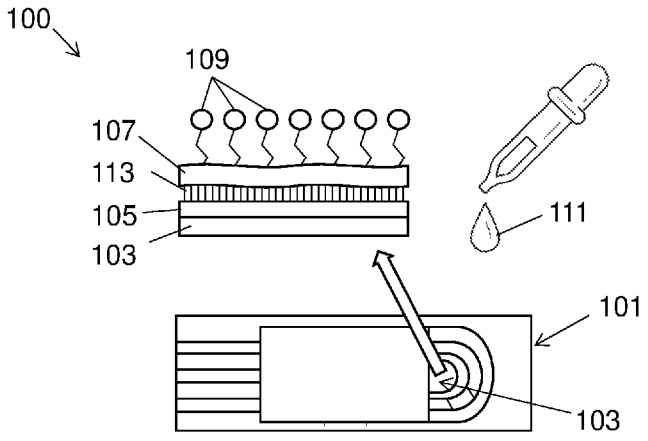


Figure 1

(57) **Abstract:** An electrochemical biosensor is provided and comprises a screen printed electrode which includes a working electrode modified with a layer of gold nanoparticles and a layer of a zeolitic imidazolate framework, the zeolitic imidazolate framework having a selected bioreceptor immobilised thereon for the detection of a target biomolecule in use. The electrochemical biosensor is suitable for incorporation in a point of care device for singe step, sensitive and selective detection of a target biomolecule, such as the SARS-CoV-2 virus. Methods of preparing the electrochemical sensor and methods of detecting a target biomolecule with the electrochemical biosensor connected in a measuring system are also provided.

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# AN ELECTROCHEMICAL BIOSENSOR

## CROSS-REFERENCE(S) TO RELATED APPLICATIONS

This application claims priority from South African provisional patent application number 2020/04795 filed on 3 September 2020, which is incorporated by reference herein.

## FIELD OF THE INVENTION

The invention relates to an electrochemical biosensor. In particular, it relates to an electrochemical biosensor that includes a surface-modified working electrode, methods for preparing the electrochemical biosensor and methods and devices for detecting a target biomolecule such as the SARS-CoV-2 virus with the electrochemical biosensor.

## BACKGROUND TO THE INVENTION

The threat to global health from COVID-19 has brought into sharp focus the need for rapid, sensitive and selective detection of viruses. A particular challenge is to identify infected, but asymptomatic individuals and to measure the extent of live virus shedding so as to enable isolation to end at the earliest safe time. The speed of this testing process could be significantly increased using Point Of Care (POC) devices that would allow testing to be carried out in the community. The development of POC devices for high performance viral and antibody detection is a challenging goal. For example, sample collection should be minimally invasive, e.g., nose/throat swab or saliva, rather than blood. The concentration of virus present in such samples can be very low especially early in the disease cycle when testing is most effective at preventing community transmission, placing extreme demands on analytical sensitivity. The “sample to answer” time needs to be short. Decreasing the limit of detection allows infections to be detected earlier and ensures that individuals are not infectious before returning to normal life.

Real time reverse Transcription PCR (rRT-PCR) is currently the gold standard of testing. This method has high sensitivity but is not suitable for large scale screening for multiple samples because of its high cost and long analysis time. It also needs skilled personal to perform and is not suitable for point-of-care testing.

Given the speed, portability, sensitivity and selectivity achieved using electrochemical detection, these sensor systems hold the promise of transformative change in clinical practice. For electrochemical sensors, the choice of electrode material is important since it can influence many aspects of the performance of electrochemical sensors. These include the double layer capacitance (influences limit of detection), the rate of heterogeneous electron transfer (influences response time, sensitivity) and the nature of the coupling chemistry required to immobilize the bioreceptors.

Accordingly, there is a need for an electrochemical biosensor with a modified working electrode that is capable of rapidly detecting a target biomolecule, in particular antigens linked to infection, with improved sensitivity.

The preceding discussion of the background to the invention is intended only to facilitate an understanding of the present invention. It should be appreciated that the discussion is not an acknowledgment or admission that any of the material referred to was part of the common general knowledge in the art as at the priority date of the application.

## SUMMARY OF THE INVENTION

In accordance with an aspect of the invention there is provided an electrochemical biosensor comprising a screen printed electrode which includes a working electrode modified with a layer of gold nanoparticles and a layer of a zeolitic imidazolate framework provided on the layer of gold nanoparticles, the layer of zeolitic imidazolate framework having a selected bioreceptor immobilised thereon for the detection of a target biomolecule in use.

The zeolitic imidazolate framework may be ZIF-8. The gold nanoparticles may have a size ranging between 5 and 20 nm, preferably 5 nm. A crosslinker may bind the zeolitic imidazolate framework to the gold nanoparticles. The gold nanoparticles may be coated with cysteamine and the crosslinker may bind the zeolitic imidazolate framework to the cysteamine coating. The crosslinker may be *bis*(sulfosuccinimidyl)suberate (BS3).

The screen printed electrode may be a screen printed carbon electrode.

The bioreceptor may be one or more antibodies configured to detect one or more target antigens. The one or more antibodies may be a SARS-CoV-2 protein antibody or a combination of SARS-CoV-2 protein antibodies configured to detect the SARS-CoV-2 virus. The SARS-CoV-2 protein antibody may be a spike protein antibody, a nucleocapsid protein antibody or a combination of

both the spike protein antibody and the nucleocapsid protein antibody.

In accordance with a second aspect of the invention, there is provided a method for preparing an electrochemical biosensor, the method comprising the steps of:

sequentially forming a layer of gold nanoparticles and a layer of zeolitic imidazolate framework on a working electrode of a screen printed electrode; and

immobilising a selected bioreceptor onto the zeolitic imidazolate framework.

The method may include the further step of modifying the surface of the gold nanoparticles so that the gold nanoparticles are configured to bind the zeolitic imidazolate framework. The surface modification of the gold nanoparticles may include coating the surface of the gold nanoparticles with cysteamine prior to depositing the gold nanoparticles onto the working electrode and depositing a solution of a crosslinker on the working electrode after the solution of cysteamine-coated gold nanoparticles has been deposited thereon. The crosslinker may be *bis*(sulfosuccinimidyl)suberate (BS3).

The step of immobilising the selected bioreceptor onto the zeolitic imidazolate framework may include depositing a solution of the bioreceptor on the surface-modified working electrode and allowing it to dry for a selected time period. The selected time period may be about 3 hours at room temperature.

The method may include the further step of incubating the working electrode in a blocking solution for a selected time period to block any unreacted bioreceptor from the bioreceptor immobilisation step. The blocking solution may be a solution of bovine serum albumin (BSA).

The bioreceptor may be an antibody or a combination of antibodies, and when the antibody is a combination of antibodies, the constituent antibodies of the combination may be immobilised sequentially or simultaneously onto the zeolitic imidazolate framework. The bioreceptor may be a SARS-CoV-2 protein antibody or a combination of two or more different SARS-CoV-2 protein antibodies configured to detect the SARS-CoV-2 virus.

In accordance with a third aspect of the invention, there is provided a method of detecting a target biomolecule, the method being carried out using the electrochemical biosensor as described above connected in a measuring system and comprising the steps of:

mixing a sample to be tested with a solution of a redox probe;

adding the mixture of the sample and the redox probe solution onto the working electrode of the screen printed electrode;

applying a voltage to the screen printed electrode; and

measuring a current flowing in the measuring system to detect changes in current which signify binding of the target biomolecule to the bioreceptor.

Differential pulse voltammetry or square wave voltammetry may be used to measure and record changes in peak current after addition of the mixture of the sample and redox probe onto the working electrode. The changes in peak current may be processed to determine a concentration of the target biomolecule detected.

The bioreceptor may be a SARS-CoV-2 protein antibody or combination of SARS-CoV-2 protein antibodies and the target biomolecule may be SARS-CoV-2 virus. The sample to be tested may be a saliva or nasopharyngeal sample. The redox probe may be a buffered solution of a 1:1 mixture of ferrocyanide/ferricyanide.

In accordance with a fourth aspect of the invention, there is provided a point of care device for detecting a target biomolecule comprising an electrochemical sensor as described above connected in a measuring system which includes a voltage source configured to apply a voltage between the working electrode and a counter electrode of the screen printed electrode and a potentiostat configured to control electric parameters and measure current flowing between the working electrode and counter electrode of the screen printed electrode, wherein a current at or above a predetermined threshold current signifies binding of the target biomolecule to the bioreceptor.

A processing module may be configured to monitor the current measured with the potentiostat to compare the current measured with a predetermined threshold value or predetermined reference values to detect binding of the target biomolecule and/or quantify a level of target biomolecule in the sample.

Embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings.

**BRIEF DESCRIPTION OF THE DRAWINGS**

In the drawings:

Figure 1 is a schematic illustration of an embodiment of an electrochemical biosensor;

Figure 2 is a flow diagram that illustrates a method for preparing an electrochemical

biosensor;

Figure 3 is a flow diagram that illustrates a method of detecting a target biomolecule with an electrochemical biosensor;

Figure 4 is a graph showing current measurements obtained using Differential Pulse Voltammetry (DPV) carried out on an electrochemical sensor having SARS-CoV-2 spike protein antibody immobilised thereon to detect SARS-CoV-2 virus and plotted against the CT values obtained using the gold standard rRT-PCR test;

Figure 5 is a graph showing current measurements obtained using Square Wave Voltammetry (SWV) with an electrochemical sensor having a mixture of SARS-CoV-2 spike protein antibody and nucleocapsid protein antibody immobilised thereon to detect SARS-CoV-2 virus and plotted against the CT values obtained using the gold standard rRT-PCR test;

Figure 6 is a graph showing current measurements obtained using SWV with an electrochemical sensor having both the SARS-CoV-2 spike protein antibody and nucleocapsid protein antibody immobilised thereon in separate and sequential immobilisation steps to detect SARS-CoV-2 virus and plotted against the CT values obtained using the gold standard rRT-PCR test; and

Figure 7 is a graph showing current measurements obtained using SWV with an electrochemical sensor having SARS-CoV-2 spike protein antibody immobilised thereon to detect SARS-CoV-2 virus and plotted against the CT values obtained using the gold standard rRT-PCR test.

## DETAILED DESCRIPTION WITH REFERENCE TO THE DRAWINGS

An electrochemical biosensor is provided which comprises a screen printed electrode having a working electrode modified with gold nanoparticles and a zeolitic imidazolate framework. A selected bioreceptor is immobilised on the surface-modified working electrode and is arranged for the detection of a target biomolecule in use. More particularly, the electrochemical biosensor (100) as illustrated in Figure 1 comprises a screen printed electrode (101) which includes a working electrode (103) modified with a layer of gold nanoparticles (105). A layer of zeolitic imidazolate framework (107) is provided on top of the layer of gold nanoparticles (105). A selected bioreceptor (109) is immobilised on the zeolitic imidazolate framework (107) for the detection of a target biomolecule in a sample (111) with the modified screen printed electrode in use and when connected in a suitably configured measuring system.

A screen printed electrode is an electrochemical measurement device with three different electrodes. A working electrode which is the electrode where the potential is controlled and where the current is measured. The working electrode serves as a surface on which the electrochemical reaction to be sensed takes place and the surface may be modified as may be required for a particular sensing application. A reference electrode is used to measure the working electrode potential. A counter or auxiliary electrode is a conductor that completes the circuit of the three-electrode cell and allows passage of current. It enables analysis of processes in which electronic transfer takes place. The screen printed electrode has a connection system arranged so that each of the three electrodes can be connected to an electronic instrument or measuring system, such as a potentiostat.

Screen printed electrodes are manufactured by printing different types of ink on plastic or ceramic substrates, allowing quick *in-situ* analysis with high reproducibility, sensitivity and accuracy. The composition of the different inks which may be used in the manufacture of the electrode (i.e. carbon, silver, gold, platinum) determines its selectivity and sensitivity. The screen printed electrode of the electrochemical biosensor may be a screen printed carbon electrode, such as the commercially available DropSens SPCE from Metrohm with the working and counter electrode material being carbon. The reference electrode material may then be silver. A carbon electrode surface of the working electrode offers a more porous platform for binding.

The gold nanoparticle layer, coating or cover that the working electrode is modified with may be formed using a suitable deposition method such as drop coating. Gold nanoparticles (AuNPs) are stable metal nanoparticles with unique optical, electronic, and catalytic activity, high biocompatibility, and an enhanced electron transfer rate. Depositing the gold nanoparticles on the surface of the working electrode of the electrochemical sensor increases its sensitivity. The gold nanoparticles may have a size ranging between 5 and 20 nm. The smaller gold nanoparticles within the range provide a larger surface area for further modification and immobilization, whereas the larger gold nanoparticles increase the conductivity of the electrode. It was found that a gold nanoparticle size of 5 nm provided a suitably large surface area for effective detection of target biomolecules, without compromising conductivity too much and thus optimizing the sensitivity of the electrochemical biosensing response of the screen printed electrodes.

Zeolitic imidazolate frameworks (ZIFs) are a class of crystalline metal-organic frameworks (MOFs) that are topologically isomorphic with zeolites. ZIFs are composed of tetrahedrally-coordinated transition metal ions (e.g. Fe, Co, Cu, Zn) connected by imidazolate linkers. ZIF's provide a support matrix or platform for the immobilization of the bioreceptor. ZIF's are porous, chemically stable, thermally stable, and have tunable pore size amongst other tunable properties.

ZIF's also have pore walls which may be modified with a wide range of functional groups because of the ready availability of substituted imidazole groups. The pores in a three-dimensional ZIF framework may also provide empty volume where hybridization of metallic nanoparticles (either naked, surface-modified or surfactant-stabilized) may occur.

The ZIF layer, coating or cover provided (i.e., grown or deposited) on the layer of gold nanoparticles of the modified screen printed electrodes serves as an immobilization platform for a selected bioreceptor. The ZIF layer provides a large surface area for immobilization of larger quantities of bioreceptor due to its surface roughness, thereby enhancing the electrode response and sensitivity of the electrochemical sensor. The ZIF layer is preferably a monolayer and bonding of a protein bioreceptor such as an antibody may occur via metal-S bonding to the metal sites on the upper surface of the ZIF monolayer. Instead of a random orientation of bioreceptors, the ZIFs interactions with the bioreceptors results in a favorable orientation of the immobilized bioreceptor on the ZIF. The ZIF also provides a stabilizing microenvironment (due to tight encapsulation) for the bioreceptor to protect it from denaturation and high temperatures, thus improving the stability and shelf-life of the electrochemical sensor.

In particular, the zeolitic imidazolate framework, ZIF-8 ($Zn_6$(2-methylimidazole)$_{12}$), may be used as an immobilization platform on the screen printed electrode of the electrochemical biosensor. ZIF-8 consists of zinc metal and 2-methylimidazole ligands coordinated in a hexagonal framework structure. Bioreceptors such as antibodies may be conjugated to the low-coordinated Zn sites located on the outer layer of ZIF-8 via Zn-S bonding, which allows one-step immobilization of antibodies with favorable orientations on ZIF-8. ZIF-8 has nanopores with a volume of about 2500 Å$^3$ a diameter of about 11.6 Å. A ZIF-8 monolayer may be formed on the gold nanoparticle layer of the modified working electrode and may have a thickness of about 5 µm.

A suitable crosslinker (113) may link or bind the zeolitic imidazolate framework to the gold nanoparticles, as illustrated in Figure 1. For example, the gold nanoparticles may be surface-modified or coated with cysteamine prior to deposition thereof on the electrode. Thereafter, the crosslinker may be deposited on the cysteamine-AuNPs. After incubating the cysteamine-AuNPs and crosslinker coated electrode in a suspension of the zeolitic imidazolate framework, the crosslinker binds the ZIF to the cysteamine coating on the AuNPs. The crosslinker may be glutaraldehyde or *bis*(sulfosuccinimidyl)suberate (BS3), but the softer linker, BS3, is preferred over glutaraldehyde to prevent denaturation of the bioreceptor and improve the stability and shelf-life of the electrochemical biosensor.

The bioreceptor may be one or more antibodies configured to detect one or more target antigens or one or more antigens configured to detect one or more target antibodies. Another alternative is for the bioreceptor to be one or more antigens and the target biomolecule to be the same or a similar antigen, but in the presence of a fixed concentration of antibody added to the working electrode in a competitive assay.

The one or more antibodies may be a SARS-CoV-2 protein antibody or a combination of SARS-CoV-2 protein antibodies configured to detect the SARS-CoV-2 virus. The SARS-CoV-2 protein antibody may be a spike protein antibody (recombinant spike protein antibody), a nucleocapsid protein antibody or a combination of both the spike protein antibody and the nucleocapsid protein antibody. When the antibody is the SARS-CoV-2 nucleocapsid protein antibody, the biosensor may be better able to detect variants of the SARS-CoV-2 virus as most variants known to date tend to vary in the structure of the spike protein.

