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(54) Title: HYDROGEN SULFIDE DETECTING APPARATUS

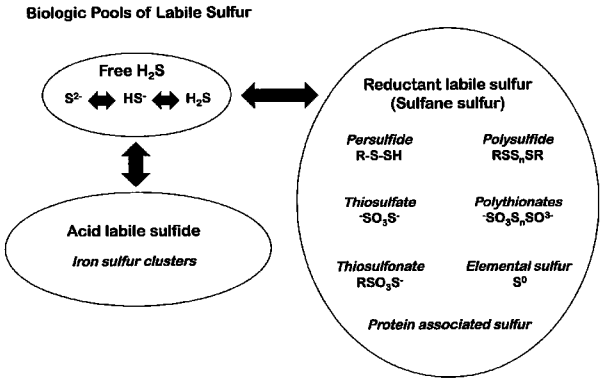


Fig. 1

(57) Abstract: A hydrogen sulfide (H_2S) detecting apparatus for measuring the concentrations of hydrogen sulfide species in a given sample is disclosed. The hydrogen sulfide detecting apparatus can comprise a plurality of reaction chambers separated from a plurality of trapping chambers by a (H_2S) permeable membrane, with the reaction chambers and trapping chambers each having buffer component(s) and/or reactive agents that expose the incoming sample to a particular pH and chemical environment in order to allow for the selective liberation and trapping of hydrogen sulfide from the sample.

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HYDROGEN SULFIDE DETECTING APPARATUS

I. CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. US 61/806,017, filed March 28, 2013, which is hereby incorporated by reference.

II. THE NAMES OF THE PARTIES TO A JOINT RESEARCH AGREEMENT

[0002] The claimed invention was made by, on behalf of, and/or in connection with the following parties to a joint university corporation research agreement: Louisiana Tech University and the Board of Supervisors of Louisiana State University and Agricultural and Mechanical College. The agreement was in effect on and before the date the claimed invention was made, and the claimed invention was made as a result of activities undertaken within the scope of the agreement.

III. FIELD OF THE INVENTION

[0003] The present invention relates to a novel apparatus and methods for the measurement of hydrogen sulfide in its various bioavailable forms.

IV. BACKGROUND OF THE INVENTION

[0004] Hydrogen sulfide (H_2S) is a colorless gasotransmitter (gaseous signaling molecule) that plays a vital role in numerous cellular functions within the human body. For instance, over the past decade, the role of H_2S beyond a toxicant and environmental pollutant has evolved to encompass several biochemical functions that are important in various physiological and pathological responses such as cardiovascular (dys)function, neurological (dys)function, gastrointestinal (dys)function, immune (dys)function, and several other molecular and cell biology responses.

[0005] For instance, H_2S has been discovered to have significant potential to contribute to the detection and treatment of cardiovascular disease, including atherosclerosis and peripheral arterial disease. Because of decreased oxidative modification of low-density lipoprotein (LDL), H_2S has also been shown to play a significant role in atherosclerosis by having noted effects on monocyte recruitment, transformation into tissue macrophages, and foam cell formation. Further, H_2S has been shown to inhibit

hypochlorite and hemin-mediated atherogenic modification of LDL. Plasma H_2S levels have also been shown to be lower in atherosclerotic plaque, and treatment with sodium hydrosulfide (NaHS) decreases both aortic plaque and intercellular adhesion molecule-1 (ICAM-1). In addition, H_2S down regulates the expression of monocyte chemoattractant protein-1, a CC chemokine that binds to the C-C chemokine receptor type 2 (CCR2) and recruits monocytes into the subendothelial layer to form atherosclerotic plaque. Another critical role of H_2S in the pathogenesis of atherosclerosis is the effect of inducing apoptosis on vascular smooth muscle cells, which generates atherosclerotic plaque. Hydrogen sulfide, administered as NaHS, decreases the proliferation of vascular smooth muscle cell via a mitogen-activated protein kinase (MAPK) pathway in a dose-dependent fashion in rat models. Additional work with a rat model reveals that H_2S reduced vascular calcification. Additionally, recent studies have shown H_2S to have a direct relationship with nitrogen monoxide and carbon monoxide in peripheral arterial disease (PAD) identification.

[0006] Hydrogen sulfide arises from multiple biological sources and tissues (e.g. bacteria and organ-specific production). Endogenous biological H_2S production primarily originates from cysteine metabolism through the activity of cystathionine β-synthase and cystathione γ-lyase or through 3-mercaptopyruvate metabolism by 3-mercaptosulfurtransferase. H_2S can come from redox-dependent metabolism of polysulfides involving glutathione or other small molecular weight thiol modifiers. Lastly, H_2S also arises from different environmental sources that affect humans such as petroleum production and exploration, food and beverage processing, waste disposal and sewage treatment, agriculture and farming, and bacterial contamination and function.

[0007] Hydrogen sulfide chemistry is complex and plays several roles in modulating protein thiol function. It affects numerous biological responses involving signal transduction responses, mitochondrial respiration, gene expression, and cell survival/viability. At a physiological pH of ~7.2-7.4, H_2S predominantly (~80%) exists in its anion HS^- form with a smaller amount in the gaseous H_2S form (~20%). This is due to pKa regulation of H_2S forms in aqueous solutions as illustrated in the following equation:

$$pKa_1 = 7.04 \quad pKa_2 \geqq 13$$

$$H_2S \leftrightarrows HS^- \leftrightarrows S^{2-}$$

Due to the different pKa's, the ionic distribution is easily manipulated and, in turn, its distribution controlled in either aqueous or gas phases.

