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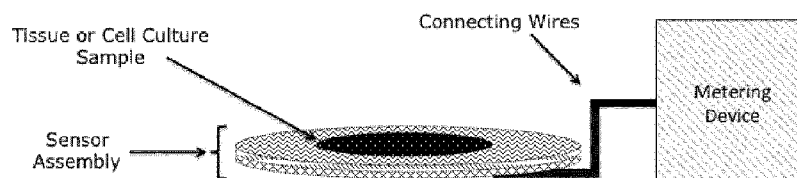


Figure 2

(57) Abstract: The present application discloses a tissue sample or cell culture sample monitoring assembly comprising a sensor assembly comprising a water-permeable sensor membrane consisting of a conducting polymer material defining an electrode of a thickness of 0.1-1,000 μm , and holding functionalizations specific to a certain cell tissue analyte, a metering device, conductor(s) electrically connecting the electrode of the sensor membrane with the metering device, and a tissue sample or a cell culture sample arranged on top of the sensor membrane of the sensor assembly, and a liquid medium receptacle wherein the sensor membrane and the tissue sample or the cell culture sample are arranged. A method of continuously or intermittently monitoring the concentration or presence of a tissue analyte in the proximity of a tissue sample or cell culture sample, a water-permeable sensor membrane and a sensor assembly are also disclosed.

SENSING MEMBRANE-BASED PLATFORM FOR TISSUE OR CELL CULTURING AND MONITORING

FIELD OF THE INVENTION

The present invention relates to a sensor membrane particularly useful as a platform for
5 tissue or cell culturing and monitoring.

BACKGROUND OF THE INVENTION

Organotypic brain slice cultures, typically prepared from immature animals, can grow and survive for several weeks or even months in vitro, and allow long-term drug treatments, as well as long-term monitoring of biological processes, as is often necessary in studies of
10 neurotoxicity, neuro-protection and neuro-repair. Therefore these cultures are an often used model in research on neurodegenerative diseases.

The concentration of energy substrates in the growth medium such as glucose, lactate or glutamine, neurotransmitters such as glutamate, GABA (gamma-aminobutyric acid) or dopamine, or cell death markers such as choline, glycerol or lactate dehydrogenase (LDH), is
15 today determined by offline methods including electrochemical techniques and high pressure liquid chromatography systems. However, this approach is both time-consuming, labor-intensive and only the accumulative concentration of the analytes released since last medium change can be measured.

For the determination of the physical release site of different analytes in a brain slice, it is
20 currently necessary to cut the brain slices and apply analyte specific dyes to these thinner slices, which is finally analysed using fluorescent microscopy. Alternatively, in order to provide information about e.g. dopamine release from a tissue sample, HPLC analysis of the medium collected after incubation for e.g. a few days is typically conducted. However, in order to measure dopamine in a culture medium using HPLC with electrochemical detection it
25 is necessary to change serum-containing or Neurobasal medium with a more simple physiological medium (e.g. Ringer's solution or Hanks balanced salt solution). In organotypic mesencephalic slice cultures dopamine levels above detection levels (about 20 femtomoles in a 50 µl sample) are reached after about 1 hour incubation in the presence of a dopamine reuptake blocker (See ref. Larsen et al. 2008 Eur. J.Neurosci. 28, 569-576).

30 Alternatively, the dopamine release can be directly measured by placing a tissue sample on an electrode. Sasso et al. (J. Nanosci. Nanotechnolo. 2012, Vol. 12, 3027-3083) recently

described self-assembled diphenylalanine peptide nanowires (PNW) used for gold sensor modifications. The peptide nanowires could be further modified by functionalization with polypyrrole (PNW/PPy) and such a modified sensor could be used as a dopamine sensor.

Moreover, the PNW modified gold surfaces were found useful for growing cells, i.e. cell

5 growth was not hindered by the peptide nanostructure. However, conventional electrodes are not particularly suited for long-term studies of tissue samples or cell cultures due to localised cell necrosis and/or inhomogeneous growth.

WO 2008/054611 A2 discloses engineered conductive polymer films to mediate biochemical interactions, in particular a functionalized conductive polymer film comprising a conductive
10 polymer and at least one receptor embedded in or absorbed to the conductive polymer. The polymer film is typically coated onto a device or an electrode.

Michalska et al., Journal of Solid State Electrochemistry, Springer Verlag, Germany, Vol. 8, No. 6, May 2004, pages 381-389, disclose PEDOT films for multifunctional membranes for electrochemical ion sensing.

