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(54) Title: AN ELECTROCHEMICAL SENSOR AND RELATED METHODS

(57) Abstract: Disclosed herein is a method of (and a sensor for) determining the presence of and/or concentration of superoxide anions in a sample of interest, the method comprising: • (a) providing an electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder, contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species, • (b) using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information, • (c) using the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest.

AN ELECTROCHEMICAL SENSOR AND RELATED METHODS

Field of the Invention

The present invention relates to an electrochemical method and device for detecting the presence and/or concentration of superoxide anions in samples of interest, and in human, animal or plant biofluids in particular.

Background

Reactive oxygen species such as superoxide have a variety of roles in the human body, including and most importantly in immune defence. They are produced during the "oxidative burst" response of activated neutrophils, through the one-electron reduction of oxygen that is catalysed by NADPH oxidase; in turn this takes place during phagocytosis. In addition, superoxide has been found to be involved in processes such as the inactivation of iron-sulphur containing proteins as well as DNA damage and signal transduction. Numerous methods exist for sensing this anion radical, the most commonly used ones being flow cytometry, polarography, the use of N-halopiperidines and spin traps.

One of the most widely used detection methods for superoxide in biological samples is colorimetric, through the reduction of the pale yellow nitroblue tetrazolium chloride (NBTC) to its pale blue formazan. Such nitroblue tetrazolium salts were first synthesised by von Pechmann and Ruge. Given their striking colour when reduced, they were quickly exploited in the detection of reducing substances and particularly superoxide.

The so-called NBT test was developed by Baehner, during his investigation into chronic granulomatous disorder. His experiments were a practical demonstration of a phagocytic cell being able to reduce the salt to its pale blue formazan only if it is able to produce the superoxide radicals needed to fight off infections. In contrast, neutrophils isolated from patients suffering from chronic granulomatous disorder were unable to reduce the compound due to the absence of NADPH oxidase, i.e. due to the absence of superoxide.

In recent years, electrochemical sensing methods have received a lot of attention, due to their speed of response and high sensitivity and reliability. Other advantages of some electrochemical sensors include their low detection limit, low cost and compatibility for miniaturisation. Concerning the detection of superoxide, most electrochemical sensors that have been developed are enzymatic and use cytochrome c or superoxide dismutase because of the high selectivity that these enzymes ensure. Both are usually immobilised on the electrode surface, either through binding on thiol groups or the utilisation of sono-gels. Between the two, superoxide dismutase is often preferred, mainly due to the structure of the heme group in cytochrome c resembling that of peroxidase, which is not specific for superoxide. However, these enzyme-based devices have an important limitation; their inherent lack of stability, which results in the responses being a function of variables such as temperature and pH and in the sensor being destroyed by non-standard conditions.

Researchers have addressed this issue through alternative modification procedures. Kim et al modified a glassy carbon electrode with a Pt-MWCNT film while Lin et al used Au nanoparticles in their film instead. Yuasa et al synthesised a Fe-porphyrin polymerised film. Even though these results are both important and encouraging, the syntheses and characterisation assays involved are complicated and time-consuming.

Brief Description of the Figures

The Figures illustrate results from the Examples below.

Figure 1 shows the reduction of NBTC on a glassy carbon macroelectrode (0.095 mM NBTC in phosphate buffer solution containing 0.1 M KCl at pH = 6.97), at variable scan rates (25 – 800 mV s^{-1}) and 298 K, with a Randles-Ševčik plot depicted in the inlay. The solution used is de-oxygenated prior to this study.

Figure 2 shows a Tafel plot for the forward peak of the 100 mV s^{-1} NBTC reduction response on the glassy carbon macroelectrode (0.095 mM NBTC phosphate buffer solution containing 0.1 M KCl at pH = 6.97 and 298 K). Calculated α = 1.03. The average value, α_{ave}, obtained by averaging the calculated α values of each scan rate was 0.98 ± 0.05. The solution used is de-oxygenated prior to this study.

Figure 3 shows the reduction of NBTC on a carbon fibre microelectrode (0.078 mM NBTC in phosphate buffer solution containing 0.1 M KCl at pH = 6.97), at variable scan rates (25 – 800 mV s^{-1}) and 298 K. The solution used is de-oxygenated prior to this study.

Figure 4 shows the steady state and peak current values obtained through the simulation of the microelectrode results depicted in Figure 3, for $D=7.2x10^{-6}$ cm^2 s^{-1}, and the number of electrons transferred in the reduction of NBTC is 4 for each scan rate (grey circles), overlaid with the experimentally obtained ones (black squares).

Figure 5 shows 100 mV s^{-1} cyclic voltammetric responses for the oxidation of the diformazan adsorbed on the glassy carbon surface. The scans were obtained in a deoxygenated phosphate buffer solution (0.1 M KCl, pH = 6.97, 298 K) for increasing holding times, at -0.35 V (vs. SCE), in an identical solution that also contained 0.1 mM NBTC. Inlay: The area of the peak increases with increasing holding time, reflecting the increasing amount of diformazan adsorbing on the surface and being oxidised in the blank solution. The plateau indicates that a monolayer is formed at holding times of 40s or larger.

Figure 6 shows the reduction of O_2 on the glassy carbon macroelectrode in an O_2 saturated (1.24 mM) phosphate buffer solution containing 0.1 M KCl, at pH = 6.97, at variable scan rates (25 – 800 mV s^{-1}) and 298 K.

Figure 7 shows a Tafel plot for the forward peak of the 100 mV s^{-1} O_2 reduction response on the glassy carbon macroelectrode (1.24 mM O_2 phosphate buffer solution containing 0.1 M KCl at pH = 6.97 and 298 K). Calculated $\alpha = 0.30$. The average value, α_{ave}, obtained by averaging the calculated α values of each scan rate, was 0.26 ± 0.06.

Figure 8 shows the Randles-Ševčik plot for the results presented in Figure 6, giving $D=1.8 \times 10^{-5}$ cm^2 s^{-1}.

Figure 9 shows 100 mV s^{-1} cyclic voltammetric responses for the reduction of NBTC in a deoxygenated 0.095 mM NBTC phosphate buffer solution containing 0.1 M KCl at pH = 6.97 (labelled) and an identical solution that had previously been saturated with O_2 (1.24 mM - labelled). The grey line is an oxidative scan, in the first solution, indicating

that the peak observed at +0.80 V (vs. SCE) is due to the oxidation of the diformazan and not the oxidation of the starting material. Responses were recorded on the glassy carbon macroelectrode, at 298 K.

Figure 10 shows 100 mV s^{-1} cyclic voltammetric responses for the reduction of NBTC on a carbon paste electrode. The scans were obtained in a deoxygenated phosphate buffer solution (0.1 M KCl at pH = 6.97, 298 K) for increasing pre-concentration times in an identical solution that also contained 0.147 mM NBTC. Inlay: The current increases with increasing pre-concentration time, reaching a plateau at 90s; the paste is then substantially loaded with NBTC.

Figure 11 shows 100 mV s^{-1} cyclic voltammetric responses for the oxidation of diformazan generated *in* the pasting liquid. The scans were recorded on a carbon paste electrode, in a deoxygenated phosphate buffer solution (0.1 M KCl at pH = 6.97, at 298 K). The paste electrode was first immersed in the 0.22 mM superoxide solution for 40s and was then pre-concentrated with NBTC for increasing pre-concentration times. Inlay: The peak area increases with increasing pre-concentration time with NBTC, reaching a plateau at the optimum NBTC pre-concentration time of 90s.

Figure 12 shows 100 mV s^{-1} cyclic voltammetric responses for the oxidation of diformazan generated *in* the pasting liquid, for varying superoxide concentrations. The scans were recorded using a carbon paste electrode, in a deoxygenated phosphate buffer solution (0.1 M KCl at pH = 6.97, at 298 K). The paste electrode was immersed in the 0.22 mM superoxide solution for 40s and then almost equilibrated with NBTC (optimum pre-concentration time: 90s). The concentration of superoxide was varied between 0 and 1.88 nM (in particular at concentrations of0 nM, 0.059 nM, 0.12 nM, 0.24 nM, 0.47 nM, 0.94 nM, 1.41 nM, 1.88 nM). Inlay: The current decreases with decreasing superoxide concentration. The practical limit of detection, in this particular context, was determined as being 0.059 nM. The slope gave a value of 1792 A M^{-1} for the sensor sensitivity.

Summary of the Invention

In a first aspect, there is provided a method of determining the presence of and/or concentration of superoxide anions in a sample of interest, the method comprising:

(a) providing an electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder, contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species,

(b) using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information,

(c) using the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest. In an embodiment, step (b) involves using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the second species is oxidised or reduced and obtaining electrochemical information.

In a second aspect, there is provided an electrochemical sensor for determining the presence of and/or concentration of superoxide anions in a sample of interest,

wherein the sensor comprises an electrically conducting substrate having on a surface thereof a paste comprising organic liquid binder and electrically conductive particles dispersed in the binder,

the sensor being capable of being used in a step (a) involving:

contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated

into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species,

the sensor being adapted to (i) use the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information, and then (ii) use the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest.

In a third aspect, there is provided an electrically conducting substrate for use as a working electrode, the electrically conducting substrate having on a surface thereof a paste comprising organic liquid binder and electrically conductive particles dispersed in the binder, wherein the binder further comprises a first species and/or a second species, wherein the first species is capable of reacting with superoxide to form the second species, and either the first and/or second species can be detected in an electrochemical test.

In a fourth aspect, there is provided a method of diagnosis of chronic granulomatous disorder, involving the method of determining the presence and/or concentration of superoxide anions in a sample of interest, as described in the first aspect.

The inventors have devised the first electrochemical analogue of the NBT test. The method described herein gives immediate quantitative results, as opposed to the traditional qualitative NBT test, and is easy to use. The technique, therefore, allows the concentration of superoxide anions in a sample of interest to be determined, which is useful in many situations, for example in medicine as a diagnostic test for chronic granulomatous disorder. It has been found that the method can be used to detect much lower concentrations of superoxide in a sample than some other tests of the prior art.

Detailed Description

The present invention provides the first to the fourth aspects mentioned above. Optional and preferred features of the various aspects are described below. Unless

otherwise stated, any optional or preferred feature may be combined with any other optional or preferred feature, and with any of the aspects of the invention mentioned herein.

Paste Electrode

The electrically conducting substrate has on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder. The electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder may be termed a working electrode for brevity herein. However, this indicates that it is suitable for use as a working electrode and does not imply that, during step (a), it is connected to an electrical source or that any potential is applied to it, although it may, if desired be connected to an electrical source and/or a potential is applied to it, during step (a). If connected to an electrical source during step (a), preferably step (a) is carried out under open circuit conditions.

The substrate may comprise any suitable electrically conducting material, e.g. a metal, an alloy of metals, and/or carbon. The substrate may comprise a transition metal, for example a transition metal selected from any of groups 9 to 11 of the Periodic Table. The substrate can comprise a metal selected from, but not limited to, rhenium, iridium, palladium, platinum, copper, indium, rubidium, silver and gold. If the substrate comprises carbon, the carbon may be selected from edge plane pyrolytic graphite, basal plane pyrolytic graphite, a glassy carbon, boron doped diamond, highly ordered pyrolytic graphite, carbon powder and carbon nanotubes.

The electrically conductive particles dispersed in the binder may comprise any suitable material. The electrically conductive particles dispersed in the binder may comprise a material selected from a metal or carbon. The electrically conductive particles dispersed in the binder may comprise or be nanoparticles. The electrically conductive particles may comprise metal nanoparticles and/or carbon nanoparticles. The electrically conductive particles may comprise a transition metal, for example a transition metal selected from any of groups 9 to 11 of the Periodic Table. The substrate can comprise a metal, which may be in the form of metal nanoparticles, selected from, but not limited to, rhenium, iridium, palladium, platinum, copper, indium,

rubidium, silver and gold. In a preferred embodiment, the particles comprise, consist essentially of or consist of carbon. At least some of the particles may have a diameter of less than 500 μm, optionally less than 200 μm, optionally less than 100 μm, less than 50 μm, less than 40 μm. The diameter of the particles may be measured, for example, by a scanning electron microscope.

The organic liquid binder can be selected from a phthalate, mineral (paraffin) oils, aliphatic and aromatic hydrocarbons, silicone oils and greases, halogenated hydrocarbons, (tricresyl phosphate) TCP, (nitrophenyl octyl ether) NPOE, diphenyl ether, glycerol, ionic liquid, as well as other suitable materials. In an embodiment, the organic liquid binder is a phthalate, which may be selected from an alkyl phthalate. The alkyl phthalate may be a polyalkyl phthalate, including, but not limited to, a di-alkyl phthalate. The or each of the alkyl(s) in the alkyl phthalate, e.g. the di-alkyl phthalate, may be a C1 to C12 alkyl, e.g. a C4 to C12 alkyl, e.g. a C6 to C10 alkyl. In some embodiments, the organic liquid binder comprises a di-alkyl phthalate selected from di-octyl phthalate and di-iso-nonyl phthalate.

If the organic liquid binder comprises an ionic liquid, the ionic liquid may be a room-temperature ionic liquid. Generally, ionic liquids are non-aqueous, organic salts comprising ions where the positive ion is charge-balanced with a negative ion. Ionic liquids have low melting points, often below 100°C, undetectable or very low vapour pressure, and good chemical and thermal stability. The cationic charge of the salt is localized over hetero atoms, such as nitrogen, phosphorous, sulphur, arsenic, boron, antimony, and aluminium, and the anions may be any inorganic, organic, or organometallic species. The ionic liquid may be selected from, but is not limited to, imidazolium ionic liquids, pyridinium ionic liquids, tetra alkyl ammonium ionic liquids, and phosphonium ionic liquids. Imidazolium, pyridinium, and ammonium ionic liquids have a cation comprising at least one nitrogen atom. Phosphonium ionic liquids have a cation comprising at least one phosphorus atom. The ionic liquid may comprise a cation selected from alkyl imidazolium, di-alkyl imidazolium, and combinations thereof. In an embodiment, each of the alkyl groups independently contain from one to ten carbon atoms. Dialkyl imidazolium ionic liquids have a cation comprising two alkyl groups extending from a five membered ring of three carbon and two nitrogen atoms, most commonly from the two nitrogen atoms of this five membered ring; the two alkyl groups may each independently be selected from C1 to C10 alkyl groups, optionally

from C1 to C6 alkyl groups, optionally from methyl, ethyl, propyl, butyl, pentyl and hexyl. In an embodiment, the dialkyl imidazolium ionic liquids have a 1-alkyl-3-methyl-imidazolium cation, wherein alkyl may be selected from C1 to C10 alkyl groups, optionally from C1 to C6 alkyl groups, optionally from methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl and decyl. The ionic liquid cation may be selected from 1-methyl-3-methylimidazolium, 1-ethyl-3-methylimidazolium, 1-propyl-3-methylimidazolium, 1- butyl-3-methyl imidazolium, 1-pentyl-3-methyl imidazolium, 1-hexyl-3-methyl imidazolium, and combinations thereof.