[0008] Hydrogen sulfide is very reactive within biological or environmental systems, resulting in sulfide equivalents being present in three different volatile sulfur pools as shown in Figure 1. These three pools—free H_2S, acid labile H_2S, and sulfane sulfur species—are important in regulating the amount of bioavailable sulfur. Free hydrogen sulfide is found dissolved in plasma and other tissue fluids. At mammalian body conditions (i.e., pH 7.4 and temperature of 37 °C), 18.5% of free hydrogen sulfide exists as H_2S gas, and the remainder is almost all hydrosulfide anion (HS-) with a negligible contribution of S^{2-}. Sulfane sulfur refers to divalent sulfur atoms bound to another sulfur, though they may bear an ionizable hydrogen at some pH values. Examples of these bound sulfurs include thiosulfate $S_2O_3^{2-}$, persulfides R-S-SH, thiosulfaonates R-S(O)-S-R', polysulfides R-S_n-R, polythionates $S_nO_6^{2-}$, and elemental sulfur S^0. Acid labile sulfide, the other major bioavailable pool, consists of sulfur present in iron-sulfur clusters contained in iron-sulfur proteins (non-heme), which are ubiquitous in living organisms, and include a variety of proteins and enzymes, including without limitation, rubredoxins, ferredoxins, aconitase, and succinate dehydrogenase. The acid labile sulfides readily liberate free H_2S in acid conditions (pH < 5.4), and the process of acid liberation may also release hydrogen sulfide from persulfides, which have traditionally been classified as sulfane sulfur. This acid labile sulfur pool has been postulated to be a reversible sulfide sink and may be an important storage pool that regulates the amount of bioavailable free hydrogen sulfide.

[0009] H_2S equivalents are readily mobilized from these pools based on changes in pH, O_2 concentration, and oxidative/reductive chemistry that affect biological and biochemical responses. Thus, detection of H_2S availability from these distinct pools is important for clinical pathophysiology diagnosis, environmental source identification, and any other organic or inorganic chemistry uses.

[0010] Unfortunately, a significant barrier to the study of hydrogen sulfide's role in human health and disease has been the lack of precise methodology and testing means for the accurate and reproducible measurement of hydrogen sulfide both *in vivo* and *in vitro*. A variety of methods to measure free H_2S have been employed, but with divergent results. These methods include a spectrophotometric derivatization method resulting in methylene blue formation, variations of this methylene blue method using high

performance liquid chromatography, sulfide ion-selective electrodes, polarographic sensors, gas chromatography, and high-performance liquid chromatography (HPLC) in conjunction with fluorimetric based methods using monobromobimane (MBB) to derivatize free H_2S. The complexity of analytical H_2S measurement, especially in living organisms, reflects the fact that hydrogen sulfide is a reactive gas and exists in the organism in in the three different volatile sulfur pools shown in Figure 1. Due to a lack of reliable, accurate analytical detection methods available to quantify H_2S and its various forms, there is great disagreement regarding precise amounts and sources of H_2S metabolism in biological and biochemical settings. Therefore, there is a great need for an apparatus and associated methodology that can be used to accurately and conveniently measure H_2S in its various bioavailable forms.

V. SUMMARY OF THE INVENTION

[0011] The invention disclosed herein is directed to a hydrogen sulfide (H_2S) detecting apparatus for measuring the concentrations of hydrogen sulfide species in a given sample. In a particular embodiment exemplifying the principles of the invention, the hydrogen sulfide detecting apparatus can comprise a plurality of reaction chambers separated from a plurality of trapping chambers by a H_2S permeable membrane, with the reaction chambers and trapping chambers each having buffer component(s) and/or reactive agents that expose the incoming sample to a particular pH and chemical environment in order to allow for the selective liberation and trapping of hydrogen sulfide from the sample.

[0012] In a preferred embodiment, the H_2S detecting apparatus features three reaction chambers, namely: a free sulfide reaction chamber having a pH from about 7.0 to about 7.5; an acid labile sulfide reaction chamber having a pH from about 2.6 to about 6.0; and a total sulfide reaction chamber having a pH from about 2.6 to about 6.0 and a reducing agent. Three corresponding trapping chambers can be positioned adjacent to the plurality of reaction chambers such that H_2S gas released from the reaction chambers will diffuse across the H_2S-permeable membrane and into the corresponding trapping chamber. The trapping chambers each have an alkaline environment with a pH from about 9.5 to about 10.0 in order to re-dissolve and trap the hydrogen sulfide gas. Detection can then be accomplished by one of the three following methods: (a) electrochemical, (b) fluorescence, or (c) colorimetric.

[0013] The H_2S detecting apparatus of the present invention can also feature a cap, an injection chamber, and a base. The cap can be positioned adjacent to the injection chamber to allow a test sample to be injected through the cap and into the injection chamber. The injection chambers can be in fluid communication with the reaction chambers via a plurality of inlets. The base can be positioned adjacent to the plurality of trapping chambers. The base can be transparent to enable fluorimetric or colorimetric detection of H_2S in the adjacent trapping chambers. The base can also feature a plurality of electrode systems to enable electrochemical detection of H_2S in the adjacent trapping chambers.

[0014] The H_2S detecting apparatus of the present invention enables the simultaneously detection of free H_2S, acid labile amounts of H_2S, bound sulfane sulfur available H_2S, and overall total bioavailable H_2S from a single test sample. The concentration of H_2S in the various pools can be calculated as follows: the free H_2S and total H_2S concentrations will be equal to the detected concentrations in the free sulfide trapping chamber and total sulfide trapping chamber, respectively. The acid labile H_2S amount can be determined by subtracting the amount measured in the free sulfide trapping chamber from that of the acid labile sulfide trapping chamber. Lastly, the bound H_2S concentration can be determined by subtracting the acid labile trapping chamber concentration from the total sulfide trapping chamber concentration.

[0015] The above summary is not intended to describe each illustrated embodiment or every possible implementation. These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawings.