A method for preparing an electrochemical biosensor is also provided and illustrated in Figure 2. The method comprises the steps of sequentially forming or depositing a layer of gold nanoparticles and a layer of zeolitic imidazolate framework on a working electrode of a screen printed electrode (201) and immobilising a selected bioreceptor onto the zeolitic imidazolate framework (203). Sequential immobilisation of the gold nanoparticle and zeolitic imidazolate framework layers on the electrode results in the immobilised bioreceptor being better orientated for binding target biomolecules and the sensor thus being more sensitive, in comparison to a configuration in which the gold nanoparticles are incorporated in the zeolitic imidazolate framework.

The screen printed electrode may be a screen printed carbon electrode. The working electrode may for example have a surface area of about 7 $mm^2$. The size and/or surface area of the working electrode may be varied to optimise the sensitivity of the biosensor and/or to miniaturise it as may be required for incorporation into a point of care device. Accordingly, the amounts of reagents used to modify the surface as specified herein below may be appropriate scaled.

Gold nanoparticles having a diameter between 5 and 20 nm, preferably 5 nm, may be used to form the gold nanoparticle layer.

The method may include the further step of modifying the surface of the gold nanoparticles (207) so that the gold nanoparticles are configured to bind the zeolitic imidazolate framework. The surface modification of the gold nanoparticles may include modifying or coating the surface of the gold nanoparticles with cysteamine prior to depositing the gold nanoparticles onto the working electrode. A 0.01 to 0.02 M solution of cysteamine, preferably a 0.02 M solution of cysteamine

may be used to form the cysteamine-gold nanoparticle composite. A select volume of the cysteamine-gold nanoparticle composite suspension may be drop coated on the working electrode and left to dry at room temperature for about 4 hours. 7 µL of cysteamine-gold nanoparticle composite suspension (1.54 mg/ml) is an appropriate amount for a working electrode with a 7 $mm^2$ surface area. An optional washing step may follow deposition of the gold nanoparticles, but it is preferred not to wash the electrode to avoid loss of gold nanoparticles. Instead, the volumes and types of solvents used to form the layer are optimised to try and alleviate the need for washing.

Following deposition of the cysteamine-gold nanoparticle composite on the working electrode, a solution of a crosslinker is deposited on the working electrode. The crosslinker may be any suitable crosslinker with functional groups arranged to covalently bind to the cysteamine-gold nanoparticle composite and to the ZIF. *Bis*(sulfosuccinimidyl)suberate (BS3) is an amine-to-amine crosslinker which binds to the amine groups on the surface of the cysteamine-gold nanoparticle composite and the amine groups on the ZIF. Deposition of a BS3 solution with a concentration of 6 mg/ml of was found to be optimal. 5 µL of the 6 mg/ml BS3 solution may be drop coated on a working electrode with a 7 $mm^2$ surface area. Thereafter the electrode may be washed with deionised water, preferably three times, and left to dry for about 3 hours at room temperature.

The layer of ZIF formed on the gold nanoparticles, and preferably crosslinked to the cysteamine modified gold nanoparticles, may be a ZIF-8 layer. 1 µL of 0.1 % ZIF-8 may be drop coated or otherwise deposited on a 7 $mm^2$ working electrode surface. With drop coating, the working electrode is left to dry for an hour at room temperature. The ZIF gives the electrode surface an overall positive charge due to the metal centres being in an ionised form ($Zn^{2+}$). Antibodies generally have a negative surface charge so a positive charge on the electrode surface helps to bind antibodies in a stable orientation.

Bioreceptors may be immobilised on the zeolitic imidazolate framework by adding a solution of the bioreceptor dropwise on the working electrode and allowing it to dry for a selected time period and at a selected temperature. The bioreceptor may be an antibody or a combination of antibodies, and when the antibody is a combination of antibodies, the constituent antibodies of the combination may be immobilised sequentially or simultaneously onto the zeolitic imidazolate framework. The antibodies may be selected to detect a particular disease or infection and may for example be SARS-Cov-2 protein antibodies. The bioreceptor may be a single type of SARS-CoV-2 protein antibody configured to detect the SARS-CoV-2 virus. For example, 5 µL of either a 10 µM SARS-CoV-2 spike protein antibody or a 10 µM SARS-CoV-2 nucleocapsid protein

antibody alone may be immobilised on the electrode surface and left to dry for three hours at room temperature. Alternatively, a combination of two or more different SARS-CoV-2 protein antibodies, such as the spike and nucleocapsid proteins may be used. Each of the different protein antibodies may be immobilised separately, i.e., 5 µL of 5 µM SARS-CoV-2 spike protein antibody may be immobilized on the electrode surface and dried for three hours at room temperature followed by immobilization of 5 µL of 5 µM SARS-CoV-2 nucleocapsid protein antibody for three hours at room temperature. The combination may also be immobilised simultaneously by forming a premix of the two different antibodies to obtain a 1:1 v/v 10 µM antibody mixture, for example, that may have been mixed for about 4 hours prior to immobilising 5 µL of the 10 µM premixture on the 7 $mm^2$ Au/ZIF modified working electrode, preferably the Au-Cys/BS3/ZIF-8 working electrode. The amount of antibody immobilised per unit surface has been optimised to ensure the immobilised antibody is suitably orientated to bind the target antigen on the virus, thereby yielding a better electrode response and improved sensitivity.

It is preferred not to wash the electrode after immobilisation of the bioreceptor on the ZIF, thus relatively small volumes of bioreceptor solution are used.

The method may include the further step of incubating the working electrode in a blocking solution (205), i.e., adding a blocking solution onto the working electrode and having the surface of the working electrode immersed therein, for a selected time period to block any unreacted bioreceptor by saturating excess protein binding sites on the working electrode after having immobilised bioreceptor onto the zeolitic imidazolate framework, thereby reducing any potential background. The blocking solution may be a solution of bovine serum albumin (BSA). 5 µL of a 1% BSA solution may be added to a 7 $mm^2$ working electrode, for example, and the electrode incubated in the solution for about three hours. At this stage of the method of manufacture or fabrication the screen printed electrode may be packaged and stored or incorporated in a point of care device with a measuring system configured to detect a target biomolecule and/or determine the concentration of the target biomolecule.

A redox probe solution may be prepared to be provided in a kit with the electrochemical biosensor or the point of care device. The redox probe solution may be prepared using a 1:1 ferrocyanide/ferricyanide mixture (5 mM) in a 1 M KCl or $KNO_3$ solution.

A method of detecting a target biomolecule is further provided and illustrated in Figure 3, the method being carried out using the electrochemical biosensor connected in a measuring system and comprising the steps of mixing a sample to be tested with a solution of a redox probe (301); adding the mixture of the sample and the redox probe solution onto the surface modified working

electrode of the screen printed electrode (SPE) (303); applying a voltage to the screen printed electrode (305); and measuring the current flowing in the measuring system (307) to detect changes in current which signify binding of the target biomolecule to the bioreceptor.

The bioreceptor may be a SARS-CoV-2 protein antibody or combination of SARS-CoV-2 protein antibodies and the target biomolecule may be SARS-CoV-2 virus. The sample to be tested may be a saliva or nasopharyngeal sample. The sample volume may be between 10 and 30 µL, but preferably about 10 µL as a smaller sample volume allows more redox probe solution to be added to the working electrode to enhance the conductivity. The redox probe may be a buffered solution of a 1:1 mixture of ferrocyanide/ferricyanide, such as 5 mM ferrocyanide/ferricyanide in 1 M KCl.

Upon binding of the target biomolecule to the bioreceptor, a change in current is measurable and occurs due to the extra coverage of the electrode surface with the bulky bioreceptor-biomolecule complex. The extra coverage hinders the access of the redox probe to the electrode surface and affects the electron transfer efficiency which either leads to an increase or decrease in current depending on the type of redox probe used and the charge on the electrode surface. In the event of a ferro/ferricyanide redox couple being used, the biorecognition binding event between the bioreceptor and biomolecule (i.e. the antibody and the virus) results in a decrease in current. The larger the decrease in peak current measured relative to a blank or control measurement with just the redox probe solution, the higher the concentration of the biomolecule (i.e. the higher the viral load) in the sample.

Differential pulse voltammetry or square wave voltammetry (SWV) may be used to detect and record changes in peak current after addition of the mixture of the sample and redox probe. The change in peak current recorded from the differential pulse voltammetry or square wave voltammetry may be processed to determine the concentration of the target biomolecule detected.