In one embodiment, the working electrode (i.e. the combination of the electrically conducting substrate and the paste) is a carbon paste electrode, being an electrode as described wherein the electrically conductive particles are or comprise carbon particles. In an embodiment, the electrically conductive particles comprise a material selected from graphite powder, acetylene black, carbon black, and carbon nanoparticles, including, but not limited to, carbon nanotubes. Carbon paste electrode are known to the skilled person. These electrodes are simple to manufacture, provide a surface for electron exchange which can be renewed easily, but have advantages when used in the method of the present invention as described herein, e.g. very low levels of superoxide can be detected in a sample.

The carbon paste can be prepared by mixing, e.g. hand-stirring, the carbon and organic liquid binder until the mass appears uniformly wetted, forming a paste-like consistency. Some prior art have described a typical consistency of a carbon paste to be that of peanut butter.

The electrically conducting substrate may be or may form part of a holder for the paste. The holder for the carbon paste may comprise an aperture or cavity in which the paste resides, and wherein the paste is in contact with the electrically conducting substrate. The holder for the carbon paste may comprise a non-electrically conducting material, e.g. selected from glass and plastic, e.g. polytetrafluoroethylene (PTFE), having therein a cavity and/or aperture in which the paste resides, the paste being in contact with the electrically conducting substrate, which may, for example, comprise or be copper. In an embodiment, the non-electrically conducting material is in the form of a tube having an interior cavity, wherein at least part of the interior cavity, preferably at one end of the tube, is filled with the paste, and the paste is in contact with the electrically conducting

substrate, which may extend away from the paste (optionally within the same interior cavity of the holder), to allow electrical contact of the electrically conducting substrate to a circuit.

For further information on carbon-paste electrodes see Svacara, Kalcher, Walcarius and Vytras: *Electroanalysis with Carbon Paste Electrodes*, CRC Press, USA, 2012, which is incorporated herein by reference in its entirety.

Step (a)

The method of the present invention involves a step (a) of providing the electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder, contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species.

As indicated above, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste. In this part of the method, the superoxide anions, if present in the sample, are transferred from the sample to the paste. Such superoxide anions may be dissolved or otherwise dispersed within the organic liquid binder of the paste and/or adsorbed onto the surface of the electrically conducting particles within the paste.

The sample of interest may be or comprise a biomaterial, e.g. a biofluid, which may have been drawn from an animal or a plant. The sample of interest may be or comprise a biomaterial, e.g. a biofluid, which may have been drawn from a human. The sample of interest may be a liquid sample. Optionally, the sample of interest is a biological sample, which may be selected from a sweat sample, a blood sample, saliva and a urine sample. The blood sample may be selected from a whole blood sample, a plasma sample and a serum sample. The sample of interest may comprise white blood

cells, or biomaterial drawn from white blood cells; and in an embodiment, the white blood cells are or comprise neutrophils.

Optionally, before contacting with the working electrode, the sample of interest may be diluted, e.g. with a liquid medium such as water, or concentrated, the diluted or concentrated sample used in the method, and the dilution or concentration is taken into account when calculating the concentration of the superoxide anion in the undiluted or unconcentrated sample.

The present inventors have found that, surprisingly, the method seems to be more effective if the paste on the electrically conducting substrate is contacted with the sample, and then the first species is incorporated into the paste. In an embodiment, the paste is contacted with a source of the first species, which is preferably after the paste has been contacted with the sample of interest. The source of the first species may comprise, consist essentially of or consist of the first species. In an embodiment, the source of the first species comprises a carrier medium, which will be termed a first carried medium herein, wherein the first species is dispersed within, e.g. suspended and/or dissolved within, the carrier medium. The first carrier medium is preferably an aqueous carrier medium, and optionally the first carrier medium further comprises a buffer. The buffer may be selected from a phosphate buffer, including, but not limited to potassium phosphate monobasic, potassium phosphate dibasic, a boric acid buffer, a glycine buffer and buffers comprising salts of bicarbonate. The first carrier medium, which may be an aqueous carrier medium, may have a pH of from 5 to 9, optionally from 6 to 8, optionally from 6.5 to 7.5.

The paste on the working electrode may be contacted with the source of the first material for a predetermined time, which may be termed a first predetermined time herein. The first predetermined time may be for a period in which the amount of the first species in the paste has equilibrated, or has neared equilibration, with the first species in the source of the first species, i.e. a time such that the concentration of the first species in the paste no longer is increasing.

Optionally the paste of the working electrode is contacted with the source of the first species for a period of at least 10 seconds, optionally for a period of at least 30

seconds, optionally for a period of at least 50 seconds, optionally for a period of at least 70 seconds.

Optionally, the amount of the first species within the source of the first species is 0.05 mM to 500 mM, optionally 0.1 mM to 200 mM.

In an embodiment, the first species is or comprises a tetrazole moiety, e.g. NBTC, and the first carrier medium is an aqueous carrier medium, that may comprise a buffer and/or may be at a pH of from 5 to 9, e.g. 6 to 8, and the amount of the tetrazole moiety within the source of the first species is 0.05 mM to 500 mM, optionally 0.1 mM to 200 mM, and optionally the paste of the working electrode is contacted with the source of the first species for a period of at least 10 seconds, optionally for a period of at least 30 seconds, optionally for a period of at least 50 seconds, optionally for a period of at least 70 seconds. A tetrazole moiety may be termed a tetrazole herein for brevity.

The paste may be contacted with the sample for a predetermined time; the predetermined time may be a time of at least 5 seconds, optionally at least 10 seconds, optionally at least 20 seconds. The paste may be contacted with the sample for a predetermined time of from 5 seconds to 5 minutes, optionally a predetermined time of from 5 seconds to 2 minutes, optionally a predetermined time of from 5 seconds to 1 minute, optionally a predetermined time of from 20 seconds to 1 minute, optionally a predetermined time of from 30 seconds to 50 seconds.

The First and Second Species

In step (a) of the method, the superoxide anions react with the first species within the paste to form a second species. Preferably, in step (a) of the method, the superoxide anions react with the first species within the paste to reduce the first species to form a second species. The first species is preferably a species that is reduced by superoxide, for example in the organic liquid binder, e.g. at a temperature of from 20 °C to 50 °C, optionally a temperature of from 20 °C to 40 °C, optionally a temperature of from 20 °C to 30 °C and/or without a potential being applied to the electrically conducting substrate having the paste thereon.

The first species may be an organic compound having an oxidisable or reducible group linked to a delocalised electron system, e.g. an aryl group, where the compound is either reduced on contact with superoxide anions by reduction of the reducible group or oxidised by oxidation of the oxidisable group. For example, the first species may be an aryl compound having an oxidisable or reducible group on one or more rings of the aryl compound. The first species may, for example, comprise a phenyl moiety having and oxidisable or reducible group on the phenyl ring or a naphthyl moiety having an oxidisable or reducible group on one or both of rings of the naphthyl moiety. The oxidisable or reducible group may be selected from, for example an optionally substituted tetrazole, $N(R)_2$ (wherein each R is alkyl, for example C1 to C10 alkyl, for example C1 to C5 alkyl, for example C1 to C3 alkyl, for example methyl, ethyl or propyl), amino, nitro, OH, COOH, -(C=O)H, -(C=O)R (wherein each R is alkyl, for example C1 to C10 alkyl, for example C1 to C5 alkyl, for example C1 to C3 alkyl, for example methyl, ethyl or propyl). The oxidisable or reducible group may be selected from, for example a substituted tetrazole ring, wherein the tetrazole ring has a nitro group thereon, or an aryl group thereon, e.g. a phenyl group, substituted with a nitro group.

In an embodiment, the first species can be, or can comprise, a nitrogen-containing heterocycle, preferably an aromatic heterocycle, and/or or a nitroxide-containing moiety. In another embodiment, the first species can be, or can comprise, a tetrazole. A tetrazole is a compound that contains a tetrazole ring. Tetrazole includes, but is not limited to, a tetrazolium salt. A tetrazolium salt may comprise a cation containing a tetrazole ring, and an anion; the anion can be any suitable anion, e.g. a halide. In an embodiment, the first species can be, or can comprise, a nitrogen-containing heterocycle, e.g. a tetrazole ring, having one or more substitutents, wherein at least one of the substitutents is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group. In an embodiment, the first species can be, or can comprise, a plurality of nitrogen-containing heterocycles, e.g. a plurality of tetrazole rings, each heterocycle having one or more substitutents, wherein at least one of the substitutents is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group, and preferably the nitrogen-containing heterocycles are linked to one another by an aryl system, e.g. a biphenyl group, wherein each ring of the biphenyl group may have one or more substituents, e.g. oxyalkyl, e.g. oxymethyl; and optionally

each of the nitrogen-containing heterocycles rings has an aryl substituent, e.g. a phenyl substituent.

In an embodiment, the first species is selected from a nitroblue tetrazolium halide, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide, 5-methyl-phenazinium methyl sulfate, 1-methoxy-5-methyl-phenazinium methyl sulfate, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2Htetrazolium inner salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, and 2,3,5-triphenyl-2H-tetrazolium chloride. In an embodiment, the first species is a tetrazole and the second species is a formazan. The halide of the nitroblue tetrazolium halide may be selected from chloride, bromide and iodide.

Tetrazolium salts have been described in earlier publications, including Biotechnology Annual Review, Volume 11, ISSN: 1387-2656, DOI: 10.1016/S1387-2656(05)11004-7, in an article by Berridge et al, which is incorporated herein by reference.

In an embodiment, the first species is selected from nitroblue tetrazolium chloride (NBTC), mitochondria-targeted hydroethidine, *p*-benzoquinone, superoxide dismutase, cytochrome c, coelenterazine, ascorbic acid, thiols, glutathione and lucigenin.

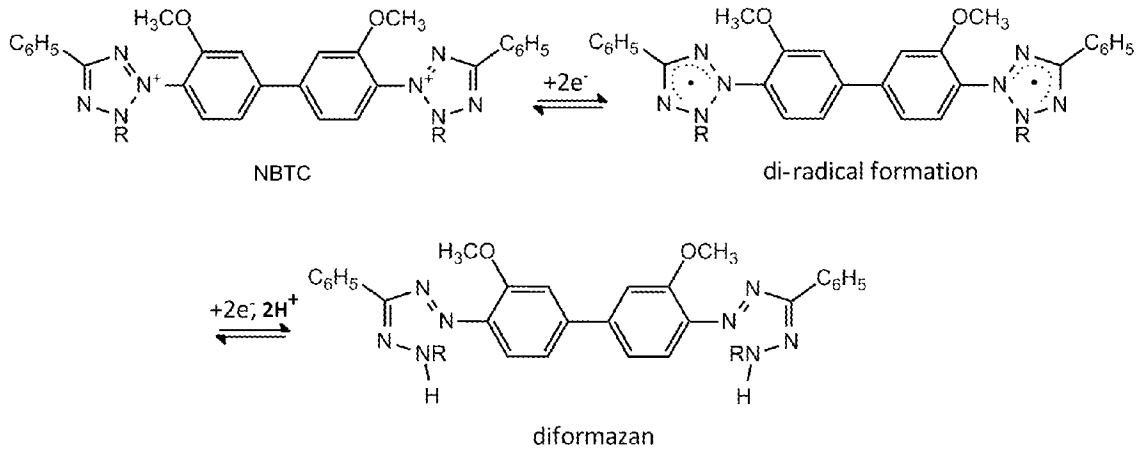
In a further embodiment, the first species can be or comprise nitroblue tetrazolium chloride (NBTC), which is shown in Scheme 1, below. NBTC is an organic dye that is electrochemically active due to the presence of two tetrazole moieties and two nitro substituents; each tetrazole ring needs two electrons and a proton to become fully reduced (see Scheme 1), while each nitro group requires four electrons and four protons for a full reduction [41]. If the first species is nitroblue tetrazolium chloride, then the second species contains a diformazan group (also shown in Scheme 1 below).

In the method of the present application, if the first species is NBTC, the reduction of its tetrazole rings is brought about by superoxide acting as a reducing agent. In the electrochemical test method, as will be described in more detail below, the electrochemical test may involve oxidation of the diformazan to form NBTC and optionally, for example if the techniques involves cyclic voltammetry, then further

reduction of the tetrazole rings of NBTC (to reform diformazan). For example, the electrochemical test may involve cyclic voltammetric scans that are carried out between +1.2 and -0.40 V (vs. SCE). This is outside of the potential window required for the reduction of the nitro groups but sufficient to reduce (and reform) the tetrazole rings.

The steps involved in the four-electron reduction of NBTC, where R: $C_6H_4NO_2$ and the counter ions in the starting material are Cl^- are shown in Scheme 1.

Scheme 1



The Electrochemical Test

Step be (b) involves using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information. The method may be used to detect electrochemical information indicative of the concentration of the first species and/or second species. A removal of an electrochemical signal in step (b), characteristic of the first species, can indicate the presence of superoxide in the sample of interest. A reduction in an electrochemical signal in step (b), characteristic of the first species, in the electrochemical test compared to the electrochemical signal in a comparable method, but which does not

involve the electrically conducting substrate, having on a surface thereof the paste, with the sample of interest, can indicate the presence of superoxide.

In an embodiment, step (b) involves using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species, preferably the second species, is oxidised or reduced and obtaining electrochemical information.

The electrochemical test may involve a voltammetry or an amperometry experiment. The voltammetry will typically involve monitoring of the current at the working electrode as the potential between the working electrode and a further electrode is changed; the further electrode may be selected from a reference electrode and a counter electrode; in some examples a counter electrode and a reference electrode are both used in the voltammetry, and a potential may be applied between the working electrode and the reference electrode or counter electrode. The voltammetry may involve a voltammetry technique selected from cyclic voltammetry, square wave voltammetry, linear sweep voltammetry, pulse voltammetry, such as normal pulse or differential pulse voltammetry; or an amperometry technique such as chronoamperometry may be used. The voltammetry may be used to obtain the electrochemical information, which may be the potential, peak current (e.g. in Amps, 'peak current' may also be termed height of the peak of current herein), area under the peak and/or peak charge at which the oxidation or reduction of the first and/or second species, preferably the first species, within the paste occurs.