VI. BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The accompanying figures, where like reference numerals refer to identical or functionally similar elements throughout the separate views, which are not true to scale, and which, together with the detailed description below, are incorporated in and form part of the specification, serve to illustrate further various embodiments and to explain various principles and advantages in accordance with the present invention:

[0017] **Figure 1** illustrates the three biological pools of sulfide found in organisms.

[0018] **Figure 2** is an exploded view of an embodiment of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention.

[0019] **Figure 3** is a top view of an embodiment of the reaction chamber of the hydrogen sulfide detecting apparatus depicted in Figure 2.

[0020] **Figure 4** illustrates representative reaction chamber conditions and the electrochemical detection methodology employed by a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention.

[0021] **Figure 5** illustrates the diffusion of hydrogen sulfide across a polydimethyl-siloxane (PDMS) membrane of different thicknesses, showing the transfer efficiency of hydrogen sulfide gas transfer from the reaction chambers to the trapping chambers of the hydrogen sulfide detecting apparatus depicted in Figure 2. The transfer efficiency was measured with high-performance liquid chromatography (HPLC) in conjunction with fluorimetric based methods using monobromobimane (MBB) to derivatize free H_2S.

[0022] **Figure 6** illustrates an embodiment of a manufacturing process for the creation of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention.

[0023] **Figure 7** is an exploded view of an alternative embodiment of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention.

VII. DETAILED DESCRIPTION OF THE INVENTION

[0024] Detailed embodiments of the present invention are disclosed herein; however, it is to be understood that the disclosed embodiments are merely exemplary of the invention, which can be embodied in various forms. Therefore, specific structural and functional details disclosed herein are not to be interpreted as limiting, but merely as a basis for the claims and as a representative basis for teaching one skilled in the art to variously employ the present invention in virtually any appropriately detailed structure. Alternate embodiments may be devised without departing from the spirit or the scope of the invention. Further, the terms and phrases used herein are not intended to be limiting; but rather, to provide an understandable description of the invention. While the specification concludes with claims defining the features of the invention that are regarded as novel, it is believed that the invention will be better understood from a consideration of the following description in conjunction with the drawing figures, in which like reference numerals are carried forward.

[0025] As used herein, the terms "a" or "an" are defined as one or more than one. The term "plurality," as used herein, is defined as two or more than two. The term "another,"

as used herein, is defined as at least a second or more. The terms "comprises," "comprising," or any other variation thereof are intended to cover a non-exclusive inclusion, such that a process, method, article, or apparatus that comprises a list of elements does not include only those elements, but may include other elements not expressly listed or inherent to such process, method, article, or apparatus. An element proceeded by "comprises ... a" does not, without more constraints, preclude the existence of additional identical elements in the process, method, article, or apparatus that comprises the element. The terms "including," "having," or "featuring," as used herein, are defined as comprising (i.e., open language). The term "coupled," as used herein, is defined as connected, although not necessarily directly, and not necessarily mechanically. As used herein, the term "about" or "approximately" applies to all numeric values, whether or not explicitly indicated. These terms generally refer to a range of numbers that one of skill in the art would consider equivalent to the recited values (i.e., having the same function or result). In many instances these terms may include numbers that are rounded to the nearest significant figure. Relational terms such as first and second, top and bottom, right and left, and the like may be used solely to distinguish one entity or action from another entity or action without necessarily requiring or implying any actual such relationship or order between such entities or actions.

[0026] As used herein, the abbreviation CA refers to chronoamperometry; the abbreviation DPV refers to differential pulse voltammetry; the abbreviation DTPA refers to diethylenetriamine pentaacetate; the abbreviation HPLC refers to high-performance liquid chromatography; the abbreviation PCR refers to polymerase chain reaction; and the abbreviation PDMS refers to polydimethyl-siloxane.

[0027] Herein various embodiments of the present invention are described. To avoid redundancy, repetitive description of similar features may not be made in some circumstances.

[0028] An embodiment of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention is shown in Figures 2 and 3. The apparatus **100** is a lab-on-a-chip device that allows H_2S in its various bioavailable forms to be measured in a reliable, analytical fashion employing unique reaction chemistry, micro-manufacturing techniques, and selective electrochemical measurement techniques. In particular, the apparatus **100** allows for rapid measurement of H_2S from the free H_2S, acid labile sulfide, and sulfane sulfur (polysulfide/persulfide) pools by featuring at least three reaction

chambers each having a particular pH and reaction chemicals present to allow for the selective liberation and trapping of H_2S from these pools. The apparatus **100** can be used for research, environmental, and clinical diagnostic purposes in determining hydrogen sulfide bioavailability in biological or other samples. The apparatus **100** is particularly useful for the detection of H_2S in plasma or any other biological or liquid sample. In a preferred embodiment, the apparatus **100** is designed for a single use; however, the apparatus **100** could be configured to be reusable in other embodiments.

[0029] Still referring to Figures 2 and 3, an embodiment of the hydrogen sulfide detecting apparatus **100** can comprise: a cap **111**; an injection chamber **121**; a plurality of reaction chambers **123, 124, 125**; a permeable membrane **131**; a plurality of trapping chambers **143, 144, 145**; and a base **151**. The cap **111** is preferably constructed out of butyl rubber and is positioned adjacent to the injection chamber **121** to allow a test sample to be injected through the cap **111** and into the injection chamber **121**. The injection chamber **121** is in fluid communication with the reaction chambers **123, 124, 125** via inlet channels **122a-c**. The permeable membrane **131** separates the reaction chambers **123, 124, 125** from adjacent trapping chambers **143, 144, 145**. The trapping chambers **143, 144, 145** are positioned adjacent to electrode systems **153, 154, 155** on the base **151**.