Differential pulse voltammetry (DPV) (also differential pulse polarography, DPP) is a voltammetry method used to make electrochemical measurements and a derivative of linear sweep voltammetry or staircase voltammetry, with a series of regular voltage pulses superimposed on the potential linear sweep or stairsteps. The current is measured immediately before each potential change, and the current difference is plotted as a function of potential. By sampling the current just before the potential is changed, the effect of the charging current can be decreased. Another type of pulse voltammetry is square wave voltammetry, which can be considered a special type of differential pulse voltammetry in which equal time is spent at the potential of the ramped baseline and potential of the superimposed pulse. The system of this measurement is usually the same as that of standard voltammetry. The potential between the working electrode

and the reference electrode is changed as a pulse from an initial potential to an interlevel potential and remains at the interlevel potential for about 5 to 100 milliseconds; then it changes to the final potential, which is different from the initial potential. The pulse is repeated, changing the final potential, and a constant difference is kept between the initial and the interlevel potential. The value of the current between the working electrode and auxiliary or counter electrode before and after the pulse are sampled and their differences are plotted versus potential. These measurements can be used to detect the presence of a target biomolecule with the use of a redox probe. The peak current measured is proportional to the concentration of the target biomolecule, i.e. in the case of the target biomolecule being SARS-CoV-2, the peak current is proportional to the viral load in the sample. The DPV scan rate may be optimized to reduce the time it takes to detect a target biomolecule. A result can be obtained within about 8 seconds with a scan rate of 0.1 V/s for example.

A point of care device for detecting a target biomolecule is provided comprising an electrochemical biosensor as described herein connected in a measuring system which includes at least a voltage source configured to apply a voltage between the working electrode and a counter electrode of the screen printed electrode and an electronic instrument such as a potentiostat configured to control electric parameters and measure the current between the working electrode and a counter electrode of the screen printed electrode, wherein an electrode response in the form of a reading of a current at or above a predetermined threshold current signifies binding of the target biomolecule to the bioreceptor.

The device components may be selected to carry out DPV or SWV and to measure a peak current during testing. The potentiostat measures the current allowing a comparison between the difference in the peak currents of a blank reading or response with just the redox probe solution and the reading or response after sample and redox probe solution are added onto the working electrode.

The point of care device or system may have its own processing module or be capable of communicating with a processing module of a mobile device. The processing module may have suitable software thereon to monitor the current flowing in the system to compare the electrode response (i.e., current measured) with a predetermined threshold value or one or more predetermined reference values to detect and/or quantify the level of target biomolecule in the sample. An output member may be in communication with the electrochemical biosensor / processing module and configured to output a result on a display member indicating a presence or a level of target biomolecule in the sample based on the electrode response (i.e. current

measured). The point of care device may be battery-operated, preferably by a rechargeable battery.

The measuring system may be a portable potentiostat configured to connect to the electrochemical sensor and carry out a selected voltammetry technique, preferably DPV or SWV, to obtain current measurements. The portable potentiostat may also be configured to connect to a mobile device via a suitable connection, such as a USB port.

The electrochemical biosensor may be in the form of a single-use disposable sensor or test strip or a multiple-use sensor or test strip. In the latter case, the sensor may be integrally formed with a sample receiving surface of a point-of-care device, such as a handheld device, and may be capable of being successively used with multiple samples. The multiple-use sensor may have a glassy carbon electrode as the working electrode which has a surface more suited to cleaning or wiping followed by remodification or immobilization of the reagents used to prepare the electrochemical sensor for detection.

**Examples**

*Electrochemical biosensor preparation*

The following fabrication steps were carried on a DropSens SPCE from Metrohm to prepare a surface-modified SPCE suitable for detecting the SARS-CoV-2 virus in spiked saliva samples:

1. 7 µL of cysteamine-gold nanoparticle composite is drop coated on carbon electrode and left to dry at room temperature for 4 hours.
2. 5 µL of 6 mg/ml BS3 solution is then drop coated and left to dry for three hours at room temperature after washing it 3 times with DI water.
3. 1 µl of 0.1 % Zeolitic Imidazolate Framework-8 is immobilised on the electrode for an hour at room temperature.
4. 5 µL of 10 µM SARS-CoV-2 spike protein antibody is immobilized on the electrode surface for three hours at room temperature.
5. A blocking step follows with µµL of 1% BSA incubated for three hours on the electrode.
6. The redox probe is prepared using 5 mM of Ferrocyanide/Ferricyanide in 1M KCl solution.

*Detection analysis in saliva samples*

A blind study was done to determine the sensitivity of the electrochemical biosensor to the SARS-CoV-2 virus. A sample population of 20 was used for this study and the results from the electrochemical biosensor were compared with the results of a standard PCR test. 20 saliva samples were analysed for detection of the SARS-CoV-2 virus causing the Covid-19 disease.

Triplicate analysis was conducted for each sample with 10 µL sample required for each analysis.

1. Palmsens software was set up on Differential Pulse Voltammetry (DPV) with the following settings: E begin = -0.3 V; E end = +0.5 V; E step = 0.01 V; E pulse = 0.02 V; t pulse = 0.02 s; scan rate = 0.1 V/s.
2. The prepared electrode (SPCE/Au-Cys/BS3/ZIF-8/Ab/BSA) was inserted in the SPCE connector and 40 µL of the Ferro/Ferri redox probe (10 µL DI water: 30 µL Ferro/Ferri) was put on the working electrode and analysed on DPV. The resultant peak was taken as the blank reading with triplicate scans taken. The 40 µL of solution was removed from the electrode to prepare for the saliva sample.
3. A premix of 10 µL of saliva sample and 30 µL of redox probe was prepared in a 2 ml plastic tube. 40 µL of this premix was then taken and put on the electrode. DPV scans were done three times for each sample on the same electrode to obtain the current reading. To analyse reproducibility, 3 premixes were analysed on different electrodes and DPV scans were taken.

The results of the current measurements with the electrochemical biosensor are plotted against the CT values obtained from a PCR test carried out on the same sample in Figure 4. The CT values are inversely proportional to the viral load measured using the PCR test. PCR CT values between 5 and 40 are considered a positive test result for Covid-19. The results showed a good linearity with a regression coefficient of 0.9663.

*Validation against PCR nasopharyngeal swabs*

This validation was undertaken to compare the electrochemical biosensor performance for different antibody combinations with the gold standard PCR technique. Square wave voltammetry was used for this analysis with sample volume in ferrocyanide at 50% vol. Square wave voltammetry measurements can be between 0 and 20 mA and thus potentially provide more data points.

1. S-N Premix at 10 µM

A combination of the SARS-CoV-2 spike protein antibody (S) and nucleocapsid protein antibody (N) was immobilised on the ZIF-8 framework of the prepared electrode (SPCE/Au-Cys/BS3/ZIF-8/Ab/BSA). The antibody mixture was prepared by mixing 10 µM of S and N antibody at the same volume ratio for 2 hours. The results of the current measurements with the electrochemical biosensor are plotted against the CT values obtained using the PCR technique in Figure 5. The S-N premix showed a 90% sensitivity. A reduction in sensitivity was observed in the region of the higher CT values. To improve sensitivity in the region with lower viral load, the volume of sample

analysed may be increased.

2. S-N Sequential at 5 µM

A combination of the SARS-CoV-2 spike protein antibody (S) and nucleocapsid protein antibody (N) was sequentially immobilised on the ZIF-8 framework of the prepared electrode (SPCE/Au-Cys/BS3/ZIF-8/Ab/BSA). In this case, the S antibody was first immobilized on the electrode and allowed to conjugate or bond for an hour. Afterward, the N antibody was immobilized on the electrode and left to conjugate or bond with the S antibody on the electrode surface. The results of the current measurements with the electrochemical biosensor (Ab = S-N sequential) are plotted against the CT values obtained using the PCR technique in Figure 6. The S-N sequential showed a 96% sensitivity. A reduction in sensitivity was also observed in the region of the higher CT values. However, the data points became expansive as CT values decreased. This caused the sensor to have a higher linear dynamic range of up to 20 data points. This was possibly due to the N antibody. A higher sample load could be introduced, or a lower concentration of S and N could be tried to optimise sensitivity at lower viral loads.

3. S alone at 10 µM

The SARS-CoV-2 spike protein antibody (S) alone was immobilised on the ZIF-8 framework of the prepared electrode (SPCE/Au-Cys/BS3/ZIF-8/Ab/BSA). The S antibody was immobilised at 10 µM. The results of the current measurements with the electrochemical biosensor (Ab = S) are plotted against the CT values obtained using the PCR technique in Figure 7. The S alone showed a 99% sensitivity. The S antibody alone had better linearity with better binding. This could have been due to a less bulk density on the electrode surface giving a less resistive route for electron transfer. The linear dynamic range is only up to 15 data points and could be increased by varying the concentrations and volume used.