The voltammetry may involve increasing the potential at the working electrode until a peak in current is observed at a certain potential, and continued until the current has dropped from this peak. The potential may be raised at a constant rate, for example at a rate of from 25 mVs^{-1} to 800 mVs^{-1}, optionally at a rate of from 50 mVs^{-1} to 200 $mVs^{-1,}$ optionally at a rate of 50 mVs^{-1} to 150 mVs^{-1}, optionally at a rate of 75 mVs^{-1} to 125 mVs^{-1}, optionally at a rate of about 100 mVs^{-1}. The voltammetry may involve increasing the potential at the working electrode from a first potential to a second potential, and, if it is a cyclic voltametry technique, cycling between these first and second potentials.

If the first species comprises a tetrazole, the electrochemical test may be carried out using a voltammetry technique that converts the second species, which results from the reduction of the tetrazole ring(s) of the tetrazole in step (a), to the first species. If the first species comprises or is a tetrazole, the electrochemical test may be carried out using a cyclic voltammetry technique that converts the second species, which results from the reduction of the tetrazole in step (a), to the first species, and then, by reversing the voltage sweep, converts the first species to the second species. If the first species comprises a tetrazole, having one or more substitutents, wherein at least one of the substitutents on the tetrazole ring is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group, the electrochemical test preferably involves oxidation of the second species to the first species such that the tetrazole ring is formed, but does not oxidise or reduce the nitro group. If the first species comprises a tetrazole, having one or more substitutents, wherein at least one of the substitutents on the tetrazole ring is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group, the electrochemical test may be carried out using a cyclic voltammetry technique that converts the second species, which results from the reduction of the tetrazole in step (a), to the first species, and then, by reversing the voltage sweep, converts the first species to the second species such that the tetrazole ring is formed, but does not result in the oxidation or reduction of the nitro groups.

If the first species is NBTC, the electrochemical test may be carried out using a voltammetry technique that converts diformazan, which results from the reduction of the tetrazole ring(s) of NBTC in step (a), to NBTC. If the first species is NBTC, the electrochemical test may be carried out using a cyclic voltammetry technique that converts the diformazan, which results from the reduction of the NBTC in step (a), to the first species, and then, by reversing the voltage sweep, converts the first species to the second species, but preferably does not result in the oxidation or reduction of the nitro groups present in NBTC and diformazan.

If the first species is NBTC, and the electrochemical test involves voltammetry, the voltammetry may involve increasing the potential at the working electrode from a first potential to a second potential, and, if it is a cyclic voltametry technique, cycling between these first and second potentials. The first potential may, for example be a potential less than (i.e. less positive than) -0.1 V, optionally a potential less than -0.2 V, optionally a potential less than -0.3 V, optionally a potential of from -0.5V to -0.2 V,

optionally a potential of from -0.5 V to -0.3 V, optionally a potential of from -0.45 V to -0.35 V, optionally a potential of around -0.4 V (all potentials being vs. SCE). The second potential may, for example, be a potential of more than (i.e. more positive than) 0.5 V, optionally a potential of more than 0.6 V, optionally a potential of more than 0.7 V, optionally a potential of more than 0.8 V, optionally a potential of more than 0.9 V, optionally a potential of more than 1 V, optionally a potential of from 0.8 to 1.5 V, optionally a potential of from 0.8 to 1.4 V, optionally a potential of from 1 to 1.4 V, optionally a potential of from 1.1 to 1.3 V, optionally a potential of about 1.2 V (all potentials being vs. SCE).

The electrochemical information may be the height or area of a peak of current measured during the electrochemical test, for example when raising the potential from the first potential to the second potential, or cycling between first and second potentials. The height or area of a peak of current measured during the electrochemical test, as described hereon, may be the height or area of a peak of current in a graph of current vs applied potential. If cycling between first and second potentials, wherein the first potential is less than (i.e. less positive than) the second potential, the height or area of a peak of current measured during the electrochemical test may be that when raising the potential from the first potential to the second potential, e.g. the peak corresponding to the oxidation of the second species.

In a preferred embodiment, the electrochemical test involves cyclic voltammetry. The electrochemical test may be carried out using a working electrode and a counter electrode, and optionally a reference electrode, in a voltammetry technique, wherein the electrochemical test involves oxidation or reduction of the second species within the paste.

As indicated above, step (a) involves, providing the electrically conducting substrate having on a surface thereof the paste, contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species. Step (a) of the method may involve contacting the paste on the electrically

conducting substrate with the sample, and then incorporating the first species into the paste. In an embodiment, step (a) of the method may involve contacting the paste on the electrically conducting substrate with the sample, and then contacting the paste with a source of the first species, wherein the source of the first species may comprise a carrier medium, which will be termed a first carried medium herein, wherein the first species is dispersed within, e.g. suspended and/or dissolved within, the carrier medium.

The electrochemical test may involve use of an electrolyte-containing medium disposed between the working electrode and a counter electrode, and, if present, the reference electrode. In the present context, the electrolyte-containing medium may be defined as an ionic conductor, which may be in the form of a liquid or solid. The electrolyte-containing medium may be in contact with the working electrode and/or the counter electrode, and, if present, the reference electrode. The electrolyte-containing medium may comprise any suitable medium for conducting electricity in an electrochemical test. The electrolyte-containing medium may comprise a liquid medium, preferably water, and the electrolyte, which may be comprise one or more salts, e.g. inorganic salts dispersed, e.g. dissolved or suspended, within the liquid medium. The inorganic salts may be salts of a metal, including, but not limited to, salts of metals, where the metal is selected from Groups 1 and 2 of the Periodic Table, and the Transition Metals (Groups 3 to 10 of the Periodic Table), and optionally the counter ions in the salts are selected from, for example, halides, phosphates, phosphites, sulphates and carbonate. The electrolyte-containing medium may comprise a liquid medium, preferably water, and optionally a buffer, which acts as the electrolyte. The buffer may be selected from a phosphate buffer, including, but not limited to potassium phosphate monobasic and potassium phosphate dibasic, a boric acid buffer, a glycine buffer and buffers comprising salts of bicarbonate. The electrolyte-containing medium, which may have a pH of from 5 to 9, optionally from 6 to 8, optionally from 6.5 to 7.5. Optionally, the electrolyte-containing medium comprises a liquid medium, which is the same as the first carrier medium of the source of the first species. The electrolyte-containing medium may be the same as, or substantially the same as, the source of the first species, except that the electrolyte-containing medium may not contain the first species.

The electrolyte-containing medium may comprise a carrier medium and the electrolyte. The carrier medium of the electrolyte-containing medium may be termed a second carrier medium herein for brevity. The second carrier medium maybe a protic or non-protic solvent. The solvent may be a polar or a non-polar solvent, dependent on the nature of the second species undergoing the electrochemical test. The solvent may be a non-polar, non-protic solvent. In some examples the solvent may be selected from water, xylene, methylene chloride, perchloroethylene, chloroform, carbon tetrachloride, chlorobenzene, acetone, 2—butanone, 2—pentanone, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, methyl propionate, ethyl propionate, a dialkylether of ethylene glycol wherein the alkyl groups contain 1 to 4 carbon atoms, a dialkylether of propylene glycol wherein the alkyl groups contain 1 to 4 carbon atoms, parafinnic solvents such as naphtha, hexane, benzene, toluene, diethyl ether, chloroform, and mixtures thereof. The solvent may comprise a protic solvent selected from water, alcohols, e.g. alkanols such as ethanol, and carboxylic acids.

The electrolyte-containing medium may comprise a solid electrolyte. The solid electrolyte may comprise a protonic conductive electrolyte polymer. The solid electrolyte may be selected from a perfluorinated ion-exchange polymer, e.g. such as that available as Nafion, or a conductive polymer selected from poly(ethylene glycol), poly(ethylene oxide), poly(propylene carbonate).

The electrolyte-containing medium may comprise or be an ionic liquid or a room-temperature ionic liquid (RTIL). Generally, ionic liquids are non-aqueous, organic salts comprising ions where the positive ion is charge-balanced with a negative ion. Ionic liquids have low melting points, often below 100°C, undetectable or very low vapour pressure, and good chemical and thermal stability. The cationic charge of the salt is localized over hetero atoms, such as nitrogen, phosphorous, sulphur, arsenic, boron, antimony, and aluminium, and the anions may be any inorganic, organic, or organometallic species. The ionic liquid may be selected from, but is not limited to, imidazolium ionic liquids, pyridinium ionic liquids, tetra alkyl ammonium ionic liquids, and phosphonium ionic liquids. Imidazolium, pyridinium, and ammonium ionic liquids have a cation comprising at least one nitrogen atom. Phosphonium ionic liquids have a cation comprising at least one phosphorus atom. The ionic liquid may comprise a cation selected from alkyl imidazolium, di-alkyl imidazolium, and combinations thereof. In an embodiment, each of the alkyl groups independently contain from one to ten

carbon atoms. Dialkyl imidazolium ionic liquids have a cation comprising two alkyl groups extending from a five membered ring of three carbon and two nitrogen atoms, most commonly from the two nitrogen atoms of this five membered ring; the two alkyl groups may each independently be selected from C1 to C10 alkyl groups, optionally from C1 to C6 alkyl groups, optionally from methyl, ethyl, propyl, butyl, pentyl and hexyl. In an embodiment, the dialkyl imidazolium ionic liquids have a 1-alkyl-3-methyl-imidazolium cation, wherein alkyl may be selected from C1 to C10 alkyl groups, optionally from C1 to C6 alkyl groups, optionally from methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl and decyl. The ionic liquid cation may be selected from 1-methyl-3-methylimidazolium, 1-ethyl-3-methylimidazolium, 1-propyl-3-methylimidazolium, 1- butyl-3-methyl imidazolium, 1-pentyl-3-methyl imidazolium, 1-hexyl-3-methyl imidazolium, and combinations thereof.

The electrochemical test may be carried out at, e.g. if an electrolyte-containing medium is used, the medium is at, a temperature of from 10 °C to 40 °C, optionally at temperature of from 15 °C to 30 °C, optionally at a temperature of from 20 °C to 30 °C. The electrochemical test may be carried out at an ambient pressure of 90 kPa to 110 kPa.

Determining the presence of or concentration of superoxide anions

As indicated, the electrochemical information may be selected from the potential, peak current, area under the peak and/or peak charge at which the oxidation or reduction of the first and/or second species, preferably the second species, within the paste occurs. The potential, peak current, area under the peak and/or peak charge at which the oxidation or reduction of the second species within the paste occurs may be determined from a graph of current vs. applied potential (i.e. potential applied to the working electrode, e.g. vs the reference electrode). In step (c), the method involves using the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest. The presence of superoxide anions in the sample of interest may be indicated by electrochemical information indicative of the oxidation or reduction of the second species in the electrochemical test, optionally the oxidation of the second species to the first species. For example, the presence of the second species may be indicated by the presence of a peak that had been determined in a calibration process to be indicative of the

oxidation or reduction of the second species. If the first species was, for example, a tetrazole, and, in step (a), the tetrazole is reduced by superoxide to the second species, the presence of a peak at a potential that is characteristic of oxidation of the second species (optionally to the first species), would indicated the presence of superoxide in the sample of interest.

The determining of the concentration of the superoxide anions in the sample of interest may be carried out by using a predetermined relationship between the electrochemical information and known concentrations of superoxide in a reference sample, and the predetermined relationship may have been determined by a calibration process. The reference sample can be a sample suitable for calibrating the concentration of the superoxide anion in the sample of interest. The reference sample and the sample of interest may be similar to one another, for example in that they contain the same liquid medium, e.g. water, optionally in approximately the same amount, and optionally any other components, aside from the superoxide anion, in the same amounts.

In the method of the present invention, the calibration process may be carried out before, during, or after any of steps (a) and (b) of the method, to determine a relationship between the concentration of the superoxide anion in the reference sample and the electrochemical information. The calibration process may involve carrying out a step (a) that is otherwise the same as step (a) of the method of the present invention, except that the reference sample is used in place of the sample of interest, and contains a known concentration of superoxide anions, then carrying out step (b), which will be otherwise the same as step (b) of the method of the present invention to determine the electrochemical information, and then, if desired, repeating steps (a) and (b), with the reference sample containing different known concentrations of the superoxide anion, and then establishing a relationship between the concentration of the superoxide anions in the reference sample and the electrochemical information. In step (a) of the method of the present invention, the concentration of the first species within the paste should be the same as the concentration of the first species within the paste in the calibration process. The concentration of the first species within the paste may be controlled by contacting the paste with the source of the first species for a predetermined amount of time. In step (a) of the method of the present invention, the paste may contacted with the source of the first species (e.g. after contacting the paste with the sample) for a first predetermined amount of time, which is the same as the

predetermined amount of time of the contacting of the source of the first species in the calibration process, and the source of the first species is the same in both the step (a) of the present invention and step (a) of the calibration process (i.e. in terms of the nature and quantity of the components in the source of the first species), and preferably the conditions of contacting the paste with the source of the first species is the same in both the step (a) of the present invention and step (a) of the calibration process. The length of time of contacting the paste with the sample of interest is preferably be the same as the length of time of contacting the paste with the reference sample in the calibration process.

In an embodiment, the electrochemical information is the height of a peak of current, which may correspond to the oxidation of the second species (optionally to the first species), and the predetermined relationship may be represented by formula (a)

$$I = C + n[\text{superoxide anion}] \qquad \text{formula (a)}$$

wherein I is the height of a peak of current (e.g. in Amps), C is a constant, and n is a coefficient, and [superoxide anion] is the concentration of the superoxide anion in the sample of interest (or in the calibration process, the concentration of the superoxide anion in the reference sample). C and n may be determined in the calibration process. From determining I in the sample of interest, and using the formula above, i.e. (I-C)/n, the concentration of superoxide anion in the sample of interest, [superoxide anion], can be determined. The present inventors have found that the variation of peak height (optionally of the peak that corresponds to the oxidation of the second species, optionally to the first species) can vary linearly with anion concentration within useful ranges.

In an alternative embodiment, if desired, higher order polynomials may be used for the determined relationship between the peak of current at the first potential and the concentration of superoxide anion in the reference sample. For example, the relationship may be expressed by a second degree polynomial of formula (b)

$$I = C + n[\text{superoxide anion}] + m[\text{superoxide anion}]^2 \qquad \text{formula (b)}$$

wherein I, C, n and [superoxide anion] are as defined above and m is a further coefficient. Again, C, n and m may be determined in a calibration process by measuring I over a range of known superoxide anion concentrations in the reference sample. Higher degree polynomials relating I and [superoxide anion] can also be used, such as third degree polynomials, fourth degree polynomials, and so on. Other functional forms can be used and the most appropriate can be determined by the skilled person. However, in many circumstances, the relationship between I and [superoxide anion] has been found to be sufficiently linear that formula (a) can be used and is adequate for determination of the concentration in a sample of interest.