[0030] In a preferred embodiment, the injection chamber **121** comprises a single piece PDMS-molded (polydimethyl-siloxane) chamber which is evacuated of air and adapted to receive fluid injected directly into it. A first inlet channel **122a** connects the injection chamber **121** to free sulfide reaction chamber **123**; a second inlet channel **122b** connects the injection chamber **121** to the acid labile sulfide reaction chamber **124**; and a third inlet channel **122c** connects the injection chamber **121** to the total sulfide-reaction chamber **125**. In this arrangement, the reaction chambers **123, 124, 125** are reproducibly filled with uniform volumes from a single injection while minimizing diffusion of buffer components and reaction products between the chambers.

[0031] Each reaction chamber **123, 124,** and **125** preferably comprises interdigitated microchannels of PDMS with dried or powder-coated buffer components and/or reactive agents that expose the incoming sample to a particular pH and chemical environment in order to allow for the selective liberation and trapping of hydrogen sulfide. Figure 4 illustrates representative reaction chamber conditions of the hydrogen sulfide detecting apparatus **100**. Free or volatilized hydrogen sulfide is derivatized in alkaline conditions,

preferably pH >7.0. The derivatization preferably occurs under low oxygen conditions, preferably <5% oxygen, more preferably <2% oxygen, and most preferably ≤1% oxygen. Accordingly, in a preferred embodiment, the free sulfide-reaction chamber **123** is at neutral pH environment (pH from about 7.0 to about 7.5). Meanwhile, the release of hydrogen sulfide from the acid labile pool generally requires a pH less than 5.4. Thus the determination of acid labile sulfide involves acidification of the sample, preferably pH <4.0, more preferably from about pH 2.0 to about pH 3.0, and most preferably about pH 2.6, thereby causing release of free hydrogen sulfide from the acid labile pool. Accordingly, the acid labile sulfide-reaction chamber **124** is preferably at an acid pH environment (pH from about 2.6 to about 6.0). Lastly, the total sulfide-reaction chamber **125** is preferably at an acidic pH environment (pH from about 2.6 to about 6.0) with a reducing agent present such as 1 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP). The total labile sulfide amount, including the sulfane sulfur component along with the acid-labile and free sulfide, is determined by using a reducing agent with an acid solution. The reducing agent is preferably TCEP, which cleaves disulfide bonds to liberate the sulfane sulfur atom. While dithiothreitol (DTT) could also be used, TCEP is preferred because it is water soluble, non-volatile, reduces disulfide bonds more rapidly and has been shown to be very stable across a wider range of pH (2.0-9.5) than DTT. TCEP does not have a thiol moiety and has the additional advantage of not requiring thiol removal prior to reaction with MBB. By contrast DTT contains a thiol moiety and has been reported to have small amounts of sulfide contaminants.

[0032] The permeable membrane **131** is positioned between the reaction chambers **123, 124, 125** and the corresponding trapping chambers **143, 144, 145**. The H_2S permeable membrane **131** is preferably silicone-based or may comprise blended materials such as silicone-polycarbonate blends. The thickness of the H_2S permeable membrane **131** may vary between about 75 μm to about 500 μm or greater depending on device construction, application, and required mechanical strength. Other constructions may utilize membrane materials that include silicone and additive compounds for increased specificity of hydrogen-sulfide permeability. These include, but are not limited to, the combination of silicone and polycarbonate for membranes or dimethyl silicone. Other membrane base materials may be utilized which include but are not limited to composite membranes with silicone or PDMS coating on micro-porous cellulose structure. Membrane fabrication may be completed via microfabrication or other techniques.

Preferential techniques include spinning membrane polymer in liquid form onto a flat surface like a silicon-nitride wafer. The membrane may be subsequently released following curing, the removal of entrapped air bubbles and solidification. Other techniques include but are not limited to Reactive Ion Etch (RIE) processes. This includes the deposition of the liquid polymer membrane atop a wafer, and then patterning and removing the wafer substrate to release the membrane for use.

[0033] The trapping chambers **143, 144, 145** are positioned beneath the reaction chambers **123, 124, 125** such that H_2S gas released from each reaction chamber will diffuse across the permeable membrane **131** and into the corresponding trapping chamber. All three trapping chambers **143, 144, 145** are filled with an alkaline solution (100 mM NaOH, pH from about 9.5 to about 10) to trap and re-dissolve the hydrogen sulfide gas which diffuses across the permeable membrane **131**. In a preferred embodiment, the trapping chambers are constructed out of PDMS. However, other materials and construction processes may be utilized, including but not limited to solid casting, RIE patterning of silicon, and 3-D printing of non-porous chambers using 3-D printing material.

[0034] The base **151** is preferably constructed out of plastic. However, it may be constructed out of other base materials including but not limited to silicon, silicon nitride, or metallic materials. In the embodiment depicted in Figures 2 and 3, the base **151** features interdigitated electrode systems **153, 154, 155** for electrochemical detection of H_2S in the adjacent trapping chambers **143, 144, 145**. The electrode systems **153, 154, 155** can each feature a working electrode, a counter electrode, and a reference electrode. The working electrodes can be constructed out of inert metals such as gold, silver or platinum, are preferably medical grade carbon fibers (6 μm diameter; 12 Ω-cm). Other embodiments may include use of graphene or similar thin-sheet materials for electrode application. The working electrodes can be fixed in the base **151** longitudinally so as to allow exposure to the sample in the trapping chambers **143, 144, 145**. The counter electrode is preferably constructed out of an electrochemically inert material such as gold, platinum, or carbon and can be fixed in the base **151** parallel to the working electrode (preferably with 1 mm - 5 mm separation) in order to detect electrons produced by the oxidation reaction occurring in the trapping chambers **143, 144, 145**. The reference electrode is preferably constructed out of platinum or Ag/AgCl wire in electrical contact with the specimen. Alternatively, and as depicted in Figure 7, the base

151 can be constructed out of a transparent material (e.g., plastic) to allow H_2S levels in the trapping chambers **143, 144, 145** to be detected via fluorescence, chemiluminescence and colorimetric detection. In another alternative embodiment, the base **151** can be constructed out of a transparent material while also featuring electrode systems, thereby providing a user the option of detection methods.