15 Marti et al., Journal of Materials Chemistry, Royal Society of Chemistry, UK, Vol. 20, No. 47, 2010, pages 10652-10660, discloses nanostructured conducting poly(N-methylpyrrole) polymers for dopamine detection.

US 2003/0215940 discloses a multi-well assembly for growing cultures in-vitro.

Hence, there is a need for sensors and methods that are able to overcome the above-
20 mentioned short-comings.

SUMMARY OF THE INVENTION

In their continuing work to develop membrane-based sensors, the present inventors have realised that the previous constructions for analysis of tissue samples and/or cell culture samples do not effectively allow for continuous measurement of e.g. dopamine release and
25 for suitable conditions for tissue and cell survival, such as stable gas and nutrients medium concentrations. For example, previous HPLC methods require sampling (thereby eliminating a truly continuous monitoring of e.g. dopamine release) and a limited choice for growth media. On the other hand, the arrangement of tissue and cell culture samples directly on an electrode does not allow for reliable growth of the tissue sample or cell culture samples.

So, in a first aspect the present invention relates to a tissue sample or cell culture sample monitoring assembly, cf. claim 1.

The invention further provides a method of monitoring the concentration or presence of a cell tissue analyte, cf. claim 4.

- 5 Furthermore, the invention relates to a water-permeable sensor membrane, cf. claim 5, and to a sensor assembly, cf. claim 9.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a schematic for the water-permeable sensor membrane of a conducting polymer material.

- 10 Figure 2 illustrates the tissue sample or cell culture sample monitoring assembly, with the biological sample (tissue or cell culture) directly on the single layer sensor assembly connected with the external equipment via an electrical connection to the sensor membrane.

DETAILED DISCLOSURE OF THE INVENTION

- 15 The present inventors have now realised that a particular sensor membrane construction allows for continuous monitoring of release of analytes while at the same time providing excellent conditions for tissue samples or cell culture samples.

Sensor membrane

- 20 As described above, the present invention i.a. provides a water-permeable sensor membrane consisting of a conducting polymer material defining an electrode and having a thickness of 0.1-1,000 μm , said membrane holding functionalizations specific to a certain cell tissue analyte.

- 25 Although it should be understood that the water-permeable sensor membrane *consists of* only one layer, it will be appreciated that the membrane in its practical use may be arranged on a support, e.g. on a separate non-conductive water permeable scaffold (e.g. a net or the like), or as a conductive support in itself for the immobilization of analyte-relevant sensing components such as enzymes or other biocatalysts.

In one embodiment, the sensor membrane is arranged on a porous material. In one variant, the sensor membrane is arranged on a membrane insert conventionally used for tissue or cell culturing, e.g. a Biopore™ inserts from Milipore (e.g. a PTFE membrane with a pore size of about 0.4 µm).

- 5 The sensor membrane should be overall water-permeable so as to allow an aqueous medium to penetrate the membrane from the bottom face and reach the upper surface where a tissue sample or a cell culture sample is intended to be arranged, as well as allowing the same aqueous medium to penetrate the membrane from the top face to the underlying surface for efficient medium exchange, needed to guarantee optimal cell or tissue growth conditions.
- 10 In the present context, the term "water-permeable" is intended to mean having the ability to allow aqueous solutions, holding relevant biological components needed for the tissue or cell culturing, to pass through the material.

Typically, a suitable water-permeability is present when the pore size is in the range of 0.05-100 µm, e.g. 0.08-50 µm, or 0.1-10 µm, such as 0.2-3 µm.

- 15 Moreover, the sensor membrane should also be conductive so as to allow for electron transfer between the redox components in the analyte and external equipment (e.g. a potentiostat or other electrical components).

- Typically, the polymer material holds functionalizations specific to a certain cell tissue analyte. The cell tissue analyte for which the polymer material provides selectivity is typically
20 selected from neurotransmitters, e.g. selected from dopamine, glutamate, GABA, DOPAC, HVA and norepinephrine, as well as other analytes and metabolites like glycerol, choline (cell death markers), glucose, lactate, and pyruvate. Particularly interesting are dopamine, GABA and norepinephrine.

- In order to adapt the sensor membrane to exhibit selectivity for dopamine, the sensor
25 membrane may be composed of an electropolymerized polypyrrole film doped with polystyrene sulfonate counter-ions [Sasso et al., Analyst, 2013, Vol. 138, 3651-3659].