The voltammetry or chronoamperometry experiment may be carried out using a suitable electrochemical analytical device, for example a potentiostat.

The determining of the concentration of the superoxide anion in the sample of interest may be carried out automatically using an appropriate calculation medium, which may be a computer program. The computer program may be stored/distributed on a suitable medium, such as an optical storage medium or a solid-state medium supplied together with or as part of the electrochemical sensor, but may also be distributed in other forms, such as via the Internet or other wired or wireless telecommunication systems.

In an alternative embodiment, the electrochemical information can be compared against a database containing the electrochemical information at a range of known superoxide concentrations, e.g. in a reference sample as described herein, to give a value for the superoxide concentration in the sample of interest.

The calibration process may be an automatic calibration step carried out by an electrochemical sensor.

The sample of interest may contain superoxide anions in a concentration of at least 0.059 nM. The method may be used to calculate the concentration of superoxide anions in the sample of interest of 0.059 nM or more.

The method may be used in the diagnosis of chronic granulomatous disorder. In an aspect, there is provided a method of diagnosis of chronic granulomatous disorder,

involving the method of determining the presence and/or concentration of superoxide anions, as described herein. The sample of interest is preferably a sample that has been removed from a human body, and preferably the method of determining the presence and/or concentration of superoxide anions is carried *in vitro*. An absence of superoxide anions or a concentration below a predetermined level, as determined using the method of the present invention, in the sample of interest may indicate the presence of chronic granulomatous disorder in the subject from which the sample of interest was drawn.

Electrochemical Sensor

In an embodiment, the method may be carried out in an electrochemical sensor, e.g. an electrochemical medical sensor. The present invention also provides a electrochemical sensor for carrying out the method; the sensor may be adapted to carry out the method, e.g. programmed to carry out the method.

In an aspect, there is provided an electrochemical sensor for determining the presence of and/or concentration of superoxide anions in a sample of interest,

wherein the sensor comprises an electrically conducting substrate having on a surface thereof a paste comprising organic liquid binder and electrically conductive particles dispersed in the binder,

the sensor being capable of being used in a step (a) involving:

contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species,

the sensor being adapted to (i) use the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information, and then (ii) use the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest. The sensor may be adapted to carry out the method described herein. Step (i) may correspond to step (b) in the method, and may be as described herein for the method. Step (ii) may correspond to step (c) in the method, and may be as described herein for the method. "Adapted to" in the present context may indicate that the sensor is programmed to carry out steps (i) and (ii).

The sensor is capable of being used in a step (a), as described above. This does not necessarily imply that the sensor is adapted to, e.g. programmed to, carry out step (a). In an embodiment, step (a) may be carried out manually by a human user of the

electrically conducting substrate having on a surface thereof the paste. Alternatively, the sensor may be adapted to, e.g. programmed to, carry out step (a). In an embodiment, the sensor may be adapted to carry out any of, or all of, steps (a), (i) and (ii) in an automated way. The sensor may use the predetermined relationship between the electrochemical information and known concentrations of superoxide in a reference sample, which may have been determined by a calibration process, to determine the concentration of superoxide anions in the sample of interest. In an embodiment, the sensor is adapted to, e.g. programmed to, carry out the calibration process described herein, to determine the predetermined relationship between the electrochemical information and known concentrations of superoxide in a reference sample.

All features as described above in relation to the method are equally applicable to the sensor. For example, the electrically conducting substrate, the paste, the comprising organic liquid binder, the electrically conductive particles, the first and second species, may be as described herein for the method.

The electrochemical sensor may comprise a working electrode, a counter electrode and an electrolyte-containing medium in contact with the working electrode and the counter electrode. The working electrode is sometimes termed a sensing electrode, and in the present context is the electrically conducting substrate having on a surface thereof the paste comprising organic liquid binder and electrically conductive particles dispersed in the binder. The working and counter electrodes may be disposed opposite one another or the working and counter electrodes may be disposed on the same face of a substrate and spaced apart from one another. The sensor may further comprise a reference electrode. The working electrode, counter electrode, the electrolyte, and, if present, the reference electrode are typically in a housing.

The shape and configuration of the counter and (if present) reference electrodes is not particularly restricted. The electrodes may be in the form of points, lines, rings and flat planar surfaces. In an embodiment, the working electrode and the counter electrode are disposed opposite one another within a housing. In an alternative embodiment, the working and reference electrodes are disposed on the same face of a substrate. In an embodiment, the electrodes are disposed on the same face of the substrate and form an interlocking pattern.

In the method, in the sensor, the electrodes may each be supported on a further substrate, which may form part of a housing optionally enclosing the electrodes and any carrier medium or electrolyte that is in contact with the electrodes. The further substrate and/or housing may comprise any inert, non-conducting material, which may be selected from, but is not limited to, ceramic, plastic and glass.

The counter and, if present, reference electrodes each comprise any suitable electrically conducting material, e.g. a metal, an alloy of metals and/or carbon. The working, counter and, if present, reference electrodes may comprise a transition metal, for example a transition metal selected from any of groups 9 to 11 of the Periodic Table. The working, counter and, if present, reference electrode may each independently comprise a metal selected from, but not limited to, rhenium, iridium, palladium, platinum, copper, indium, rubidium, silver and gold.

In an embodiment, the method is carried out in an electrochemical sensor comprising a working electrode and a counter electrode, wherein the working electrode and counter electrode are used to determine the potential at which the first and/or second species, preferably the second species, is oxidized or reduced.

The electrochemical sensor may be calibrated to take into account the temperature when calculating the concentration of the superoxide anions in the sample of interest.

The electrochemical sensor may comprise a separable device that can control the electrochemical sensor such that the sensor carries out the method described herein; the separable device may carry out the step (c) as described herein. The electrochemical sensor and/or separable device may contain an appropriate computer program for controlling the electrochemical sensor and/or separable device, such that the method as described herein is carried out. The computer program may be on suitable hardware, firmware or other storage medium that may form part of the electrochemical sensor and/or the separable device.

Also provided herein is an electrically conducting substrate for use as a working electrode, the electrically conducting substrate having on a surface thereof a paste an comprising organic liquid binder and electrically conductive particles dispersed in the binder, wherein the binder further comprises a first species and/or a second species,

wherein the first species is capable of reacting with superoxide to form the second species, and either the first and/or second species can be detected in an electrochemical test. The first species may be capable of selectively reacting with or largely selectively reacting with superoxide to form the second species. "Selectively reacting with" or "largely selectively reacting with" superoxide may indicate that in the sample of interest, the superoxide reduces the first species, but none or substantially none of the other components in the sample of interest reduces the first species. For example, in a biomaterial such as a blood sample, the superoxide reduces the first species, but none or substantially none of the other components in the biomaterial reduces the first species. The electrically conducting substrate, the paste, the organic liquid binder, the electrically conductive particles, the first species and the second species may be as described above for the method. In an embodiment, the first species can be, or can comprise, a nitrogen-containing heterocycle, preferably an aromatic heterocycle, and/or or a nitroxide-containing moiety. In another embodiment, the first species can be, or can comprise, a tetrazole, and/or the second species is or comprises a formazan. In an embodiment, the first species can be, or can comprise, a nitrogen-containing heterocycle, e.g. a tetrazole, having one or more substitutents, wherein at least one of the substitutents is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group. In an embodiment, the first species can be, or can comprise, a plurality of nitrogen-containing heterocycles, e.g. a plurality of tetrazole rings, each heterocycle having one or more substitutents, wherein at least one of the substitutents is a nitro group or an aryl group, e.g. a phenyl group, substituted with a nitro group, and preferably the tetrazole rings are linked to one another by an aryl system, e.g. a biphenyl group, wherein each ring of the biphenyl group may have one or more substituents, e.g. oxyalkyl, e.g. oxymethyl; and optionally each of the tetrazole rings has an aryl substituent, e.g. a phenyl substituent.

In an embodiment, the first species is selected from a nitroblue tetrazolium halide, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide, 5-methyl-phenazinium methyl sulfate, 1-methoxy-5-methyl-phenazinium methyl sulfate, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2Htetrazolium inner salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, and 2,3,5-triphenyl-2H-tetrazolium chloride. In an embodiment, the first

species is a tetrazole and the second species is a formazan. The halide of the nitroblue tetrazolium halide may be selected from chloride, bromide and iodide.

In an embodiment, the first species is selected from nitroblue tetrazolium chloride (NBTC), mitochondria-targeted hydroethidine, *p*-benzoquinone, superoxide dismutase, cytochrome c, coelenterazine, ascorbic acid, thiols, glutathione and lucigenin.

In a further embodiment, the first species can be nitroblue tetrazolium chloride (NBTC), which is shown in Scheme 1.

Embodiments of the present invention will now be described with reference to the following non-limiting Examples and the accompanying drawings.

Examples

In the Examples below, a carbon paste electrode with an unmodified surface is used. By immersing the paste electrode in aqueous superoxide solutions of varying concentrations, different amounts of superoxide can be pre-concentrated in the pasting liquid dioctyl phthalate. NBTC is a compound with proven high specificity for superoxide. Dioctyl phthalate can be pre-concentrated with NBTC by immersing the paste electrode in an aqueous NBTC solution, which acts as the source of NBTC. NBTC can thus be reduced by superoxide in the binder material. The oxidation of the produced diformazan is then observed at ca. +0.69 V (vs. SCE) and this peak is used as the superoxide detection signal.

In the following examples, all reagents were purchased from Aldrich (Gillingham, U.K.), at the highest grade available, and were used as received, without any further purification. These were nitroblue tetrazolium chloride (NBTC), dimethyl sulfoxide (DMSO), sodium hydroxide (NaOH), potassium superoxide (KO_2), potassium ferricyanide ($K_3Fe(CN)_6$), dioctyl phthalate, graphite powder (particles < 20 μm), potassium phosphate monobasic (K_2HPO_4), potassium phosphate dibasic ($K_2H_2PO_4$) and potassium chloride (KCl). All aqueous solutions were prepared daily, at 298 K, using deionised water of resistivity of no less than 18.2 MΩ cm (25° C, Millipore UHQ, Vivendi, U.K.) as the solvent and KCl (0.1 M) as the supporting electrolyte. They were made at pH = 6.97, achievable through the use of appropriate K_2HPO_4/ $K_2H_2PO_4$ buffers and confirmed using a Hannah pH 213 pH meter. Where examples required the absence of oxygen, solutions were deoxygenated using oxygen-free nitrogen (N_2, BOC, Guildford, U.K.), in an air-tight environment, for at least 30 minutes. The measurements themselves were carried out under a light N_2 flow. For examples performed in O_2 saturated solutions, solutions were purged with O_2 (O_2, BOC, Guildford, U.K.), prior and during measurements in the same way as N_2.

Cyclic voltammetric measurements were recorded using a computer controlled Autolab potentiostat (PGSTAT 101, EcoChemie, Utrecht, Netherlands), in a home-built Faraday cage. A standard three-electrode configuration was used, with a glassy carbon macroelectrode (GC, 1.475 mm radius, IJ Cambria Scientific Ltd, Llwynhendy, U.K.), a Carbon Paste Electrode (CPE, 1.97 mm radius, 1.00 mm depth, made in-house) and a Carbon Fibre microelectrode (CF, 5.819 μm radius, BASi Technicol, West Lafayette,

IN) acting as working electrodes. A platinum wire (99.99 % GoodFellow, Cambridge, U.K.) was utilised as the counter electrode and a Standard Calomel reference electrode (SCE, Bas Inc, Japan) completed the assembly. All examples were carried out in a thermostated water bath, at a temperature of 25 ± 0.1 ^{o} C. Voltammetric simulations were achieved through the use of in-house programmes [38, 39]

The carbon paste electrode holder was made from a copper rod of radius of 1.97 mm running through a Teflon rod (for electrical contact), leaving a 1.00 mm deep cavity at the edge. The paste was made with 4.26 g of graphite powder (particles < 20 μm) and 1.40 mL dioctyl phthalate. It was then characterised in a 0.35 mM $K_3Fe(CN)_6$, phosphate buffer solution containing 0.1 M KCl. A variable scan rate experiment (25 – 800 mV s^{-1}) gave a diffusion coefficient equal to 7.2 (± 0.4) x 10^{-6} cm^2 s^{-1}, in agreement with the literature value of 7.6 x 10^{-6} cm^2 s^{-1} [40], indicating that the paste electrode could be used quantitatively.

The glassy carbon macroelectrode, as well as the carbon fibre microelectrode, was polished using diamond sprays of decreasing particle size (3.0 μm, 0.1 μm and 0.01 μm, Kemet Ltd, U.K.). Both were briefly sonicated in deionised water, between each polishing step and before a measurement was recorded, to remove any adhered diamond particles. The carbon paste electrode did not need to be polished; instead, its surface was renewed between each scan by cleaning the holder and packing fresh paste.

Cyclic voltammetric scans were, unless otherwise stated, carried out between +1.2 and -0.40 V (vs. SCE), which is outside of the potential window required for the reduction of the nitro groups but sufficient to reduce the tetrazole rings of the NBTC.

Example 1

The Synthesis of Superoxide

The UV Visible spectra of different KO_2 solutions (0.12 – 1.69 mM) in DMSO were recorded, using a Hitachi U-2001 spectrophotometer. An extinction coefficient of 1010 L mol^{-1} cm^{-1} was determined, which was in agreement with literature [36]. The superoxide used in the solutions in which the sensor was tested was synthesised using the method proposed by Hyland et al [37], whereby an air-saturated DMSO solution

containing 0.55M H_2O and 5mM NaOH was prepared. By recording the UV Visible spectrum of the resultant solution and using the previously calculated extinction coefficient, the initial superoxide concentration was determined as being 0.22 mM.

Example 2

Characterisation of the electrochemical behaviour of NBTC with a Glassy Carbon Electrode

To study the electrochemical reduction of NBTC, a cyclic voltammetry variable scan rate study (25-800 mV s^{-1}) was completed in a deoxygenated aqueous phosphate buffer solution, containing 0.095 mM NBTC and 0.1 M KCl, at pH = 6.97. A glassy carbon macroelectrode was used and, as shown in Figure 1, a clear reduction peak can be seen at around -0.25 V (vs. SCE). The oxidation of the produced formazan is observed at around +0.93 V (vs. SCE) during the reverse scan.