The electrochemical biosensor enables single step, sensitive and selective detection of a target biomolecule, such as the SARS-CoV-2 virus. It may provide for a low-cost, high sensitivity, miniaturized and on-site high throughput screening of multiple samples.

The electrochemical biosensor is preferably used as an immunosensor capable of directly measuring the presence of the SARS-CoV-2 virus in a bodily fluid sample obtained in a minimally invasive manner.

The manner in which the electrode surface is modified or fabricated provides a robust, stable

arrangement or configuration and it was found that with gold nanoparticles and ZIF-8 being fabricated in successive layers on the electrode surface, the antibodies are immobilised with a significantly improved orientation that favours antigen / virus bonding and thereby improve the sensitivity of the sensor. Optimised concentrations of reagents are used for immobilising the various layers and the bioreceptor on the screen printed electrode and in relation to the size of the electrode surface to be modified. This avoids saturation of the immobilised antibody at clinically relevant antigen/viral levels in a sample.

The electrochemical sensor preferably works on the principal of a direct assay, but it will be apparent to those skilled in the art that a competitive assay may also be carried out with the electrochemical biosensor. A biosensor working on the principle of a direct assay provides faster results and a detection method with the sensor will be less complicated and involve less steps when carried out in practice.

In the case of a competitive assay being carried out, recombinant spike protein and/or nucleoprotein may be used as the bioreceptor or biomarker for SARS-CoV-2. The biosensor may be based on indirect competition between free virus in a nasal/saliva sample and immobilized SARS–CoV-2 protein for a fixed concentration of antibody added to the sample.

The electrochemical biosensor or immunosensor may comprise an arrangement allowing a competitive assay to be carried out on a screen printed carbon electrode or glassy carbon electrode modified with zeolitic imidazolate framework and gold nanoparticles. Differential Pulse Voltammetry (DPV) and Square Wave Voltammetry (SWV) and a suitable measurement system able to carry out these techniques may be used to detect the changes in the peak current after addition of different concentrations a sample solution to be tested or assayed. The biosensor fabrication may be done by drop coating glassy carbon electrode or screen printed carbon electrode with CTAB capped gold nanorods and ZIF-8 composite (CTABAu-ZIF-8) forming a transducer followed by immobilization of the SARS–CoV-2 protein using a covalent attachment method with glutaraldehyde (GA) as a cross linker.

The sample analyzed may be a spiked sample for testing and calibration or nasal or saliva samples obtained from subjects to be tested. The target analyte, i.e., the free virus may interact with the antibody binding sites via electrostatic forces.

All the fabrication steps may be optimized for biosensor components, immobilization technique (drop coating and immersion), concentration and incubation time of linker and bioreceptor, as well as the synthesis of the CTABAu-ZIF-8 transducer.

The principle of detection in this SARS-CoV-2 virus detection method is an indirect competition between the sample's free virus and the immobilized SARS-CoV-2 protein for a fixed concentration of added antibody to the sample. A reduction in peak current for a ferrocyanide/ferricyanide redox couple confirms binding in each stage. The antibody binds to the immobilized protein and this reduces the peak current on the DPV. A reduction in peak current shows that the electrode surface is covered with the bulky antibodies. When this coverage occurs, access of the ferro/ferri cyanide redox couple to the conductive surface is limited and a reduction in the electron transfer is also observed which yields to a current decrease. The response of the biosensor is then detected by measuring the peak current changes from samples possibly containing different concentrations of SARS-CoV-2 antigen / virus.

An example of an electrochemical sensor carrying out competitive voltametric detection of SARS-CoV-2 is described herein below.

*Preparation of phosphate buffered saline (PBS) (10x = 0.1M) solution*

PBS solution was used as a supporting electrolyte and for solution preparation. About 80 g NaCl and 2 g KCl were dissolved into 800 mL distilled water. Approximately 14.4 g $Na_2HPO_4 \cdot 12H2O$ and 2.4 g $KH_2PO_4$ were then dissolved into the solution. The pH adjustment to the desired value was done using 1 M HCl or 1 M NaOH solutions followed by dilution to 1 L with distilled water and stored at room temperature.

*Synthesis of ZIF-8*

ZIF-8 was prepared by dissolving 0.026 g of (0.12 mmol) zinc nitrate hexahydrate and 0.02 g of 2-methylimidazole (0.24 mmol) in 10 ml of deionized water. The solution was kept still for 4 h at room temperature. Afterwards, the resulting ZIF-8 was taken out, washed with methanol for three times and dried at 60 °C overnight.

*Synthesis of a CTABAu-ZIF-8 composite*

Five milliliters of CTAB capped Au NRs (1 x $10^7$ M), 0.1 mL of 2-methylimidazole (0.24 mM), and 0.1 mL of $Zn(NO_3)_2 \cdot 6H_2O$ (0.12 mM) were mixed and stirred at 20 °C for 3 h. Next, the mixture solution was set at room temperature without stirring. This allowed ZIF-8 to grow on the gold nanorods surface. After 24 hours, there was centrifugation of the solid products, and they were rinsed with methanol three times and dried at 80 °C for 3 h in the oven.

*Synthesis of cysteine functionalized Au NRs encapsulated with ZIF-8*

A cysteine solution of 0.001 M was mixed with CTABAu-ZIF-8 solution at room temperature for 3 hours.

*Electrode preparation and modification*

Modified electrodes were made by drop coating a CTABAu-ZIF-8 composite on a glassy carbon electrode with a surface area of 0.071 $cm^2$. Different concentrations of the CTABAu NRs in the composites were tested to obtain the maximum response. Higher concentrations resulted in aggregation of CTABAU NRs and therefore a CTABAu-ZIF-8 composite formed with 1 x $10^7$ M Au NRs was selected for further testing. The electrodes were left to dry at room temperature before use. Prior to each modification, the GCE electrode was polished repeatedly with 1, 0.3 and 0.05 µm alumina slurries. After each polishing, electrode cleaning adopted was as follows; successive 5 minutes rinsing with doubly distilled water, ultrasonication in ethanol and finally with another doubly distilled water to remove any adsorbed substances on the electrode surface. Activation of the transducer was done with a glutaraldehyde cross linker which was added to the electrode surface to serve as a cross link for the cysteine-modified Au NRs and the SARS-CoV-2 antigens. Next, 20 µL of a 15 $\mu g.mL^{-1}$ solution of SARS-CoV-2 antigen was added to the modified electrode in PBS buffer, pH 7.4 for 2 hours at room temperature. A 1% BSA blocking solution was used for 1 hour to block any unreacted functional groups. This is followed by rinsing the modified electrode to remove excess BSA. The electrode is then stored at 4°C. A control electrode was used as reference by immobilizing only BSA thereon.

*Competitive voltametric detection of SARS-CoV-2*

A fixed antibody concentration is used to conduct an indirect competitive assay for SARS-CoV-2 analyte. Following the immobilization of SARS-CoV-2 antigens on the modified electrode, the modified electrode is incubated with a mixture of SARS-CoV-2 antigens in the range of 2 $pg.mL^{-1}$ to 20 $\mu g.mL^{-1}$ and 20 $\mu g.mL^{-1}$ of SARS-CoV-2 antibody for 20 min – 30 min. Ferrocyanide/ferricyanide ($[Fe(CN)_6]^{3-/4-}$) in 0.1M PBS, pH 7.4 is used to conduct electrochemical measurements. The response is calculated as: (Io-I)/Io%, where (Io) is the DPV current at the antigen-modified electrodes after blocking with 1% BSA and (I) is the current after the electrochemical biosensor is incubated with a mixture of SARS-CoV-2 antibody and SARS-CoV-2 antigen.

*Spiked nasal fluid/saliva testing*

The electrochemical biosensor was tested with artificial nasal fluid sample or saliva. Various concentrations (0.2 nM, 2 nM, 20 nM) of SARS-CoV-2 solutions (15 µL) are spiked in 1:100 diluted nasal fluid/saliva and mixed with 15 µL of 20 µM of the solution of antibodies. Then, 20 µL of each mixture is put on the surface of the modified electrodes containing SARS-CoV-2. This is followed

by incubation at room temperature for 30 min. Testing revealed that the analytical parameters were linear with a SARS-CoV-2 virus detection limit of 0.1 nM.

A selective and sensitive electrochemical biosensor is described capable of rapidly detecting a target biomolecule within a small sample volume. The electrochemical biosensor consists of a screen printed electrode providing a miniaturized, low cost and potentially disposable sensing platform that can readily be integrated into a point of care device. The surface of the working electrode is modified with a gold nanoparticle layer and a ZIF layer on top of it so that any type of bioreceptor capable of conjugating to the ZIF can be immobilized thereon. The gold nanoparticle layer improves conductance, whereas the surface roughness of the ZIF maximises the amount of bioreceptor that can be immobilized thereon. Moreover, the positively charged ZIF surface allows antibodies to bind in a favourable orientation which maximises the electrode response upon binding of a target antigen.