- In order to adapt the sensor membrane to exhibit selectivity for glutamate, enzymes such as glutamate oxidase, horseradish peroxidase and ascorbate oxidase wired via
poly(ethyleneglycol) diglycidyl ether (PEDGE) to an Os(bpy)₂Cl-complexed, ethylamine-
30 quaternized, redox polymer (POs-EA) may be incorporated. Reference is also made to the papers by Oldenzien and Westerink in Anal. Chem. 2005

In order to adapt the sensor membrane to exhibit selectivity for GABA, enzymes such as gabase and glutamate oxidase can be incorporated (M. Zhang and L. Mao, Frontiers in Bioscience, 10, 345-352, 2005).

Illustrative examples of suitable polymers for establishing the sensor membrane are e.g.

- 5 polymers selected from polypyrroles (PPY), polyanilines (PANI), and polythiophenes such as poly(3,4-ethylenedioxythiophene) (PEDOT).

In one embodiment, the sensor membrane is prepared from an intrinsically conducting polymer.

- 10 In another embodiment, the sensor membrane is prepared from polymers that are made conductive by doping with conductive components, e.g. carbon or metal nanoparticles.

In some embodiments, it is preferred that the sensor membrane comprises a polymer selected from conducting polymers having the ability to be electro-polymerized, i.e. to be polymerized under the influence of an electric current.

- 15 The sensor membrane typically has a thickness of 0.1-1,000 μm , e.g. 0.5-500 μm , such as 1.0-500 μm , e.g. 1.0-20 μm , or 10-80 μm , or 50-120 μm or 100-400 μm .

The polymer membrane for the single layer sensor can in principle be fabricated as described below.

- 20 In one embodiment, the sensor membrane is a porous intrinsically conducting polymer material. Examples of suitable materials are PEDOT (such as PEDOT-based polymers). When the sensor membrane is a porous intrinsically conducting polymer material, it typically has a thickness of 0.1-1,000 μm , such as 1.0-500 μm , e.g. 1.0-20 μm , 10-80 μm , 50-120 μm or 100-400 μm . Moreover, the porous intrinsically conducting polymer material typically has a porosity of at least 40 %, such as at least 50 %, e.g. between 60 % and 90 %, or between 65 % and 85 %.

- 25 When the sensor membrane is of a porous intrinsically conducting polymer material, it can be prepared by chemical polymerization casting methods [H. Allcock, M. Hofmann, S. Lvov, X.Y. Zhou, D. Macdonald. Proton Conducting Polymer Membranes, US Patent 6,759,157 (2004).].

The porosity of the intrinsically conducting polymer material can be controlled in a track etching process where a thin film of the polymer material is subjected to a high energy ion

(for instance Argon) bombardment in which process the material around individual ion impacts becomes soluble whereas the non disturbed area remains insoluble. In this way the porosity of the sensor membrane can be controlled by the ion dose and by standard photolithography the porosity can be confined into smaller areas if desired, thereby providing a suitable patterning. The individual electrodes can be defined by another photolithography patterning process where the conductivity of the polymer is increased locally to define the electrode and corresponding connections.

In another embodiment, the sensor membrane is prepared from a polymer made conducting by metal nanoparticle doping.

Illustrative examples hereof are any conductive polymers like conductive SU-8 [M. Marelli, G. Divitini, C. Collini, L. Ravagnan, G. Corbelli, C. Chisleri, A. Gianfelice, C. Lenardi, P. Milani, L. Lorenzelli. Flexible and biocompatible microelectrode arrays fabricated by supersonic cluster beam deposition on SU-8. J. of Micromech. Microeng. 21(4), 045013 (2011)] (with doped Pt or Au particles). When the sensor membrane is of a porous polymer electrode with incorporated metallic nanoparticles, it typically has a thickness of 0.1-1,000 μm , such as 1.0-500 μm , e.g. 1.0-20 μm , 10-80 μm , 50-120 μm or 100-400 μm . Moreover, a porous polymer electrode with incorporated metallic nanoparticles typically has a porosity of at least 40 %, such as at least 50 %, e.g. between 60 % and 90 %, or between 65 % and 85 %.

When the sensor membrane is of a porous polymer electrode with incorporated metallic nanoparticles, it can be prepared by standard microfabrication technique like lithography, in particular for the purpose of patterning.