A Tafel analysis (shown in Figure 2) was performed to obtain an average value of the transfer coefficient for the reduction process, α_{ave}. An average value of 0.98 ± 0.05 was generated, indicating that the first electron transfer was electrochemically reversible. Given the observed transfer coefficient and the likely stoichiometry of the electrode process (see Scheme 1 above), the following mechanism (Equations 1a and 1b) was considered, in which a reversible one-electron process is followed by a total of *(m-1)* fully driven one-electron transfers, giving an m-electron process overall:

$$NBT^{2+} + e^- \leftrightarrows NBT^{\cdot+} \qquad \text{(Equation 1a)}$$

$$NBT^{\cdot+} + (m\text{-}1)\, e^- \rightarrow \text{products} \qquad \text{(Equation 1b)}$$

The data in the inlay of Figure 1 was thus analysed assuming the following form of the Randles-Ševčik equation ([42] – Equation 2)

$$I_p = \boldsymbol{m}(2.69 \times 10^5) v^{1/2} A c D^{1/2}, \qquad \text{(Equation 2)}$$

where m is the total number of electron transfer reactions in the process giving rise to the voltammetric peak of interest, v is the scan rate (V s^{-1}), A is the electrode area (cm^2), c the surface concentration of the analyte (mol cm^{-3}) and where D is the diffusion

coefficient ($cm^2\ s^{-1}$). Depending on the value of m (=1, 2, 3 or 4), different values of D were obtained.

Example 3

Characterisation of the electrochemical behaviour of NBTC with a Carbon Fibre Microelectrode

In order to identify the correct choice, cyclic voltammetric experiments were next conducted in a deoxygenated aqueous phosphate buffer solution, containing 0.078 mM NBTC and 0.1 M KCl, at pH = 6.97, on a carbon fibre *microelectrode* for four scan rates between 100 and 800 mV s^{-1}. The results are shown in Figure 3. The steady state current was seen to increase greatly with scan rate and in-house simulation programmes [38, 39] were used to simulate the results for the microdisc voltammograms. The programmes simulated the mechanism described by Equations 1a and 1b.

Figure 4 shows that the best agreement between the theoretical and experimentally obtained current values was achieved by using the diffusion coefficient obtained for m=4, $D=7.2\times10^{-6}\ cm^2\ s^{-1}$. Thus the process likely corresponds to a four one-electron step process, where all steps after the first are indeed fully driven, as shown by Equations 3a-3d.

$$NBT^{2+} + e^- \leftrightarrows NBT^{\cdot+} \qquad \text{(Equation 3a)}$$

$$NBT^{\cdot+} + e^- \rightarrow NBT^{\cdot} \qquad \text{(Equation 3b)}$$

$$NBT^{\cdot} + e^- \rightarrow NBT^{-} \qquad \text{(Equation 3c)}$$

$$NBT^{-} + e^- \rightarrow NBT^{2-} \qquad \text{(Equation 3d)}$$

Protonation is here neglected, but see Scheme 1 above.

Example 4

Characterisation of the electrochemical behaviour of Diformazan

To complete the characterisation of the electrochemical behaviour of NBTC in this system, a 100 mV s^{-1} oxidative scan was first carried out on the glassy carbon macroelectrode, from +0.15 to +0.95 V (vs. SCE). Since the large back peak seen at

ca. +0.93 V (vs. SCE) in Figure 1 was not observed, it was concluded that the peak was in fact a consequence of the oxidation of the diformazan as opposed to the oxidation of the starting material.

Example 5

Characterisation of the adsorptive properties of Diformazan

The adsorptive properties of the diformazan were next investigated, on the glassy carbon macroelectrode, through a cyclic voltammetric transfer-holding experiment. For this, first a negative scan, from +0.20 V to -0.35 V (vs. SCE), was carried out in an aqueous phosphate buffer solution, containing 0.1 mM NBTC and 0.1 M KCl, at pH = 6.97. The potential was then held at -0.35 V (vs. SCE), for increasing holding times, after which the reverse scan was completed in a *fresh* deoxygenated aqueous phosphate buffer solution, which was of the same pH but, importantly, *only* contained 0.1 M KCl and no NBTC. As can be seen in Figure 5, the observed current increased with increasing holding time, consistent with increasing amounts of the diformazan being adsorbed and with the material being bound to the electrode surface when oxidised. The plateau seen in the inlay at 40s indicates, on the basis of the charge passed, that an apparent monolayer is formed. These observations are consistent with Equations 4a-4c.

$NBT^{2+} + 4e^- + 4H^+ \leftrightarrows NBT$	Reduction Scan	(Equation 4a)
$NBTDF \rightarrow NBTDF_{(ads)}$		(Equation 4b)
$NBTDF_{(ads)} - ne^- \rightarrow$ products	Oxidation Scan	(Equation 4c)

Example 5

Discounting the effect of Oxygen

The reduction of O_2 in an oxygen saturated (pure O_2 - 1.24 mM [43]) phosphate buffer solution, containing 0.1 M KCl at pH = 6.97, was next investigated, on the glassy carbon substrate, by cyclic voltammetry. The results are shown in Figure 6. As expected, one two-electron peak was seen in the reductive scan at around -0.81 V (vs. SCE), while no peak was observed in the reverse sweep, according to $O_2 + 2e^- \rightarrow H_2O_2$.

$$I_p = (2.99 \times 10^5)n(n' + \alpha)^{1/2}v^{1/2}AcD^{1/2} \qquad \text{(Equation 5)}$$

The average α value calculated through Tafel analysis (shown in Figure 7) was α_{ave} = 0.24 ± 0.06, showing that the first step was rate determining, and that hence n' = 0, where n' is the number of electrons transferred *before* the rate determining step (RDS). Utilising the irreversible Randles-Ševčik equation (Equation 5), where n is the number of electrons transferred and where the rest of the terms have been previously defined, D_{O2} was found to be equal to 1.8×10^{-5} cm^2 s^{-1} (see Figure 8); both α and D are in agreement with literature [44-48]. By then recording the cyclic voltammetric responses, on the glassy carbon macroelectrode, of systems in which both NBTC *and* O_2 were present in solution, it was established that the presence of O_2 did not cause a significant interference with the electrochemistry of NBTC (see Figure 9). The solutions used were once again aqueous phosphate buffer solutions, containing 0.1 M KCl, at pH = 6.97, though they contained both NBTC (0.095 mM) and O_2 (1.24 mM).

Example 6

Characterisation of the electrochemical behaviour of NBTC with a Carbon Paste Electrode

A carbon paste electrode was also used in the study of the reduction of NBTC in water, in order to take advantage of an interesting feature of such surfaces; the fact that species can dissolve and accumulate in the liquid binder if they have a high solubility and stability in it. This has been previously observed to happen with oxygen when the oil dioctyl phthalate is, as in this case, used as the pasting liquid [34], and with ferrocene when mineral oil is used as the binder [49]. In such studies, in order for any results obtained to be meaningful, it is vital to ensure that the amount of relative materials present in the paste does not vary. A cyclic voltammetry transfer experiment was hence carried out to determine the necessary pre-concentration time of the paste electrode with NBTC.

The paste electrode was thus immersed in a *deoxygenated* aqueous phosphate buffer solution, containing 0.147 mM NBTC and 0.1 M KCl, at pH = 6.97, for different pre-concentration times, under open-circuit conditions. A potential cycle between +0.85 and -0.35 V (vs. SCE) was then conducted in a fresh *deoxygenated* aqueous

phosphate buffer solution, of the same pH and which *only* contained 0.1 M KCl and no NBTC. The peak corresponding to the reduction of NBTC was seen at around -0.23 V (vs. SCE), while the peak due to the oxidation of the generated diformazan was observed at around +0.68 V (vs. SCE).

The results of this example are presented in Figure 10, where a clear increase of the peak current with pre-concentration time can be observed (see inlay). As is depicted in the inlay, the peak current reaches a plateau at 90s. It was thus concluded that NBTC does in fact dissolve and accumulate in dioctyl phthalate and that the paste is equilibrated with NBTC after 90s.

Example 7
Evaluation of the electrochemical behaviour of NBTC and Superoxide with a Carbon Paste Electrode

Having shown that NBTC can build up in dioctyl phthalate, it was surmised that this would also be the case with superoxide and hence the reduction of NBTC by superoxide might take place *in* the paste. Superoxide was thus synthesised using the method proposed by Hyland et al [37], whereby NaOH and H_2O are added to an air-saturated DMSO solution. The carbon paste electrode was first immersed in the produced 0.22 mM superoxide solution for 40s, under open-circuit conditions. Next, it was immersed in a deoxygenated aqueous phosphate buffer solution, containing 0.147 mM NBTC and 0.1 M KCl, at pH = 6.97, for different pre-concentration times, again under open-circuit conditions. For each NBTC pre-concentration time, a 100 mV s^{-1} oxidative scan was carried out, from 0.15 V up to +0.85 V (vs. SCE), in a deoxygenated phosphate buffer solution, of the same pH and which contained *only* 0.1 M KCl (no NBTC). The results are presented in Figure 11 where the peak observed at around +0.74 V (vs. SCE) corresponds to the oxidation of the now chemically produced diformazan.

Through the presence of the diformazan oxidation peak, it was concluded that the reduction of NBTC was indeed occurring *in* the paste. It was thus inferred that the superoxide radical can transfer into the paste, noting its high stability in organic media [50-51] such as dioctyl phthalate. Importantly, this interpretation is consistent with the increasing size of the diformazan oxidation peak with increasing NBTC pre-

concentration time (see inlay of Figure 11). The trend reflects the increasing amount of NBTC accumulating in the liquid binder and being reduced by superoxide, which results in increasing amounts of diformazan being oxidised. At pre-concentration times longer than 90s, as was previously observed, the paste is equilibrated with NBTC. The maximum possible amount of diformazan is then produced and it is this that leads to the plateau shown in the inlay of Figure 11. The optimum pre-concentration time with NBTC was thus identified as being 90s.

Example 8

Evaluation of the electrochemical behaviour of NBTC with different concentrations of Superoxide Anions

Lastly, the original 0.22 mM superoxide solution was diluted in water in order to test the sensor in aqueous 0.059 – 1.88 nM superoxide solutions. The above optimised method was used and, for each superoxide concentration, the paste electrode was thus immersed first in the superoxide solution for the fixed time of 40s, under open-circuit conditions, and next equilibrated with NBTC, again under open-circuit conditions. A 100 mV s^{-1} oxidative scan was then carried out from +0.15 V to 0.85 V (vs. SCE), in a deoxygenated phosphate buffer solution containing *only* 0.1 M KCl (no NBTC), at pH = 6.97. The cyclic voltammetric responses obtained are shown in Figure 12, with a signal having been observed at ca. +0.69 V (vs. SCE) for concentrations as low as 0.059 nM. As expected, the obtained peak current decreases with decreasing superoxide concentration (see inlay of Figure 12) since decreasing amounts of diformazan are formed and then oxidised. Importantly, the fact that the lowest concentration for which a signal was seen was 0.059 nM determined the *practical* limit of detection as being 0.059 nM. The slope of the calibration curve (see inlay of Figure 12) gave a value of 1.79 μA nM^{-1} for the sensor sensitivity.

It is worth noting that the obtained *practical* limit of detection is *much* lower compared to (calculated) *theoretical* values shown in Table 1, below. The sensitivity of the method results from the accumulation of superoxide over 40s and as such mimics stripping voltammetry where nanomolar concentrations are routinely observed.

Table 1

Electrode	*Calculated LOD / nM*	*Sensitivity / μA nM^{-1}*	*Reference*
MWCNTs-Pt/GC	100	6.1 x 10^{-5}	22
DTSP-CytC/Au	73	2.8 x 10^{-9}	23
PTTCA-CytC/GC	50	-	25
SOD/Pt SPE	190	3.5 x 10^{-6}	26
Au-NS/SOD	100	5.9 x 10^{-7}	27

Table 1 shows the limit of detection (LOD) and sensitivity literature values for other superoxide sensors, where MWCNTs stands for multi-walled carbon nano-tubes, DTSP for dithiobis(succinimidyl)propionate, PTTCA for poly- 5,2':5'2''-terthiophene-3'-carboxylic acid, SOD for superoxide dismuthase, SPE for screen printed electrode and Au-NS for Au nanospherical electrode. Attention is drawn to the fact that the LOD values are *calculated* and hence may not represent the lowest superoxide concentration that can be practically detected.

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Each of the above individual references is included herein by reference in its entirety.

CLAIMS

1. A method of determining the presence of and/or concentration of superoxide anions in a sample of interest, the method comprising:

(a) providing an electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder, contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species,

(b) using the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the first and/or second species is oxidised or reduced and obtaining electrochemical information,

(c) using the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest.

2. The method of claim 1, wherein, in step (a) the first species is incorporated into the paste after the contacting of the paste with the sample of interest.

3. The method of claim 1, wherein, in step (a), the first species is reduced by the superoxide anions to form the second species.

4. The method of any one of the preceding claims, wherein the first species is a tetrazole.

5. The method of claim 4, wherein the second species is a formazan.

6. The method of any one of claims 1 to 3, wherein the first species is selected from a nitroblue tetrazolium halide, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide, 5-methyl-phenazinium methyl sulfate, 1-methoxy-5-methyl-phenazinium

methyl sulfate, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt, 5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2-thiazolyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt, sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2Htetrazolium inner salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, and 2,3,5-triphenyl-2H-tetrazolium chloride, mitochondria-targeted hydroethidine, *p*-benzoquinone, superoxide dismutase, cytochrome c, coelenterazine, superoxide dismutase and lucigenin.

7. The method of any one of the preceding claims, wherein the electrochemical information is selected from the potential, the peak current, area under the peak and/or peak charge at which the oxidation or reduction of the first and/or second species within the paste occurs in the electrochemical test.

8. The method of any one of the preceding claims, wherein the presence of superoxide anions in the sample of interest is indicated by electrochemical information indicative of the oxidation or reduction of the second species in the electrochemical test.

9. The method of any one of the preceding claims, wherein the presence of superoxide anions in the sample of interest is indicated by electrochemical information indicative of the oxidation of the second species to the first species.

10. The method of any one of the preceding claims, wherein the determining of the concentration of the superoxide anions in the sample of interest is carried out by using a predetermined relationship between the electrochemical information and known concentrations of superoxide in a reference sample.

11. The method of claim 10, wherein the electrochemical information is the peak current, area under the peak and/or peak charge.