The foregoing description has been presented for the purpose of illustration; it is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Persons skilled in the relevant art can appreciate that many modifications and variations are possible in light of the above disclosure.

The language used in the specification has been principally selected for readability and instructional purposes, and it may not have been selected to delineate or circumscribe the inventive subject matter. It is therefore intended that the scope of the invention be limited not by this detailed description, but rather by any claims that issue on an application based hereon. Accordingly, the disclosure of the embodiments of the invention is intended to be illustrative, but not limiting, of the scope of the invention, which is set forth in the following claims.

Finally, throughout the specification and accompanying claims, unless the context requires otherwise, the word ‘comprise’ or variations such as ‘comprises’ or ‘comprising’ will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

**CLAIMS:**

1. An electrochemical biosensor comprising a screen printed electrode which includes a working electrode modified with a layer of gold nanoparticles and a layer of a zeolitic imidazolate framework provided on the layer of gold nanoparticles, the layer of zeolitic imidazolate framework having a selected bioreceptor immobilised thereon for the detection of a target biomolecule in use.

2. The electrochemical biosensor a claimed in claim 1, wherein the zeolitic imidazolate framework is ZIF-8.

3. The electrochemical biosensor as claimed in claim 1 or claim 2, wherein the gold nanoparticles have a size ranging between 5 and 20 nm.

4. The electrochemical biosensor as claimed in claim 3, wherein the gold nanoparticles have a size of 5 nm.

5. The electrochemical biosensor as claimed any one of claims 1 to 4, wherein a crosslinker binds the zeolitic imidazolate framework to the gold nanoparticles.

6. The electrochemical biosensor as claimed in claim 5, wherein the gold nanoparticles are coated with cysteamine and the crosslinker binds the zeolitic imidazolate framework to the cysteamine coating.

7. The electrochemical biosensor as claimed in claim 5 or claim 6, wherein the crosslinker is *bis*(sulfosuccinimidyl)suberate (BS3).

8. The electrochemical biosensor as claimed in any one of claims 1 to 7, wherein the screen printed electrode is a screen printed carbon electrode.

9. The electrochemical biosensor as claimed in any one of claims 1 to 8, wherein the bioreceptor is one or more antibodies configured to detect one or more target antigens.

10. The electrochemical biosensor as claimed in claim 9, wherein the one or more antibodies is a SARS-CoV-2 protein antibody or a combination of SARS-CoV-2 protein antibodies configured to detect the SARS-CoV-2 virus.

11. The electrochemical biosensor as claimed in claim 10, wherein the SARS-CoV-2 protein antibody is a spike protein antibody, or a nucleocapsid protein antibody or a combination of both the spike protein antibody and the nucleocapsid protein antibody.

12. A method for preparing an electrochemical biosensor, the method comprising the steps of:

sequentially forming a layer of gold nanoparticles and a layer of zeolitic imidazolate framework on a working electrode of a screen printed electrode; and

immobilising a selected bioreceptor onto the zeolitic imidazolate framework.

13. The method as claimed in claim 12, further including the step of modifying the surface of the gold nanoparticles so that it is configured to bind the zeolitic imidazolate framework.

14. The method as claimed in claim 13, wherein the surface modification of the gold nanoparticles includes coating the surface of the gold nanoparticles with cysteamine prior to depositing the gold nanoparticles onto the working electrode, and wherein a solution of a crosslinker is deposited on the working electrode after the solution of cysteamine-coated gold nanoparticles is deposited thereon.

15. The method as claimed in claim 14, wherein the crosslinker is *bis*(sulfosuccinimidyl)suberate (BS3).

16. The method as claimed in any one of claims 12 to 15, wherein the step of immobilising the selected bioreceptor onto the zeolitic imidazolate framework includes depositing a solution of the bioreceptor on the working electrode and allowing it to dry for a selected time period.

17. The method as claimed in any one of claims 12 to 16, further including the step of incubating the working electrode in a blocking solution for a selected time period to block any unreacted bioreceptor from the bioreceptor immobilisation step.

18. The method as claimed in claim 17, wherein the blocking solution is a solution of bovine serum albumin (BSA).

19. The method as claimed in any one of claims 12 to 18, wherein the bioreceptor is an antibody or a combination of antibodies, and when the antibody is a combination of

antibodies, the constituent antibodies of the combination may be immobilised sequentially or simultaneously onto the zeolitic imidazolate framework.

20. The method as claimed in claim 19, wherein the bioreceptor is a SARS-CoV-2 protein antibody or a combination of two or more different SARS-CoV-2 protein antibodies configured to detect the SARS-CoV-2 virus.

21. A method of detecting a target biomolecule, the method being carried out using the electrochemical biosensor as claimed in any one of claims 1 to 11 connected in a measuring system and comprising the steps of:

mixing a sample to be tested with a solution of a redox probe;

adding the mixture of the sample and the redox probe solution onto the working electrode of the screen printed electrode;

applying a voltage to the screen printed electrode; and

measuring a current flowing in the measuring system to detect changes in current which signify binding of the target biomolecule to the bioreceptor.

22. The method as claimed in claim 21, wherein differential pulse voltammetry or square wave voltammetry is used to measure and record changes in peak current after addition of the mixture of the sample and redox probe onto the working electrode.

23. The method as claimed in claim 21 or claim 22, wherein the bioreceptor is a SARS-CoV-2 protein antibody or a combination of SARS-CoV-2 protein antibodies and the target biomolecule is the SARS-CoV-2 virus.

24. The method as claimed in claim 23, wherein the sample is a saliva or nasopharyngeal sample.

25. The method as claimed in any one of claims 21 to 24, wherein the redox probe is a buffered solution of a 1:1 mixture of ferrocyanide/ferricyanide.

26. A point of care device for detecting a target biomolecule comprising an electrochemical sensor as claimed in any one of claims 1 to 11 connected in a measuring system which includes a voltage source configured to apply a voltage between the working electrode and a counter electrode of the screen printed electrode and a potentiostat configured to control electric parameters and measure current flowing between the working electrode

and counter electrode, wherein a current at or above a predetermined threshold current signifies binding of the target biomolecule to the bioreceptor.