In one embodiment, the functionalisations (e.g. a substance or substances) making the sensor membrane selective to a certain cell tissue analyte are incorporated in the conducting polymer membrane, in particular the functionalisations (such as substances like counter ions or enzymes) are incorporated in the conducting polymer during the fabrication of the membrane.

Alternatively, the functionalisations making the sensor membrane selective to a certain cell tissue analyte are introduced subsequent to preparation of the membrane of the (native) conducting polymer, e.g. the membrane can be functionalized by incorporation of the relevant substance or substances (e.g. a specific enzyme) after fabrication of the membrane of the (native) conducting polymer. This can e.g. be accomplished chemically by covalent attachment of the relevant functionalisations.

As a further alternative, the functionalisations may be introduced by a subsequent electro polymerization process of a thin layer (e.g. constituting at the most 20 % of the total thickness of the final membrane) of the same or similar conductive polymer as the membrane itself on top of this, but where the thin layer includes the relevant

5 functionalisations, e.g. substance or substances.

Specific embodiments of the invention

In a currently most preferred embodiment, the sensor membrane is prepared from poly(3,4-ethylenedioxythiophene) (PEDOT).

10 One currently preferred embodiment of the invention relates to a water-permeable sensor membrane consist of a poly(3,4-ethylenedioxythiophene) (PEDOT) defining an electrode and having a thickness of 0.001-1.0 μm .

Such a sensor membrane is especially suited for measuring dopamine.

15 Another currently preferred embodiment of the invention relates to a water-permeable sensor membrane consist of a polypyrrole defining an electrode and having a thickness of 0.001-1.0 μm .

Such a sensor membrane is especially suited for measuring dopamine if it e.g. is an electropolymerized polypyrrole film doped with polystyrene sulfonate counter-ions.

20 Selectivity for other analytes can be obtained if the sensor membrane is an electropolymerized film doped with enzymes such as e.g. gabase and glutamate oxidase for GABA detection or glutamate oxidase for glutamate detection (M. Zhang and L. Mao, Frontiers in Bioscience, 10, 345-352, 2005).

A sensor assembly

25 The present invention also provides a sensor assembly comprising (i) a sensor membrane as defined hereinabove, (ii) a metering device, and (iii) one or more conductors electrically connecting the electrode of the sensor membrane with the metering device.

Examples of suitable metering devices are any commercially available potentiostat (electronic hardware required to control a two- or three-electrode cell and run most electroanalytical

experiments), or simpler versions of such a device, like an amperometric detector (a detector designed to apply a potential to a channel and measures the current response).

The individual electrodes of the sensor membrane are electrically connected with the metering device, e.g. by use of, e.g., conducting glue, silver paste or conducting tape.

- 5 The metering device may further be electrically connected to a reference electrode and to a counter electrode arranged in a conventional manner.

A tissue sample or cell culture sample monitoring assembly

- 10 The present invention further provides a tissue sample or cell culture sample monitoring assembly comprising a sensor assembly as defined hereinabove, and a tissue sample or a cell culture sample arranged on top of the single layer sensor membrane.

- 15 In one embodiment, the invention provides a tissue sample or cell culture sample monitoring assembly comprising a sensor assembly comprising (i) a water-permeable sensor membrane consisting of a conducting polymer material defining an electrode and having a thickness of 0.1-1,000 μm , wherein said membrane holds functionalizations specific to a certain cell tissue
15 analyte, (ii) a metering device, (iii) one or more conductors electrically connecting the electrode of the sensor membrane with the metering device, and a tissue sample or a cell culture sample arranged on top of the sensor membrane of the sensor assembly, and further comprising a receptacle wherein the sensor membrane and the tissue sample or the cell culture sample are arranged, said receptacle further comprising a liquid medium for said
20 tissue sample or cell culture sample.

- 25 The end user will typically be able to arrange the tissue sample or cell culture sample, e.g. an organ tissue sample, on the present sensor membrane in the same way as for conventional tissue culture membranes. Examples of typical tissue samples are organ tissue samples from the brain or spine of rats, mice or other test animals along with neuronal cell lines, e.g. PC-12 cells. Furthermore the sensor assembly could be used in the study of differentiation of neuronal stem cells into for instance dopaminergic, noradrenergic or serotonergic neurons.