12. The method of claim 10, wherein the electrochemical information is the height of a peak of current, which corresponds to the oxidation of the second species, optionally to the first species, and the predetermined relationship is represented by formula (a)

$$I = C + n[\text{superoxide anion}] \qquad \text{formula (a)}$$

wherein I is the height of a peak of current, C is a constant, and n is a coefficient, and [superoxide anion] is the concentration of the superoxide anion in the sample of interest.

13. The method of any one of the preceding claims, wherein the electrochemical test involves a voltammetry or an amperometry experiment.

14. The method of claim 13, wherein the voltammetry involves a voltammetry technique selected from cyclic voltammetry, square wave voltammetry, linear sweep voltammetry and pulse voltammetry.

15. The method of any one of the preceding claims, wherein the organic liquid binder is selected from a phthalate, mineral (paraffin) oils, aliphatic and aromatic hydrocarbons, silicone oils and greases, halogenated hydrocarbons, tricresyl phosphate (TCP), (nitrophenyl octyl ether) NPOE, diphenyl ether, glycerol and ionic liquids.

16. The method of any one of the preceding claims, wherein the organic liquid binder comprises or is an alkyl phthalate.

17. The method of any one of the preceding claims, wherein the organic liquid binder comprises or is a di-alkyl phthalate.

18. The method of any one of the preceding claims, wherein the combination of the electrically conducting substrate and the paste is a carbon paste electrode.

19. The method of any one of the preceding claims, wherein the sample of interest is or comprises a biomaterial drawn from an animal or a plant.

20. The method of any one of the preceding claims, wherein the sample of interest is drawn from a human and selected from a blood sample, a sweat sample, saliva and a urine sample.

21. The method of any one of the preceding claims, wherein the sample of interest comprises white blood cells, or biomaterial drawn from white blood cells.

22. A method of diagnosis of chronic granulomatous disorder, the method involving the method of determining the presence and/or concentration of superoxide anions as defined in any one of the preceding claims.

23. An electrochemical sensor for determining the presence of and/or concentration of superoxide anions in a sample of interest,

wherein the sensor comprises an electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder,

the sensor being capable of being used in a step (a) involving: contacting the paste with the sample of interest, wherein, if superoxide anions are present in the sample of interest, at least some of the superoxide anions are incorporated within the paste, wherein a first species is present within the paste at the time of contacting the paste with the sample of interest or a first species is incorporated into the paste after the contacting of the paste with the sample of interest, the superoxide anions then reacting with the first species to form a second species,

the sensor being adapted to (i) use the electrically conducting substrate, having on a surface thereof the paste, produced in step (a) as a working electrode in an electrochemical test that involves altering the potential at a working electrode to a potential at which the second species is oxidised or reduced and obtaining electrochemical information, and then (ii) use the electrochemical information to determine the presence of and/or concentration of superoxide anions in the sample of interest.

24. The electrochemical sensor of claim 23, wherein the sensor is adapted to carry out any of, or all of, steps (a), (i) and (ii) in an automated way.

25. An electrically conducting substrate for use as a working electrode, the electrically conducting substrate having on a surface thereof a paste comprising an organic liquid binder and electrically conductive particles dispersed in the binder, wherein the binder further comprises a first species and/or a second species, wherein

the first species is capable of reacting with superoxide to form the second species, and either the first and/or second species can be detected in an electrochemical test.

26. An electrically conducting substrate of claim 25, wherein the first species is or comprises a tetrazole and/or the second species is or comprises a formazan.

27. An electrically conducting substrate of claim 26, wherein the tetrazole is or comprises a nitroblue tetrazolium halide, and/or the second species is a formazan formed from reduction of a nitroblue tetrazolium halide with superoxide.

28. An electrically conducting substrate of any one of claims 25 to 27, wherein the combination of the electrically conducting substrate and the paste is a carbon paste electrode

FIG. 1

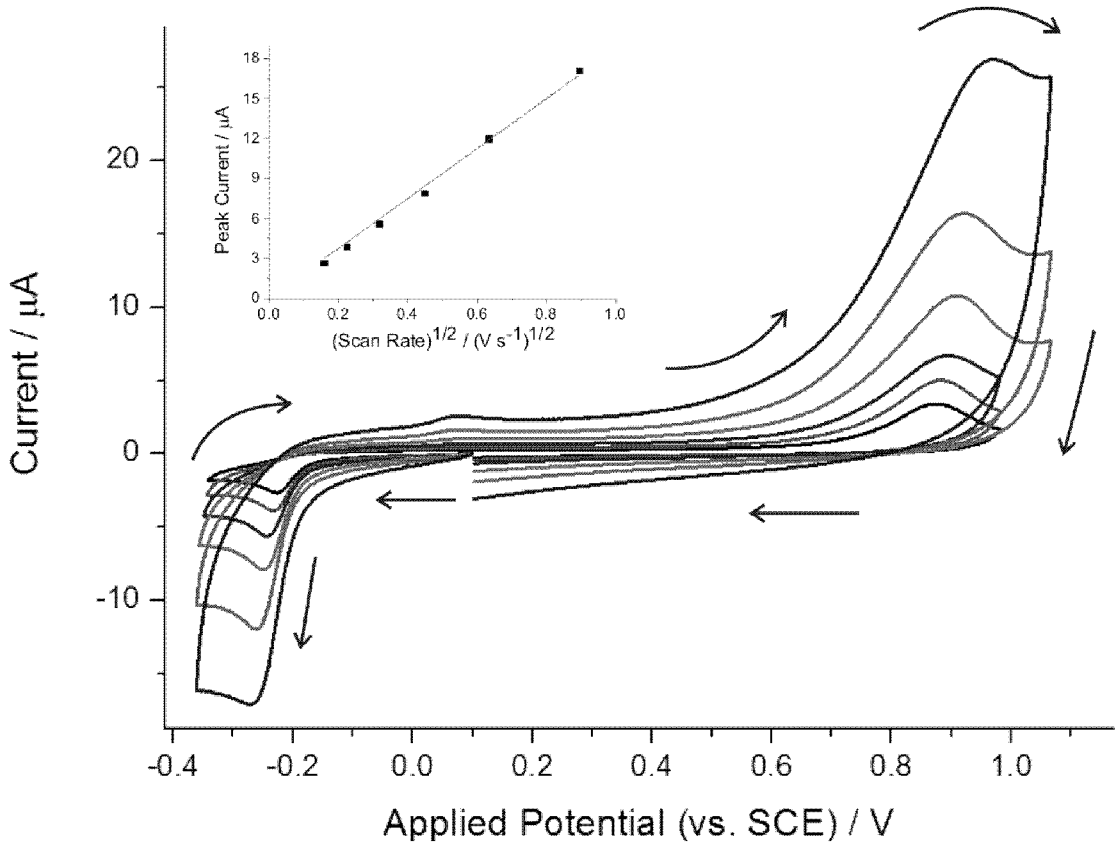

Current / µA
Applied Potential (vs. SCE) / V
Peak Current / µA
(Scan Rate)$^{1/2}$ / (V s^{-1})$^{1/2}$

FIG. 2

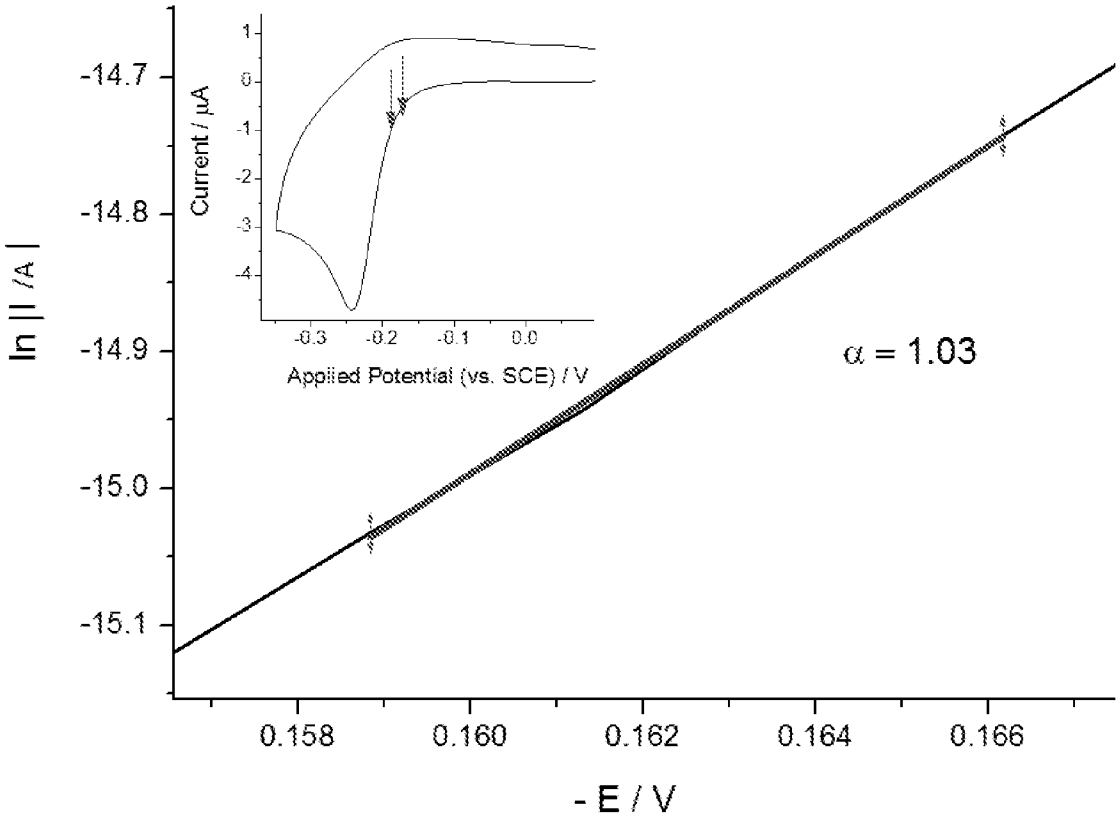

ln | I /A |
- E / V
Current / µA
Applied Potential (vs. SCE) / V
α = 1.03

FIG. 3

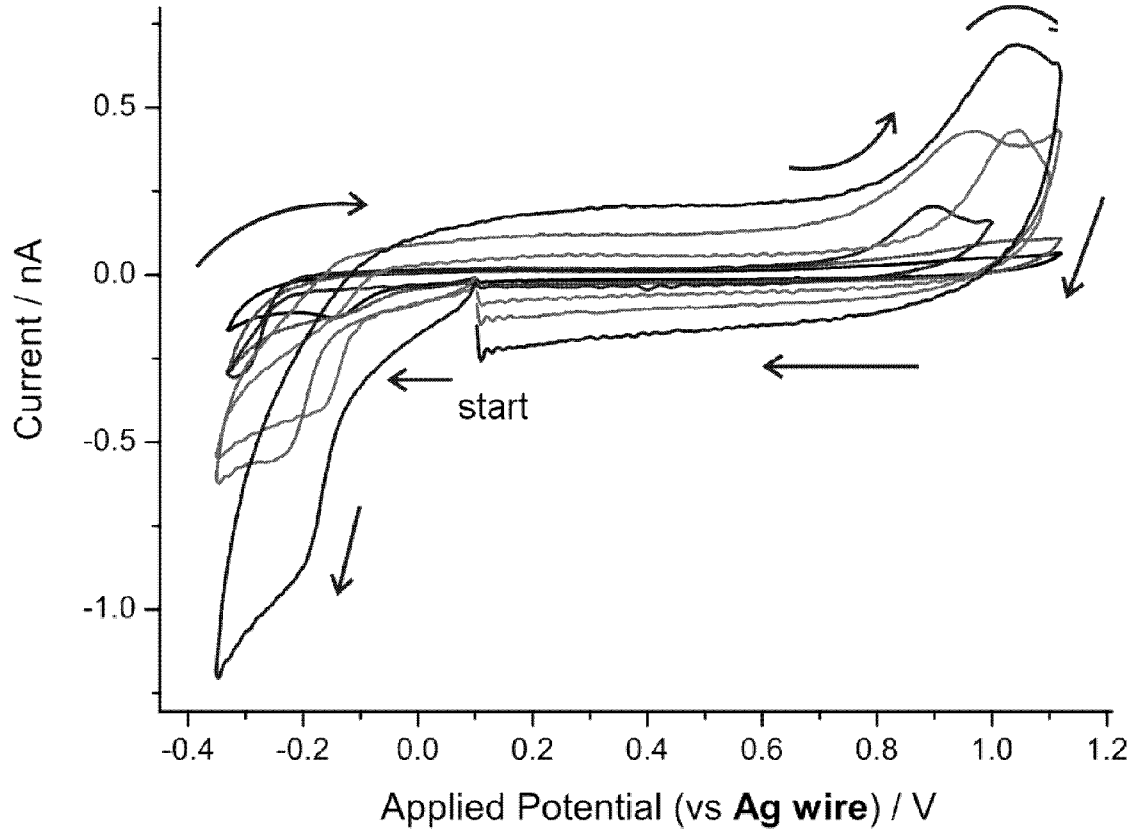

0.5
0.0
-0.5
-1.0
Current / nA
start
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Applied Potential (vs **Ag wire**) / V