[0035] In operation, a sample can be deposited into the injection chamber **121** by using a needle to penetrate the cap **111**. The sample will be transmitted in uniform volumes to the free sulfide reaction chamber **123**, the acid labile sulfide reaction chamber **124**, and the total sulfide-reaction chamber **125** via the first inlet channel **122a**, the second inlet channel **122b**, and the third inlet channel **122c**, respectively. The releasing chambers **123, 124, 125** are separated from their corresponding trapping chambers **143, 144, 145** by the H_2S permeable membrane **131**. In the free sulfide-reaction chamber **123**, only free H_2S gas will diffuse across the membrane **131** into the free sulfide trapping chamber **143**. In the acid labile sulfide-reaction chamber **124**, both the free H_2S and acid labile H_2S pools will diffuse across the membrane **131** into the acid labile sulfide trapping chamber **144**. In the total sulfide-reaction chamber **125**, H_2S from all three pools (free, acid labile, and bound sulfane sulfur) are released and will diffuse into the corresponding total sulfide trapping chamber **145**. Upon entry into the trapping chambers **143, 144, 145**, the H_2S is converted from its gaseous form into the HS^- form due to the presence of basic (pH ~9.5-10.0) conditions. The concentration of H_2S in the various pools then can be calculated as follows: the free H_2S and total H_2S concentrations is equal to that measured by the free sulfide trapping chamber **143** and total sulfide trapping chamber **145**, respectively. The acid labile H_2S amount is determined by subtracting the amount measured in the free sulfide trapping chamber **143** from that of the acid labile sulfide trapping chamber **144**. The bound H_2S concentration is determined by subtracting the acid labile trapping chamber **144** concentration from the total sulfide trapping chamber **145** concentration. In this way, the device simultaneously detects free H_2S, acid labile amounts of H_2S, bound sulfane sulfur available H_2S, and overall total bioavailable H_2S from one specimen.

Electrochemical Detection

[0036] As depicted in Figures 2 and 4, the hydrogen sulfide detecting apparatus **100** can feature a base **151** having integrated electrode systems **153, 154, 155** for the electrochemical detection of H_2S in the trapping chambers **143, 144, 145**. Contemporary

electrochemical methods used for *in vivo* and *in vitro* detection of biological compounds are chronoamperometry (CA) and differential pulse voltammetry (DPV). Both CA and DPV utilize a three-electrode system (reference, counter and working). In CA, the working electrode potential is held constant (with respect to the reference electrode) and current is measured as a function of time. Excellent temporal resolution and sensitivity is achieved with this technique. However, the origin of the current cannot be discriminated, for the measured current is a superposition of any species that is electrolyzed at or below the working electrode potential. For single species concentration determination, selective working electrodes must be used.

[0037] DPV is a hybrid of traditional cyclic voltammetry and CA. The sensitivity is similar to CA, but the temporal resolution is less. DPV has a potential applied to the working electrode that is a linearly increasing pulse train. The difference in current per pulse is recorded as a function of a linearly increasing voltage. Current is measured at two points for each pulse, the first point just before the pulse application and the second at the end of the pulse. This technique yields a curve with a peak that is directly proportional to species concentration. This allows for concentration discrimination of species in solution whose half-wave potential differs by as little as 40 to 50 mV.

[0038] H_2S has an oxidation reaction at -0.14 V producing two electrons through HS^- reaction with $Fe(CN)_6$ to yield the overall equation of: $H_2S \rightarrow S + 2H^+ + 2e^-$. Both CA and DPV can detect the electrons generated from HS^- oxidation. And since both modalities are incorporated in contemporary potentiostats, both can be used for determining optimal electrochemical detection. During CA detection, the working electrode is fixed between -0.20 - 0.30 V to oxidize H_2S/HS^-, and during DPV a range of voltages is applied. The voltages for electron detection must sweep from -0.3 V to 0.3 V with a scan rate of 5-10 mV/sec and a scan increment 2-4 mV. An example of pulsing parameters are a pulse height of 25 mV, a step/drop time of 100 ms, and a pulse width of 50 ms; although these may vary by 50% depending on chip performance.

[0039] Figure 4 illustrates one embodiment of working chamber conditions and electrochemical detection methodology. Electrochemical detection of H_2S is performed in the three separate trapping chambers: the free sulfide trapping chamber **143**; the acid labile sulfide trapping chamber **144**; and the total sulfide trapping chamber **145**. As explained above, the free sulfide reaction chamber **123** is preferably at neutral pH 7.0, the acid labile sulfide reaction chamber **124** at acid pH (pH from about 2.6 to about 6), and

the total sulfide reaction chamber **125** at acid pH (pH from about 2.6 to about 6) plus 1 mM TCEP to liberate bound H_2S. Hydrogen sulfide gas will diffuse across the permeable membrane and be trapped in the corresponding trapping chambers due to the basic conditions present (pH from about 9.5 to about 10.0). FeCN will oxidize HS- to produce 2e-. The changes in electrochemical potential can be measured using a potentiostat coupled to the embedded electrodes. The potentiostat can be coupled to the integrated electrode systems **153, 154, 155**, preferably via a copper wire that is adhered to the electrodes with a silver epoxy.