Advantageously, the monitoring assembly further comprises a receptacle wherein the sensor membrane and the tissue sample or cell culture sample are arranged, said receptacle further comprising a liquid medium for said tissue sample or cell culture sample.

The receptacle may be in the form of conventional trays capable of holding a plurality (e.g. 6 or 12) of individual sensor membranes. Each well holds a sensor membrane with the tissue sample or cell culture sample and a liquid medium for the tissue sample or cell culture sample. Such a tray is typically equipped with a lid.

5 *A method*

Still further, the present invention provides a method of continuously or intermittently monitoring the concentration or presence of a cell tissue analyte in the proximity of a tissue sample or cell culture sample, said method comprising the step of:

- 10 a) providing a monitoring assembly as defined hereinabove (i.e. the single layer membrane), and
- b) measuring the concentration or presence of the cell tissue analyte by electrochemical techniques.

In one interesting embodiment, the monitoring is conducted continuously, hence the accurate development of the concentration (or presence) of the cell tissue analyte can be monitored.

- 15 Measurement can be done either by amperometry, where then the current response can be related to the amount of analyte (e.g. dopamine) released.

- As an alternative to a continuous measurement, it is of course also possible to obtain several point measurements over a period of time (i.e. virtually continuous in the sense of achieving a data point e.g. every 10 min. for a period of e.g. several weeks), using other
- 20 electrochemical techniques like cyclic voltammetry or linear sweep voltammetry or electrochemical impedance spectroscopy, or even several amperometric measurements e.g. every 10 min. or the like.

EXAMPLES

Example 1

- 25 The third conducting polymer layer can be prepared by electropolymerization from a solution containing the monomer precursor, by applying a constant potential to the sensor membrane relative to the polymerization potential, e.g. 0.7 V for 10 seconds in the case of polypyrrole.

The monomer solution can contain counter-ion dopants or enzymes needed for the specificity and sensitivity increase with respect to a specific analyte, e.g. polystyrene sulfonate ions in the case of dopamine or gabase and glutamate oxidase for GABA detection.

- 5 The characterization of the sensor membrane can be achieved by standard electrochemical techniques. The sensor membrane can be used as working electrode in a standard 3-electrode setup electrochemical cell, having a Pt counter electrode and a Ag/AgCl reference electrode immersed in an electrolyte solution and with all electrodes connected to an external potentiostat. Cyclic voltammetry can be used to study the sensor membrane response to the specific analytes intended for the detection as well as standard redox couples, e.g. potassium
- 10 ferri/ferrocyanide, by swiping the potential in a window relevant to the oxidation and reduction of each analyte, e.g. 0.2-0.8 V, and by observing how the anodic and cathodic current responses obtained change with respect to potential sweep rate, e.g. ranges of 10-250 mV/s, and concentration of the analyte, e.g. ranges of 0.01-10 mM for standard redox couples and 0.05-800 nM for analyte solutions. Amperometry can be used to investigate the
- 15 sensor membrane current response to subsequent additions of analytes with varying concentrations at constant potentials close to the oxidation/reduction potentials resulting from the cyclic voltammetry investigation. An amperometric calibration can be achieved by constructing a plot of current response vs. analyte concentration, yielding statistical values such as minimum detection limit and the concentration range of linear response.
- 20 The membrane assembly can be utilized as standard tissue or cell culture membrane. Therefore the preparation of the experiments is similar to the preparation of normal culture experiments. First the membrane is placed in a 6 well plate, which is filled with medium so that the bottom part of the membrane is in contact with the culture medium and the top part with a thin layer of the medium withdrawn from below due to capillary forces. The tissue or
- 25 cell culture is placed on the membrane and the 6 well plate moved to an incubator that controls the temperature and humidity. All of the preparation steps must be performed in a sterile laminar flow bench.
- The analyte detection from cells or tissue can be obtained by amperometric measurements at potentials close to the oxidation/reduction potential peaks of specific analytes, e.g. -0.1 V,
- 30 0.3 V or 0.6 V, by connecting the sensor membrane to a potentiostat in the setup discussed above, with the electrolyte solution being the culture medium. Upon the release of analyte from the cells or tissue, a change will appear in the current trace that can yield information about the analyte and the mechanics of the release.