FIG. 4

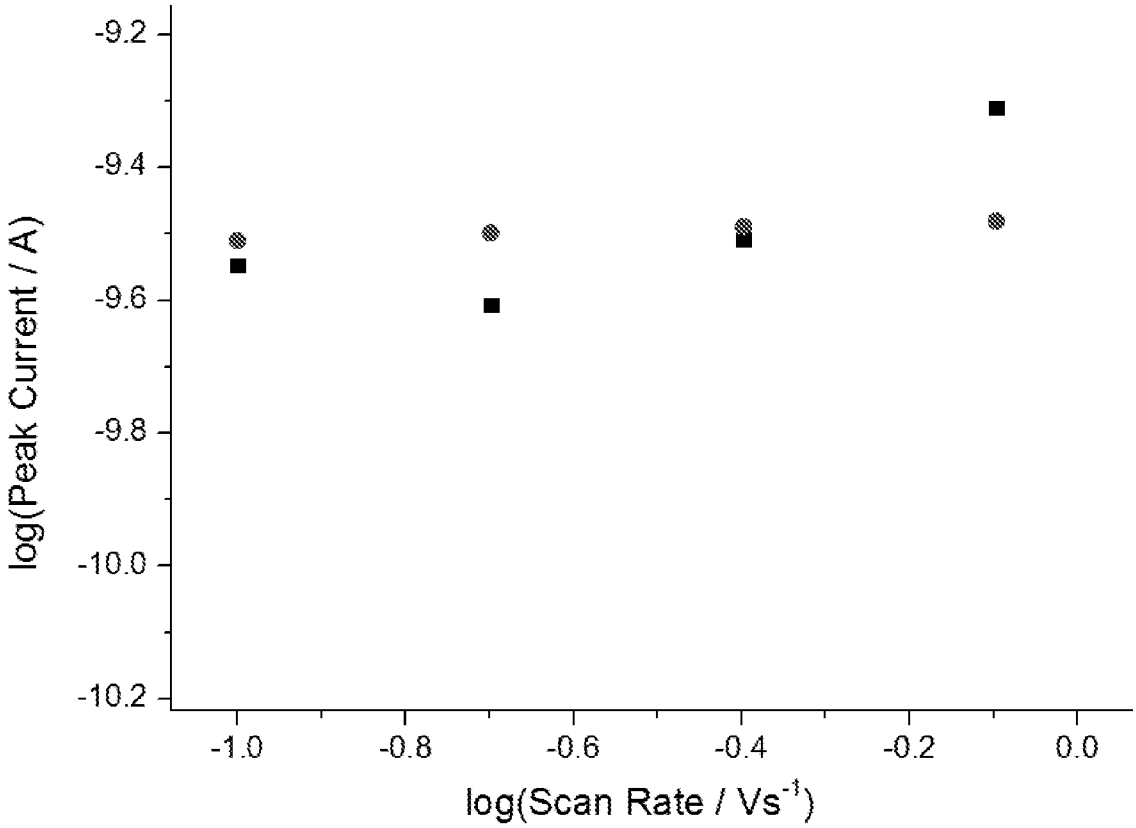

-9.2
-9.4
-9.6
-9.8
-10.0
-10.2
log(Peak Current / A)
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
log(Scan Rate / Vs^{-1})

FIG. 5

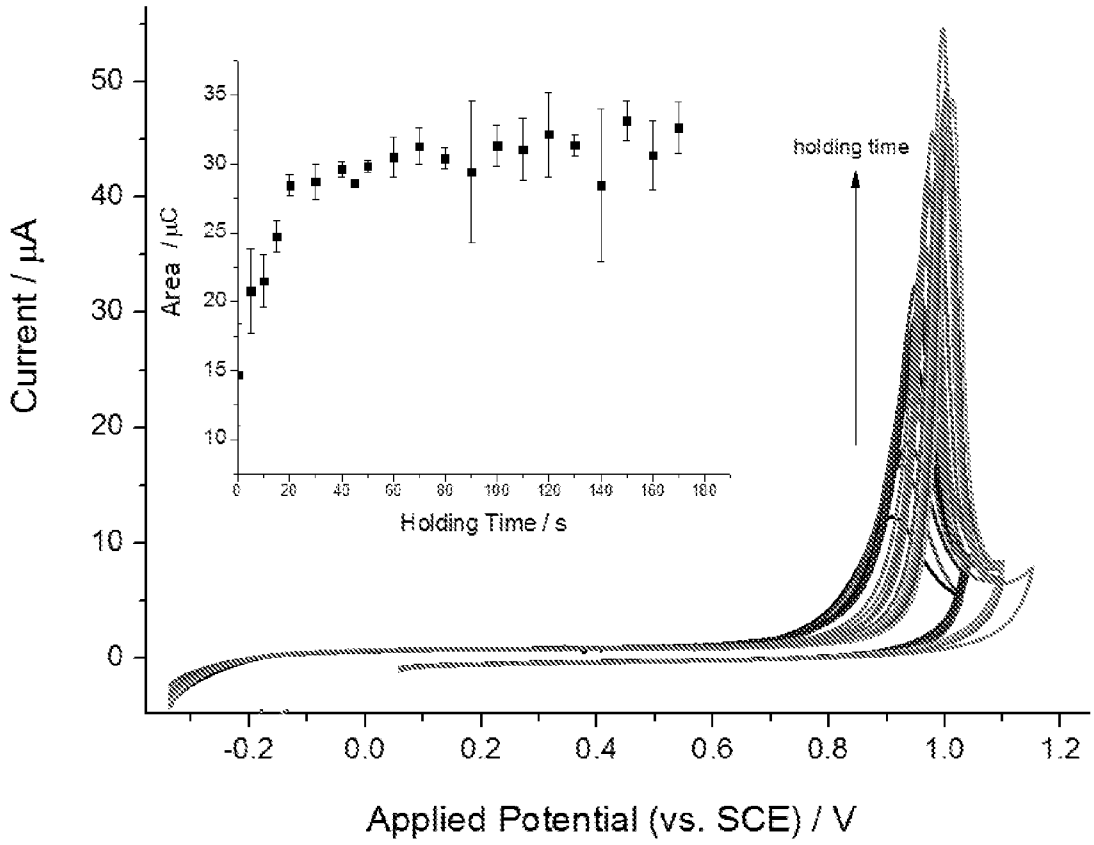

Current / µA
Applied Potential (vs. SCE) / V
holding time
Area / µC
Holding Time / s

FIG. 6

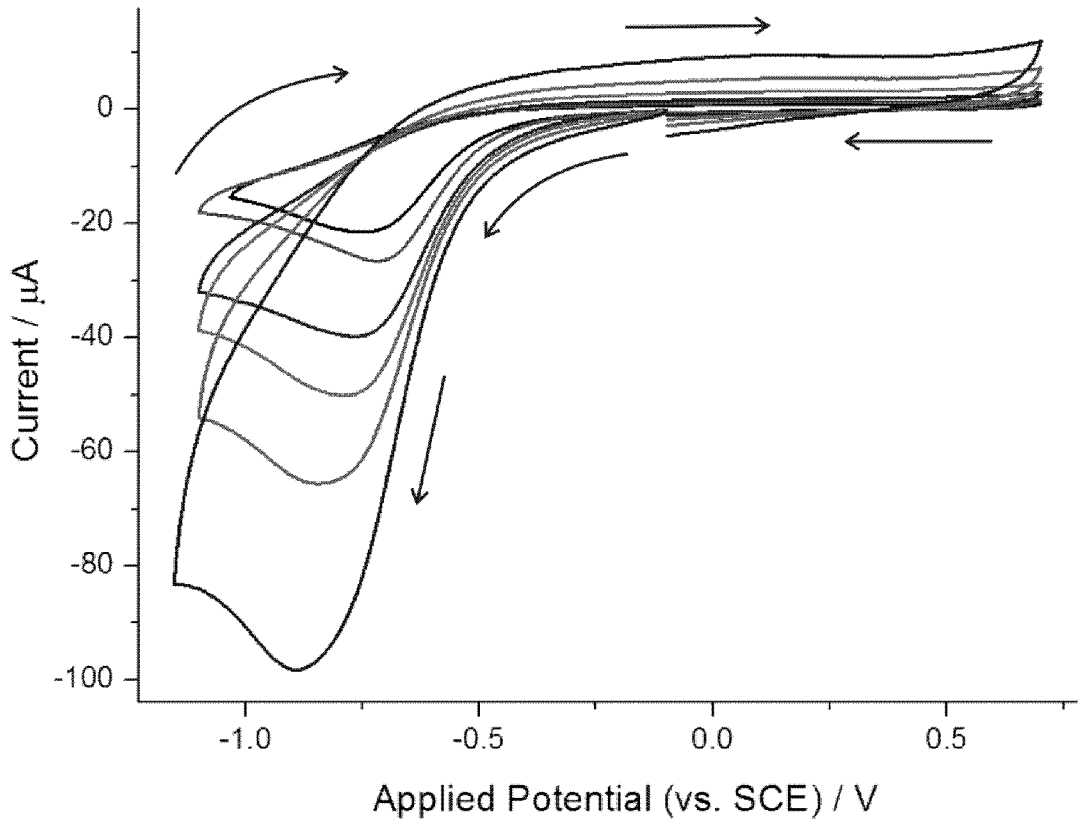

Current / µA
Applied Potential (vs. SCE) / V

FIG. 7

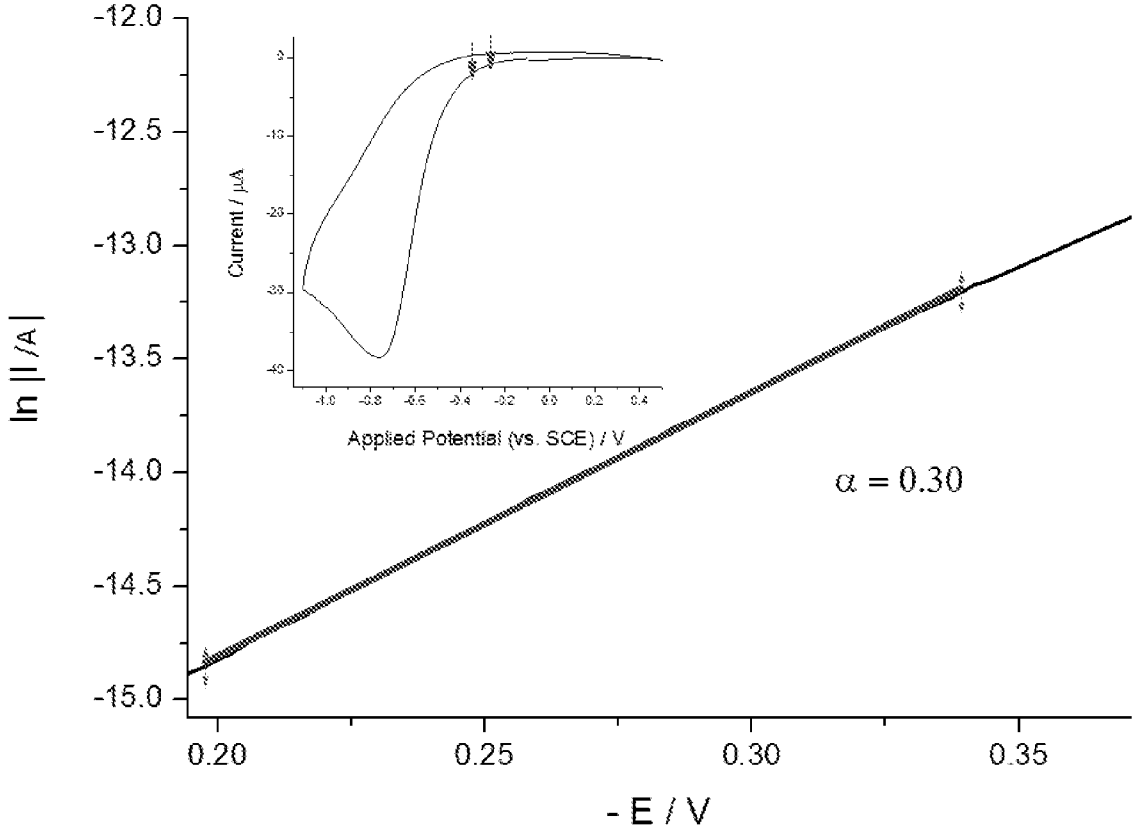

-12.0
-12.5
-13.0
-13.5
-14.0
-14.5
-15.0
ln |I /A|
0.20
0.25
0.30
0.35
- E / V
α = 0.30
Current / μA
Applied Potential (vs. SCE) / V

FIG. 8

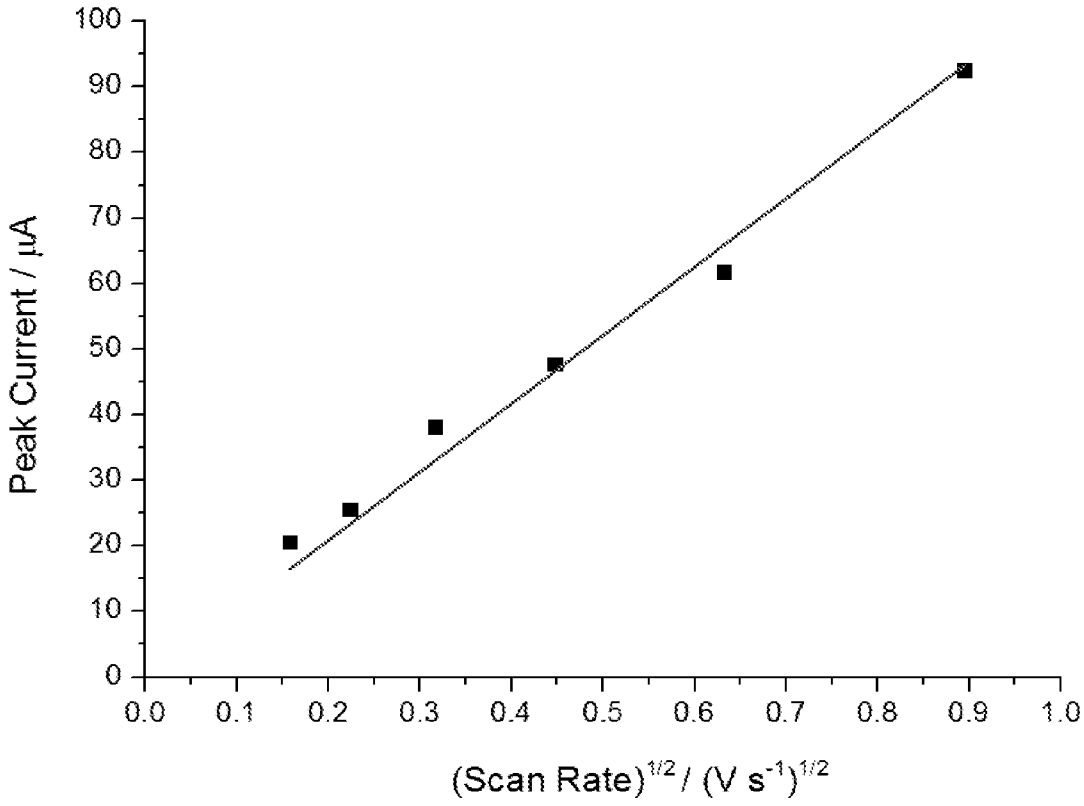

100
90
80
70
60
50
40
30
20
10
0
Peak Current / μA
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
(Scan Rate)1/2 / (V s-1)1/2