5

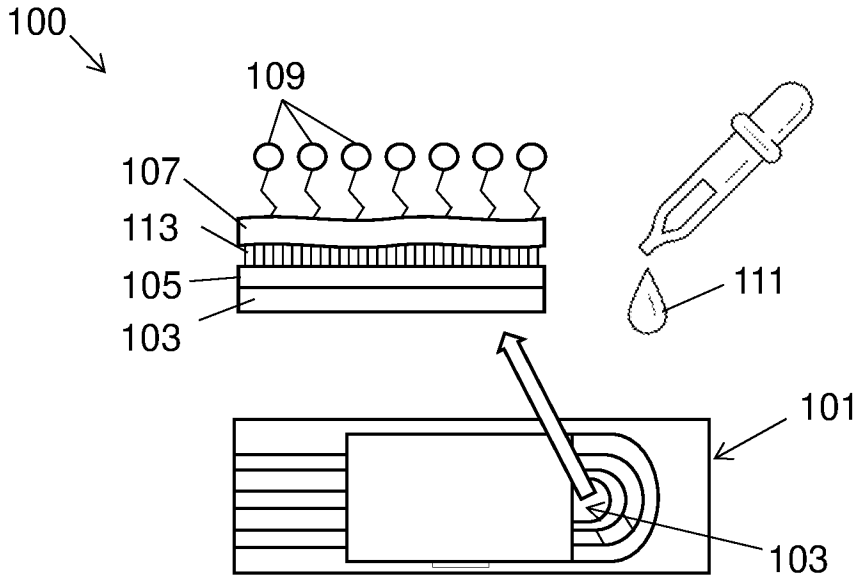


Figure 1

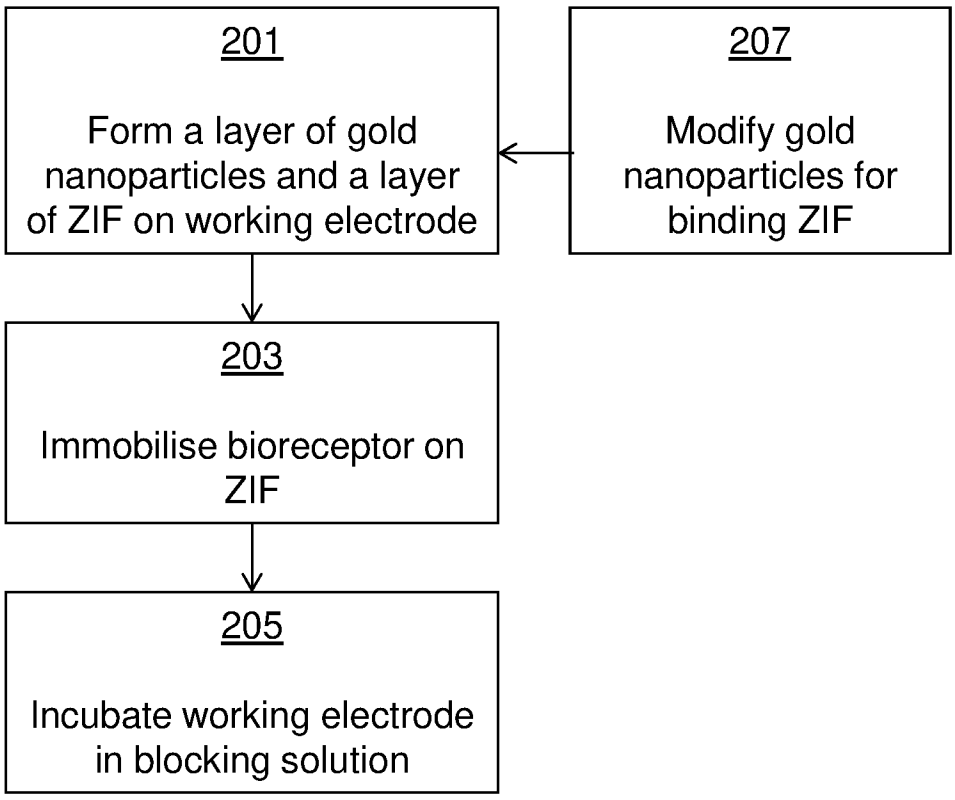


Figure 2

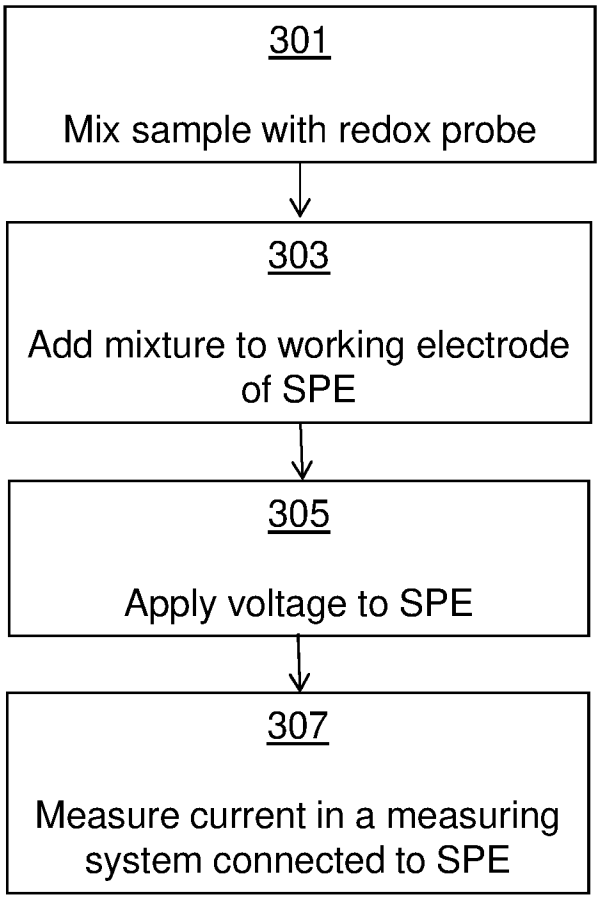
301
Mix sample with redox probe
303
Add mixture to working electrode of SPE
305
Apply voltage to SPE
307
Measure current in a measuring system connected to SPE