Fluorescence, chemiluminescence and colorimetric detection

[0040] As depicted in Figure 7, the hydrogen sulfide detecting apparatus **100** can alternatively feature a transparent base **151** to allow H_2S levels in the trapping chambers **143, 144, 145** to be detected via fluorescence, chemiluminescence and colorimetric dyes. Dyes such as bimane compounds, including but not limited to dibromobimane, monobromobimane, benzodithiolone, and dansyl azide, can be used in conjunction with fluorescence excitation and emission spectrometry to detect sulphide. Hydrogen sulphide reaction with electron-poor aromatic or other electrophilic chemicals can produce color shifts in the visible light spectrum. For example nitrobenzofurazan thioether compounds can react to form nitrobenzofurazan thiol with a shift in absorbance spectrum at 534 nm. Hydrogen sulphide contained within the trapping chambers **143, 144, 145** can be detected by chemiluminescence through reaction with ozone or other electrophilic compounds to stimulate photon release.

Device Fabrication

[0041] A hydrogen sulfide detecting apparatus exemplifying the principles of the present invention can be fabricated utilizing a variety of materials and techniques. One preferred method is to fabricate in layers via PDMS. Alternate polymer materials, apart from PDMS may be utilized that include SU-8 polymers or similar structures. Additives to the base material may be employed, such as polyethylene oxide (PEO). These additives can increase the capillary action of the devices. Other methods include but are not limited to the use of silicon or metals such as copper. For example, a suitable microfabrication procedure would be to utilize bulk micro-machined silicon wafers that serve as the device substrate. Alternate fabrication processes may be utilized including layer-by-layer deposition through advanced printing and processing, but not limited to 3D

printing. Casting via mold-and-pour could also be used to generate the appropriate structures given non-permeable materials.

[0042] In a preferred embodiment, the hydrogen sulfide detecting apparatus of the present invention is constructed in layers utilizing PDMS construction in combination with other polymer materials. For example, the hydrogen sulfide detecting apparatus 100 depicted in Figure 2 is comprised of five layers. Referring to Figure 2, the first layer **110** can comprise a butyl rubber cap **111**. The second layer **120**, which is bonded to the first layer **110**, can be cast from a reusable mold to form the injection chamber **121**, the plurality of reaction chambers **123**, **124**, **125**, and inlet channels **122a-c**. The third layer **130** can comprise a PDMS membrane **131** and is bonded to the second layer **120**. The thickness of the membrane **131** may be varied depending on the material used and fabrication technology employed. The fourth layer **140** is bonded to the third layer **130** and comprises a plurality of trapping chambers **143**, **144**, **145** filled with a trapping buffer (e.g., 100 mM NaOH, pH from about 9.5 to about 10). In the preferred construction, the fourth layer **140** is a 1 mm thick section of PDMS that has trapping chambers **143**, **144**, **145** cut out of the PDMS material and aligned with the reaction chambers **123**, **124**, **125**. The fifth layer **150** preferably consists of a plastic base **151** with interdigitated electrode systems **153**, **154**, **155** for electrochemical detection of the test specimen. The electrode systems **153**, **154**, **155** can be printed on the surface via microfabrication techniques and aligned with the trapping chambers **143**, **144**, **145** formed by cutouts in the fourth layer **140**. The fifth layer **150** preferably is longer than the fourth layer **140**, allowing access to the electrodes on the apparatus **100**. For example, the first layer **110** can be 10 nm x 2 nm, the second, third, and fourth layers **120**, **130**, **140** can be 40 nm x 2.5 nm, and the fifth layer **150** can be 50 nm x 2.5 nm. Other embodiments of the chip design could feature either increased or reduced dimensions to enable detection of larger or smaller volumes, respectfully. Finally, the fifth layer **150** is bonded to the fourth layer **140** and air is evacuated from the injection and releasing chambers.

[0043] Figure 6 illustrates an exemplary process **200** for manufacturing the second layer **120** of the hydrogen sulfide detecting apparatus **100**. In the preferred PDMS fabrication, a silicon nitride wafer is provided in step **201**. In step **202**, the silicon nitride wafer is spin-coated with 500 μm thick SU-8 photoresist and soft baked. Next, the wafer is exposed through a lithographic mask and baked post exposure (step **203**). The photoresist is then developed and rinsed in step **204**. In step **205**, uncured PDMS is

poured onto the mold and cured. In the preferred PDMS polymer construction, the cast is removed in step **206** and the injection chamber is cut out through the entire thickness of the cast in step **207**. The required chemical reaction buffers for the acid labile (acid pH from about 2.6 to about 6.0) and total sulfide (acid pH plus 1 mM TCEP) can be coated by evaporation of concentrated solutions on to the surface of the respective chambers **124**, **125** to complete the second layer **120**. Final height of PDMS material should be high enough to encapsulate the designed channels with heights to 2000 microns. Design features include high surface areas consisting of, but not limited to, capillary channels that range from 1 to 400 microns in width with heights variable from 10 to 2000 microns. However, one skilled in the art will appreciate that channel height is determined based on required sample volumetric size. Additionally, one skilled in the art will recognize that the foregoing process may also be utilized for manufacturing the fourth layer **140** of the hydrogen sulfide detecting apparatus **100**.

Examples

Example 1

[0044] The transfer efficiency of H_2S across 75 μm and 150 μm PDMS membranes was demonstrated using an embodiment of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention. A sample was introduced into a single acid reaction chamber separated by a 75 μm PDMS membrane from an alkaline trapping chamber containing 10 mM monobromobimane (MBB). This experiment was repeated with a 150 μm PDMS membrane. The H_2S transfer efficiency over time was measured by RP-HPLC detection of sulfide dibromane (SDB). Figure 5 illustrates the diffusion of H_2S across a PDMS membrane of different thicknesses, utilizing fluorescent detection by HPLC. The transfer efficiency of H_2S from the acid reaction chamber into the trapping chamber is depicted as measured using a MBB detector for both a 75-μm membrane and a 150-μm membrane. The sodium sulfide volatilized the sulfide anion into H_2S gas, which diffused across the membrane and was trapped in a separate chamber at pH 9.5 with 0.1 mM DTPA. Sample aliquots were taken from the trapping chamber at specified times. The amount of sulfide was detected using fluorescent HPLC analysis as described in PCT/US2013/031354, which is incorporated herein by reference. As shown in Figure 5, an approximate 15% transfer efficiency occurred within 10 minutes using the 75-μm membrane, while an approximate 50% transfer efficiency occurred within 10 minutes using the 150-μm membrane. The transfer efficiency of H_2S across the

permeable membrane can be utilized to calibrate the hydrogen sulfide detecting apparatus **100**.