CLAIMS

1. A tissue sample or cell culture sample monitoring assembly comprising a sensor assembly comprising (i) a water-permeable sensor membrane consisting of a conducting polymer material defining an electrode and having a thickness of 0.1-1,000 μm , wherein said
5 membrane holds functionalizations specific to a certain cell tissue analyte, (ii) a metering device, (iii) one or more conductors electrically connecting the electrode of the sensor membrane with the metering device, and a tissue sample or a cell culture sample arranged on top of the sensor membrane of the sensor assembly, and further comprising a receptacle wherein the sensor membrane and the tissue sample or the cell culture sample are arranged,
10 said receptacle further comprising a liquid medium for said tissue sample or cell culture sample.
2. The monitoring assembly according to claim 1, wherein the cell tissue analyte is selected from dopamine, glutamate, GABA, DOPAC, HVA, norepinephrine, glycerol, choline, glucose, lactate, and pyruvate.
- 15 3. The monitoring assembly according to any one of the preceding claims, wherein the conducting polymer is selected from polypyrroles (PPY), polyanilines (PANI), and polythiophenes like poly(3,4-ethylenedioxythiophene) (PEDOT).
4. A method of continuously or intermittently monitoring the concentration or presence of a tissue analyte in the proximity of a tissue sample or cell culture sample, said method
20 comprising the step of:
 - a. providing a monitoring assembly as defined in any one of the claims 1-3,
 - b. measuring the concentration or presence of the cell tissue analyte by electrochemical techniques.
5. A water-permeable sensor membrane consisting of a conducting polymer material
25 defining an electrode and having a thickness of 0.1-1,000 μm , said membrane holding functionalizations specific to a certain cell tissue analyte.
6. The sensor membrane according to any one of the preceding claims, wherein the cell tissue analyte for which the polymer material provides selectivity is selected from dopamine, glutamate, GABA, DOPAC, HVA, norepinephrine, glycerol, choline, glucose, lactate, and
30 pyruvate.

7. The sensor membrane according to any one of the preceding claims, which comprises a polymer selected from conducting polymers having the ability to be electropolymerized.
8. The sensor membrane according to any one of the preceding claims, wherein the conducting polymer is selected from polypyrroles (PPY), polyanilines (PANI), and
5 polythiophenes like poly(3,4-ethylenedioxythiophene) (PEDOT).
9. A sensor assembly comprising (i) a sensor membrane according to any one of the claims 5-8, (ii) a metering device, and (iii) one or more conductors electrically connecting the electrode of the sensor membrane with the metering device.

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(1)

Conducting Polymer Material



Figure 1

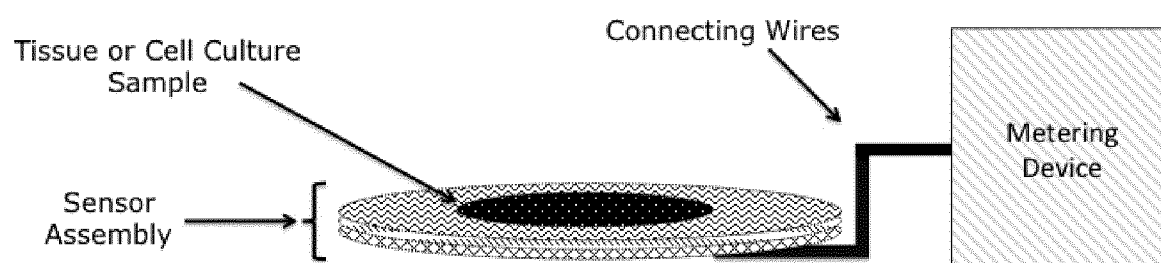
(2)

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

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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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2008/054611 A2 (HARVARD COLLEGE [US]; PARKER KEVIN KIT [US]; O'GRADY MEGAN [US]) 8 May 2008 (2008-05-08) cited in the application claims 1,10	1-9
Y	POTTER STEVE M ET AL: "A new approach to neural cell culture for long-term studies", JOURNAL OF NEUROSCIENCE METHODS, vol. 110, no. 1-2, 30 September 2001 (2001-09-30), pages 17-24, XP002714410, ISSN: 0165-0270 figure 2	1-9



Further documents are listed in the continuation of Box C.



See patent family annex.

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MICHALSKA A ET AL: "PEDOT films: multifunctional membranes for electrochemical ion sensing", JOURNAL OF SOLID STATE ELECTROCHEMISTRY SPRINGER-VERLAG GERMANY, vol. 8, no. 6, May 2004 (2004-05), pages 381-389, XP002689607, ISSN: 1432-8488 the whole document -----	1-9
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