Figure 3

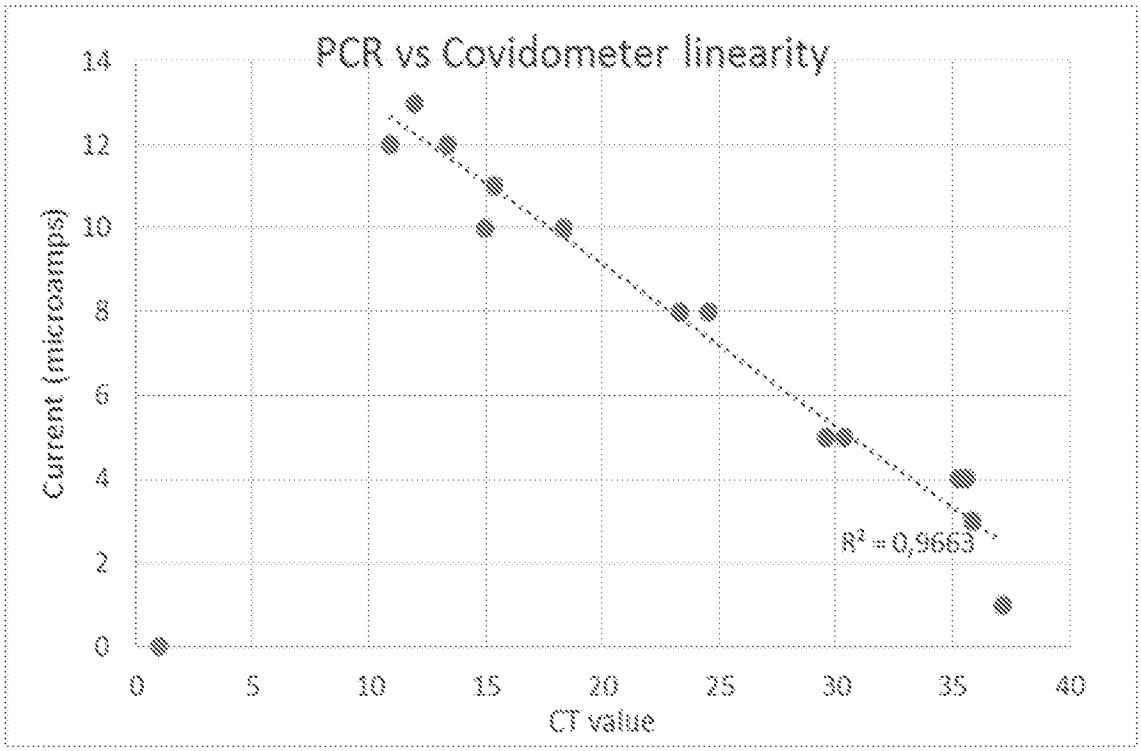


Figure 4

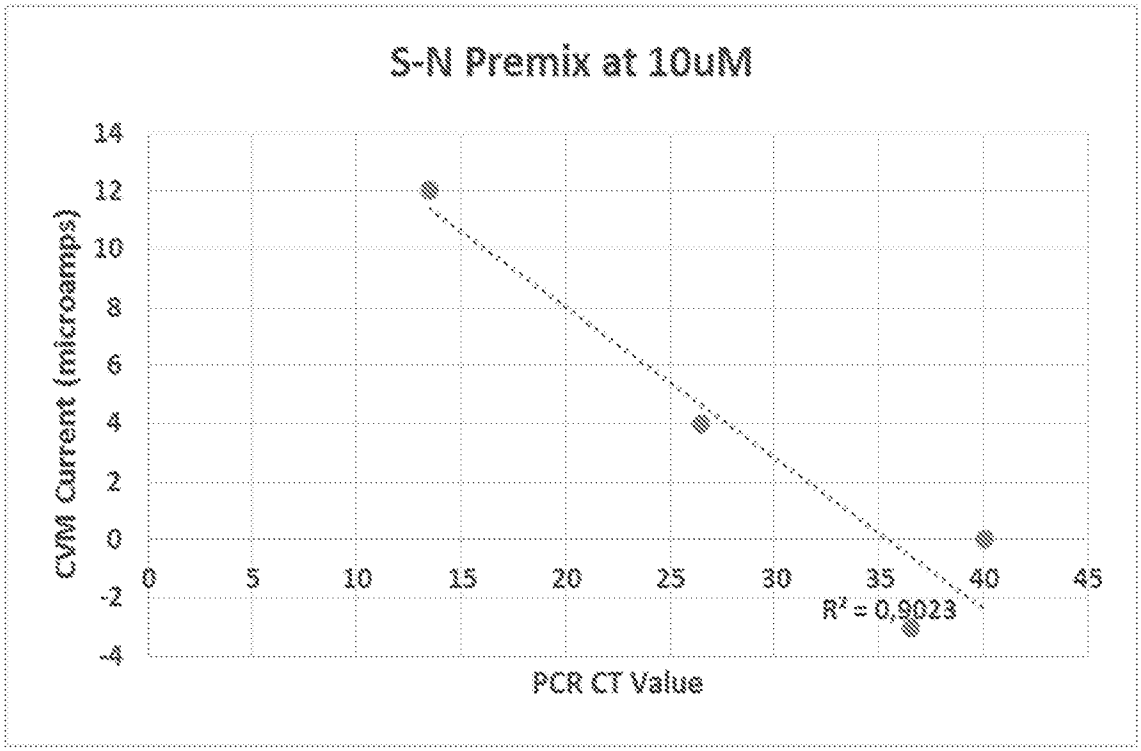


Figure 5

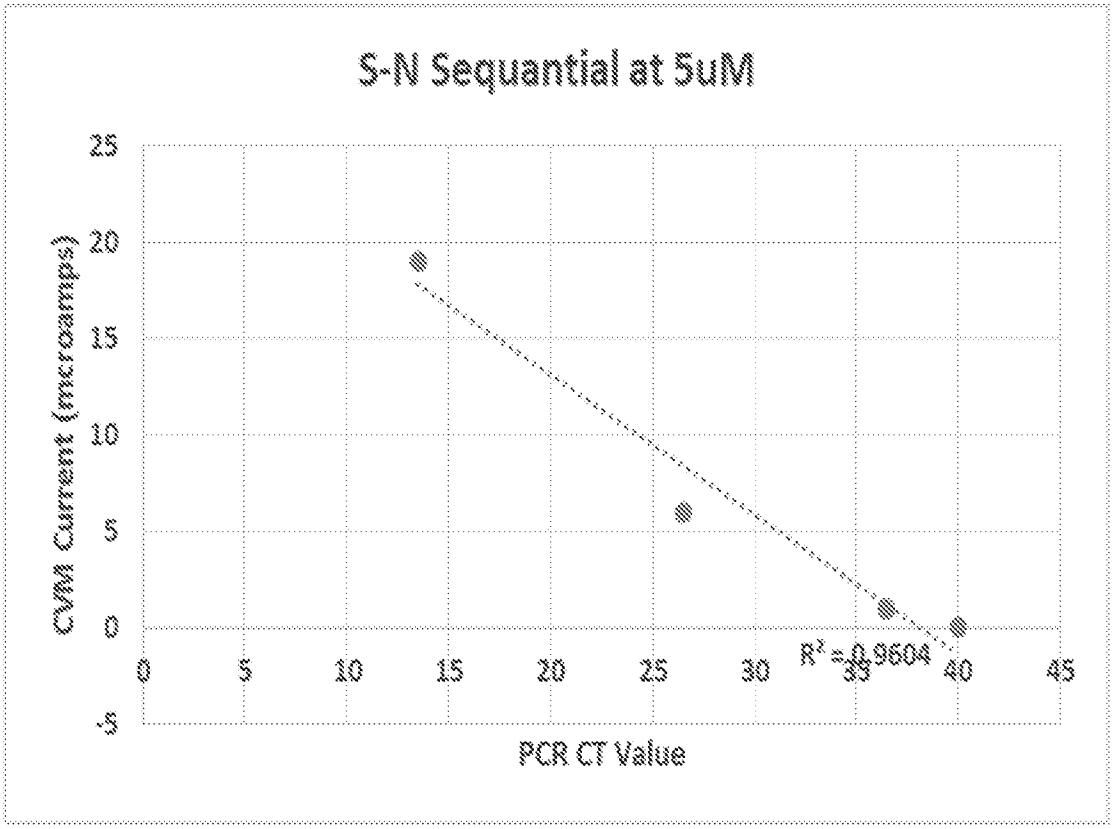


Figure 6

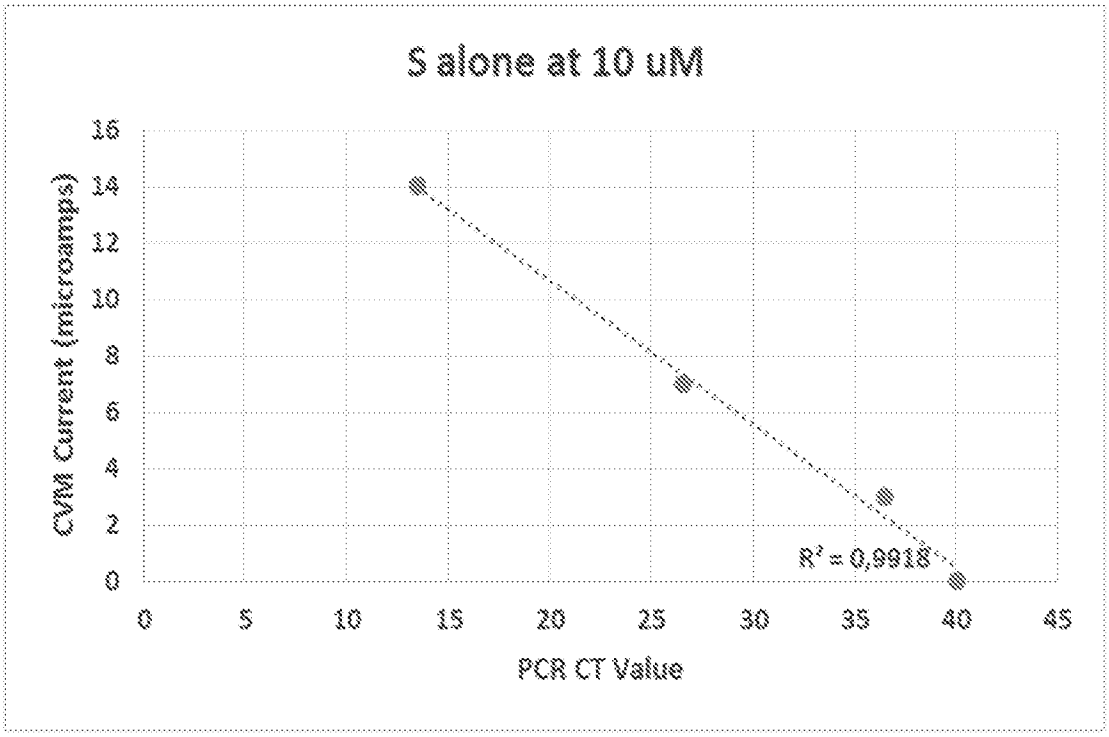


Figure 7

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 2021/058069

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***G01N 33/53*** (2006.01); ***G01N 27/327*** (2006.01); ***G01N 33/543*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIAP, EPODOC, PUBMED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CN 109490385 A (SHANGHAI PUDONG HOSPITAL) 19 March 2019 (19.03.2019). (Abstract and Translation) [online 05.11.2021] from the TXPMTCEB / EPO Database claims, abstract | 1-26 |
| A | CN 110441296 A (UNIV JINAN) 12 November 2019 (12.11.2019). (Abstract and Translation) [online 05.11.2021] from the TXPMTCEA / EPO Database abstract, claims | 1-26 |
| A | CN 108918623 A (UNIV HAINAN NORMAL) 30 November 2018 (30.11.2018). (Abstract and Translation) [online 05.11.2021] from the TXPMTCEA / EPO Database claims, abstract | 1-26 |
| A | CN 110672688 A (FOSHAN NANHAI DISTR LISHUI ECONOMY PROMOTION BUREAU, UNIV SUN YAT SEN) 10 January 2020 (10.01.2020). (Abstract and Translation) [online 05.11.2021] from the TXPMTCEA / EPO Database. claims,abstract | 1-26 |

Further documents are listed in the continuation of Box C. | [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 08 November 2021 (08.11.2021) | 10 November 2021 (10.11.2021) |
| Name and mailing address of the ISA/AT<br>Austrian Patent Office<br>Dresdner Straße 87, A-1200 Vienna<br>Telephone No. +43 (1) 53424 342 | Authorized officer<br>Görner Wolfram<br>Telephone No. +43 1 534 24 558 |

| Patent document cited in search report | Patent family member(s) | Publication date |
|---|---|---|
| CN 109490385 (A) | | |
| | CN 109490385 (A) | 2019-03-19 |
| | CN 109490385 (B) | 2020-12-11 |
| CN 110441296 (A) | | |
| | CN 110441296 (A) | 2019-11-12 |
| CN 108918623 (A) | | |
| | CN 108918623 (A) | 2018-11-30 |
| CN 110672688 (A) | | |
| | CN 110672688 (A) | 2020-01-10 |