Example 2

[0045] An embodiment of a hydrogen sulfide detecting apparatus exemplifying the principles of the present invention can be used to determine the concentration of H_2S in a specimen using electrochemical, fluorescence, or colorimetric detection methods. In such instances, a blood sample will be obtained from a subject and placed into vacutainer tubes containing lithium heparin (BD Biosciences, Cat. No. 367886), which is then immediately centrifuged at 4°C at 1500 RCF for 4 minutes to separate the plasma from the red blood cells. The plasma sample will then be injected into the injection chamber **121** of the apparatus **100** via a 26-gauge needle and 1cc syringe. The sample will be pulled into the injection chamber **121** which is evacuated of air by wicking action, where it will be further pulled into the three parallel reaction chambers **123, 124, 125** for free sulfide, acid labile+free sulfide, and total sulfide detection respectively. The buffer components that coat the chambers will dissolve in the plasma sample, providing the correct pH and chemical concentrations necessary for the reactions to occur at room temperature. After approximately 15 minutes, hydrogen sulfide will be liberated from each of the reaction chambers **123, 124, 125**; will diffuse across the membrane **130**; and will be trapped in the alkaline buffer in the respective trapping chambers **143, 144, 145**. Detection can then be accomplished by one of the three following methods: (a) electrochemical, (b) fluorescence, or (c) colorimetric.

[0046] If the electrochemical method is to be employed, the apparatus **100** will be connected to a potentiostat such as the VersaStat 4 (Princeton Applied Research), with one lead each for the working electrode, counter electrode, and reference electrode. A method such as differential pulse voltammetry (DPV) will be used to acquire a signal that is a measure of hydrogen sulfide concentration in the plasma sample. Typical settings for the DPV parameters are 25 mV for pulse height, 50 msec for pulse width, 1 mV for step height, and 100 msec for step width. Peak currents will be obtained for each chamber and converted into sulfide concentrations based on a calibration function (See Example 1).

[0047] If a fluorescence method is to be employed, the apparatus **100** will have a fluorescent dye such as dibromobimane (DBB) dissolved in solution in the trapping chambers **143, 144, 145**. After reaction between dye and hydrogen sulfide in the trapping

chambers **143, 144, 145**, fluorescence will be measured using appropriate excitation and emission wavelengths. If DBB dye is used these are 358 nm and 484 nm respectively. Fluorescence will be quantified and converted to sulfide concentrations by means of a calibration function (See Example 1).

[0048] If a colorimetric method is to be used, the apparatus 100 will have a compound such as nitrobenzofurazan thioether dissolved in solution in the trapping chambers **143, 144, 145**. Upon reaction with sulfide, it will form nitrobenzofurazan thiol, with a shift in the absorbance spectrum at 534 nm as previously noted. Absorbance will be quantified and converted to sulfide concentrations by means of a calibration function (See Example 1).

[0049] Free sulfide, acid-labile sulfide, bound sulfane sulfur, and total sulfide can then be calculated as follows. Free sulfide and total sulfide concentrations will be equal to that measured in the free sulfide and total sulfide trapping chambers **143, 145** respectively. The acid labile sulfide concentration will be equal to that measured in the "acid labile + free sulfide" chamber **144** minus the concentration in the free sulfide chamber **143**. The bound sulfane sulfur concentration will be found by subtracting the concentration measured in the "acid labile + free sulfide" chamber **144** from that measured in the total sulfide chamber **145**.

[0050] The foregoing description and accompanying drawings illustrate the principles, exemplary embodiments, and modes of operation of the invention. However, the invention should not be construed as being limited to the particular embodiments discussed above. Many modifications of the embodiments described herein will come to mind to one skilled in the art having the benefit of the teaching presented in the foregoing descriptions and the associated drawings. Accordingly, it should be appreciated that variations to those embodiments can be made by those skilled in the art without departing from the scope of the invention.

What is claimed is:

1. A hydrogen sulfide (H_2S) detecting apparatus comprising:
    a. an injection chamber;
    b. a plurality of reaction chambers in fluid communication with the injection chamber;
    c. a plurality of trapping chambers positioned adjacent to the plurality of reaction chambers;
    d. a H_2S-permeable membrane positioned between the plurality of reaction chambers and the plurality of trapping chambers.

2. The hydrogen sulfide detecting apparatus of claim 1, wherein the plurality of reaction chambers comprise: a free sulfide reaction chamber; an acid labile sulfide reaction chamber; and a total sulfide reaction chamber.

3. The hydrogen sulfide detecting apparatus of claim 2, wherein the free sulfide reaction chamber has a neutral pH environment with a pH from about 7.0 to about 7.5.

4. The hydrogen sulfide detecting apparatus of claim 3, wherein the acid labile sulfide reaction chamber has an acidic environment with a pH from about 2.6 to about 6.0.

5. The hydrogen sulfide detecting apparatus of claim 4, wherein the total sulfide reaction chamber has an acidic environment with a pH from about 2.6 to about 6.0 and further comprises a reducing agent.

6. The hydrogen sulfide detecting apparatus of claim 5, wherein the reducing agent is selected from the group consisting of: tris(2-carboxyethyl)phosphine hydrochloride and dithiothreitol.