FIG. 9

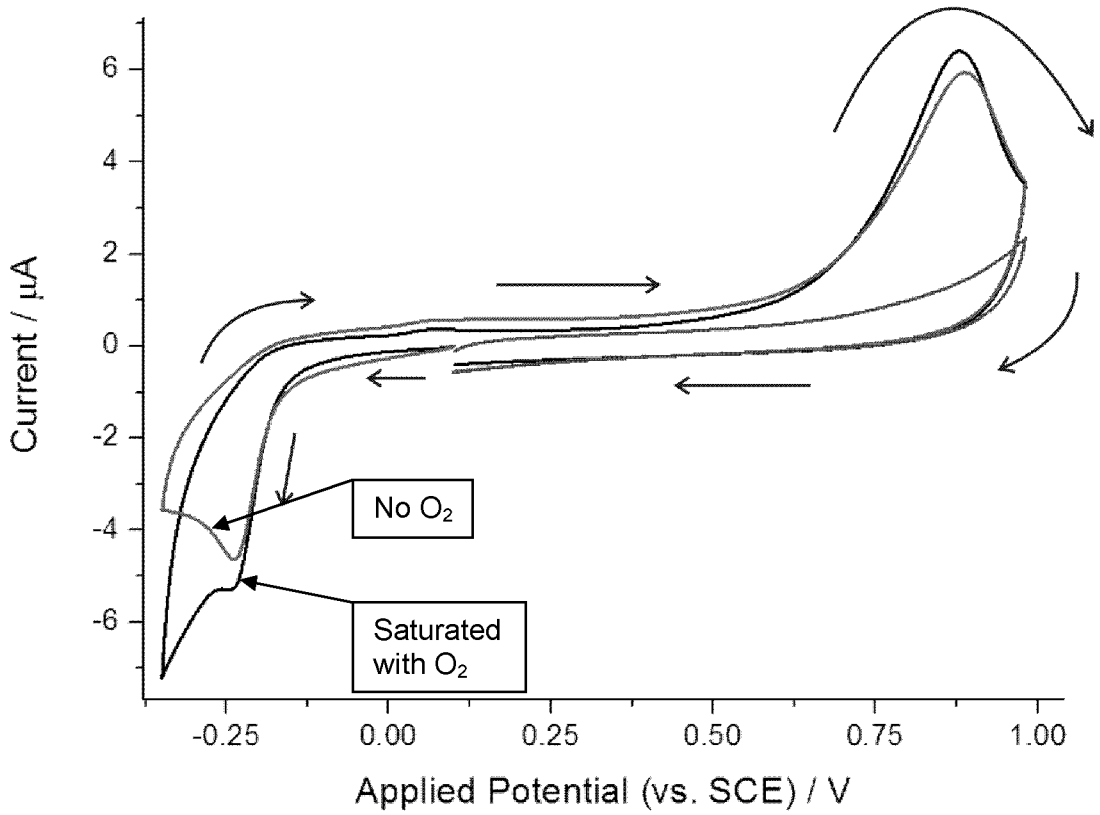

Current / µA
6
4
2
0
-2
-4
-6
No O_2
Saturated with O_2
-0.25
0.00
0.25
0.50
0.75
1.00
Applied Potential (vs. SCE) / V

FIG. 10

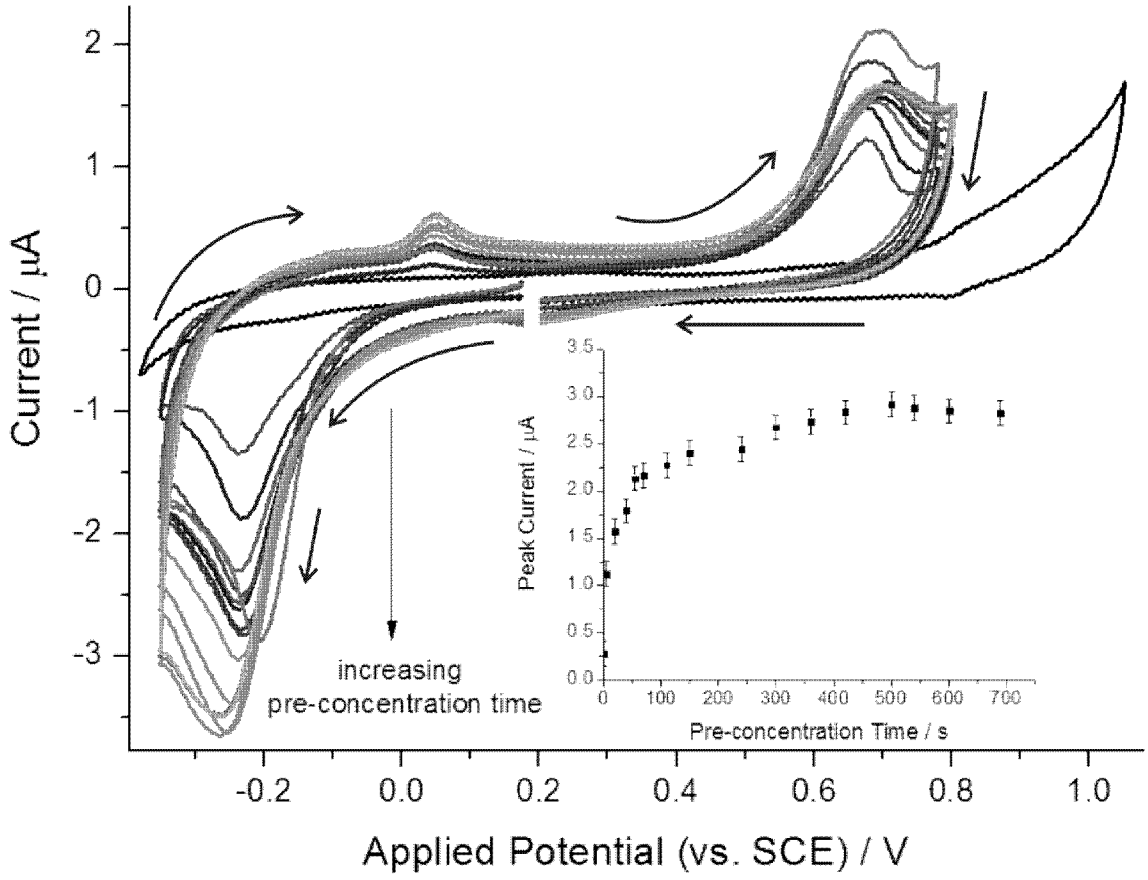

Current / µA
2
1
0
-1
-2
-3
increasing pre-concentration time
Peak Current / µA
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
100
200
300
400
500
600
700
Pre-concentration Time / s
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Applied Potential (vs. SCE) / V

FIG. 11

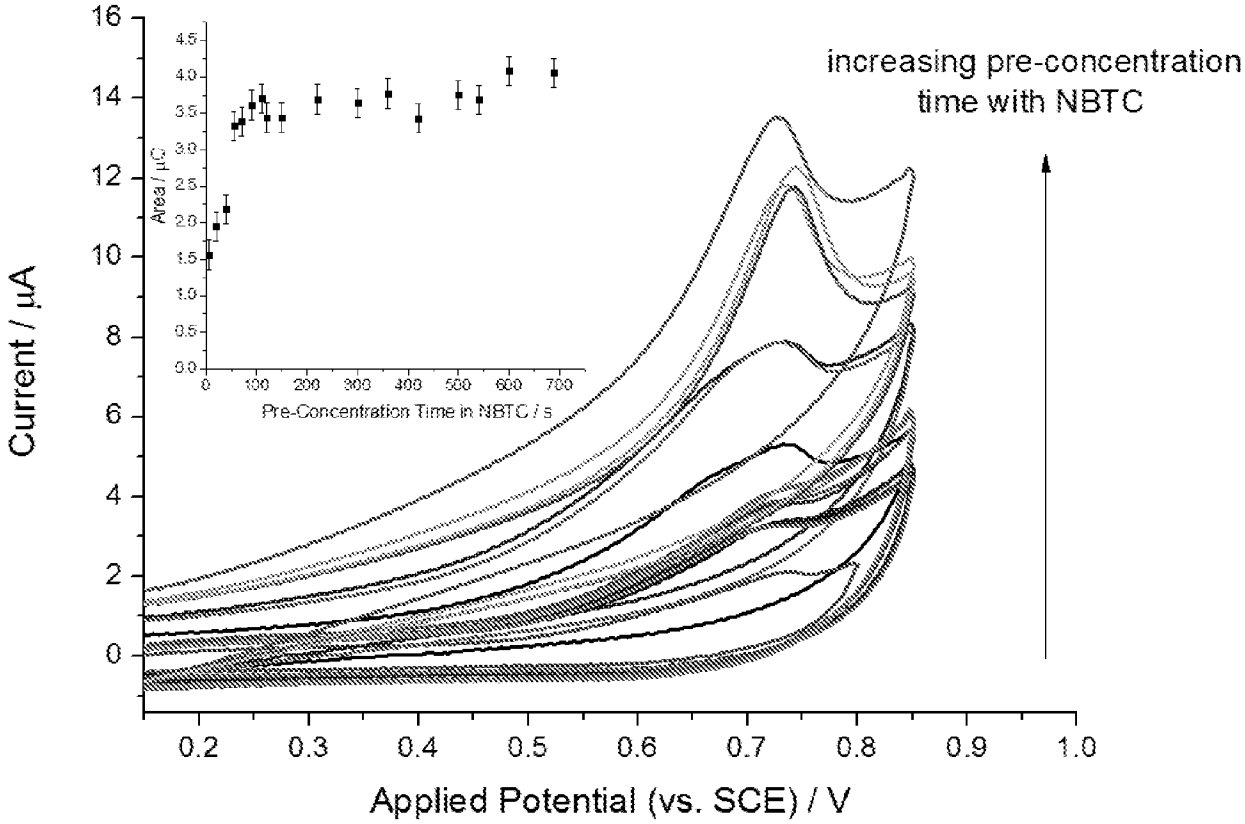
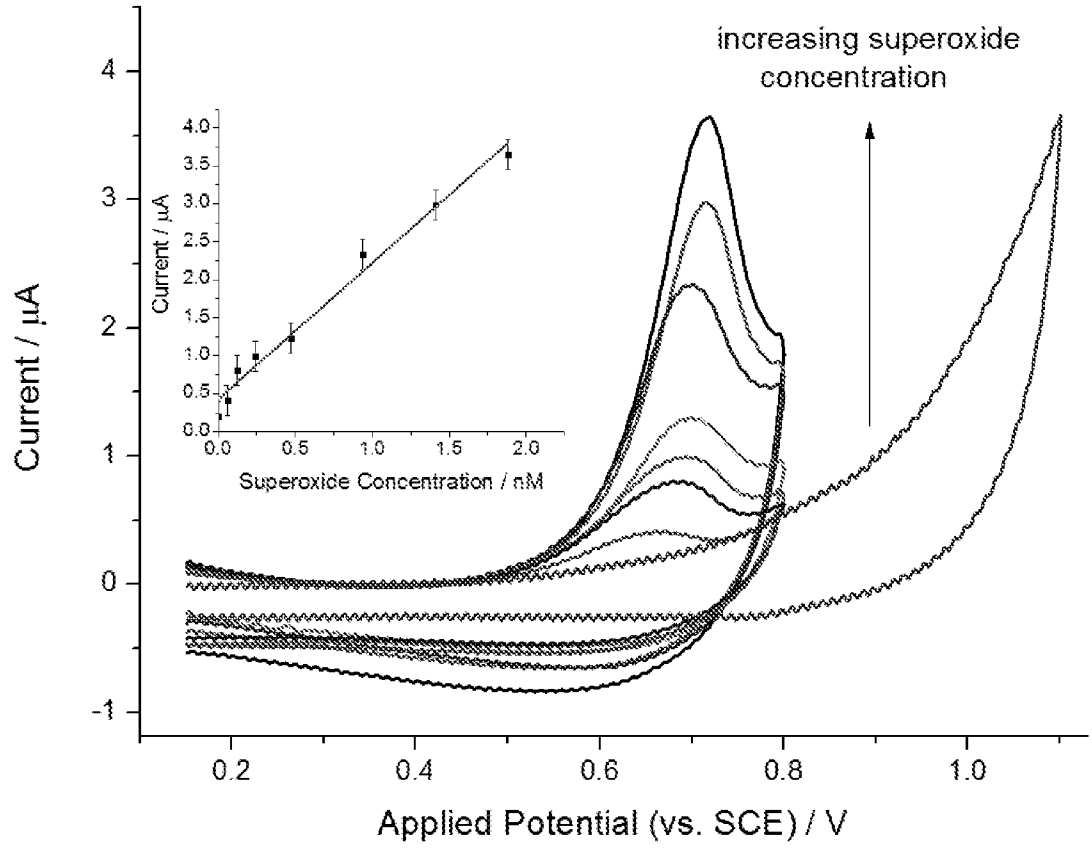

increasing pre-concentration time with NBTC
Current / µA
Applied Potential (vs. SCE) / V
Area / µC
Pre-Concentration Time in NBTC / s

FIG. 12


increasing superoxide concentration
Current / µA
Applied Potential (vs. SCE) / V
Current / µA
Superoxide Concentration / nM

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2014/053448

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N27/30 G01N27/48
ADD.

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)
EPO-Internal, WPI Data, INSPEC, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HYUNG SIM CHOI ET AL: "A Quantitative Nitroblue Tetrazolium Assay for Determining Intracellular Superoxide Anion Production in Phagocytic Cells", JOURNAL OF IMMUNOASSAY AND IMMUNOCHEMISTRY, vol. 27, no. 1, 1 January 2006 (2006-01-01), pages 31-44, XP055166359, ISSN: 1532-1819, DOI: 10.1080/15321810500403722 abstract ----- -/--	1-28

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

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"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
3 February 2015	18/02/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Komenda, Peter

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E D KINGSLEY ET AL: "Short Communication SOME OBSERVATIONS ON CARBON PASTE ELECTRODES IN AC VOLTAMMETRY", ANALYTICA CHIMICA ACTA, vol. 206, 1 January 1988 (1988-01-01), pages 385-390, XP055166493, 'EXPERIMENTAL'	23,24
X	KAREL VYTRAS ET AL: "Carbon paste electrodes in electroanalytical chemistry", JOURNAL OF THE SERBIAN CHEMICAL SOCIETY, vol. 74, no. 10, 1 January 2009 (2009-01-01), pages 1021-1033, XP055166542, ISSN: 0352-5139, DOI: 10.2298/JSC0910021V the whole document	23-25,28
X	EMIR TURKUS&BREVE;IC ET AL: "AMPEROMETRIC DETERMINATION OF GLUCOSE WITH AN MnO 2 AND GLUCOSE OXIDASE BULK-MODIFIED SCREEN-PRINTED CARBON INK BIOSENSOR", ANALYTICAL LETTERS, vol. 34, no. 15, 31 December 2001 (2001-12-31), pages 2633-2647, XP055166749, ISSN: 0003-2719, DOI: 10.1081/AL-100108410 'EXPERIMENTAL PART'	23-25,28