7. The hydrogen sulfide detecting apparatus of claim 5, wherein the plurality of trapping chambers comprise:

a. a free sulfide trapping chamber positioned adjacent to the free sulfide reaction chamber such that such that H_2S gas released from the free sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the free sulfide trapping chamber;

b. an acid labile sulfide trapping chamber positioned adjacent to the acid labile sulfide reaction chamber such that such that H_2S gas released from the acid labile sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the acid labile sulfide trapping chamber; and

c. a total sulfide trapping chamber positioned adjacent to the total sulfide reaction chamber such that such that H_2S gas released from the total sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the total sulfide trapping chamber.

8. The hydrogen sulfide detecting apparatus of claim 7, wherein the plurality of trapping chambers each have an alkaline environment.

9. The hydrogen sulfide detecting apparatus of claim 7, wherein the plurality of trapping chambers each have an alkaline environment with a pH from about 9.5 to about 10.0.

10. The hydrogen sulfide detecting apparatus of claim 8, further comprising a base positioned adjacent to the plurality of trapping chambers.

11. The hydrogen sulfide detecting apparatus of claim 10, wherein the base comprises a plurality of electrode systems to enable electrochemical detection of H_2S in the adjacent plurality of trapping chambers.

12. The hydrogen sulfide detecting apparatus of claim 11, wherein the plurality of electrode systems comprise: a first electrode system positioned adjacent to the free sulfide trapping chamber; a second electrode system positioned adjacent to the acid labile sulfide trapping chamber; and a third electrode system positioned adjacent to the total sulfide trapping chamber.

13. The hydrogen sulfide detecting apparatus of claim 12, wherein the plurality of electrode systems each comprise a reference electrode, a counter electrode, and a working electrode.

14. The hydrogen sulfide detecting apparatus of claim 10, wherein the base is transparent to enable fluorimetric or colorimetric detection of H_2S in the adjacent plurality of trapping chambers.

15. The hydrogen sulfide detecting apparatus of claim 10, further comprising an injection chamber in fluid communication with the plurality of reaction chambers via a plurality of inlet channels, wherein the plurality of inlet channels comprise:
    a. A first inlet channel connecting the injection chamber to the free sulfide reaction chamber;
    b. a second inlet channel connecting the injection chamber to the acid labile sulfide reaction chamber; and
    c. a third inlet channel connecting the injection chamber to the total sulfide reaction chamber.

16. The hydrogen sulfide detecting apparatus of claim 13, further comprising a cap positioned adjacent to the injection chamber to allow a test sample to be injected through the cap and into the injection chamber.

17. A hydrogen sulfide (H_2S) detecting apparatus comprising:
    a. an injection chamber;
    b. a plurality of reaction chambers in fluid communication with the injection chamber, the plurality of reaction chambers comprising:
        i. a free sulfide reaction chamber having a pH from about 7.0 to about 7.5;
        ii. an acid labile sulfide reaction chamber having a pH from about 2.6 to about 6.0; and
        iii. a total sulfide reaction chamber having a pH from about 2.6 to about 6.0 and further having a reducing agent.

c. a plurality of trapping chambers positioned adjacent to the plurality of reaction chambers, wherein the plurality of trapping chambers each have an alkaline environment with a pH from about 9.5 to about 10.0;

d. a H_2S-permeable membrane positioned between the plurality of reaction chambers and the plurality of trapping chambers; and

e. a base positioned adjacent to the plurality of trapping chambers.

18. The hydrogen sulfide detecting apparatus of claim 17, wherein the plurality of trapping chambers comprise:

a. a free sulfide trapping chamber positioned adjacent to the free sulfide reaction chamber such that such that H_2S gas released from the free sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the free sulfide trapping chamber;

b. an acid labile sulfide trapping chamber positioned adjacent to the acid labile sulfide reaction chamber such that such that H_2S gas released from the acid labile sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the acid labile sulfide trapping chamber; and

c. a total sulfide trapping chamber positioned adjacent to the total sulfide reaction chamber such that such that H_2S gas released from the total sulfide reaction chamber will diffuse across the H_2S-permeable membrane and into the total sulfide trapping chamber.

19. The hydrogen sulfide detecting apparatus of claim 18, wherein the base comprises a plurality of electrode systems to enable electrochemical detection of H_2S in the adjacent plurality of trapping chambers, wherein the plurality of electrode systems comprise: a first electrode system positioned adjacent to the free sulfide trapping chamber; a second electrode system positioned adjacent to the acid labile sulfide trapping chamber; and a third electrode system positioned adjacent to the total sulfide trapping chamber.

20. A method for measuring in a sample the concentrations of free hydrogen sulfide, of acid-labile sulfide, and of sulfane-bound sulfur, the method comprising:

a. providing a hydrogen sulfide (H_2S) detecting apparatus having: an injection chamber; a plurality of reaction chambers in fluid communication with the injection chamber; a plurality of trapping chambers positioned adjacent to the plurality of reaction chambers, the plurality of trapping chambers defining a free sulfide trapping chamber, an acid labile sulfide trapping chamber, and a total sulfide trapping chamber, with the plurality of trapping chambers each having an alkaline environment; and a H_2S-permeable membrane positioned between the plurality of reaction chambers and the plurality of trapping chambers;

b. depositing a sample into the injection chamber;

c. detecting the concentration of H_2S in the free sulfide trapping chamber to determine the free hydrogen sulfide concentration in the sample;

d. detecting the concentration of H_2S in the acid labile sulfide trapping chamber and the total sulfide trapping chamber;

e. determining the acid labile sulfide concentration by subtracting the detected concentration of H_2S in the free sulfide trapping chamber from the detected concentration of H_2S in the acid labile sulfide trapping chamber; and

f. determining the sulfane-bound sulfur concentration by subtracting the detected concentration of H_2S in the acid labile sulfide trapping chamber from the detected concentration of H_2S in the total sulfide trapping chamber.

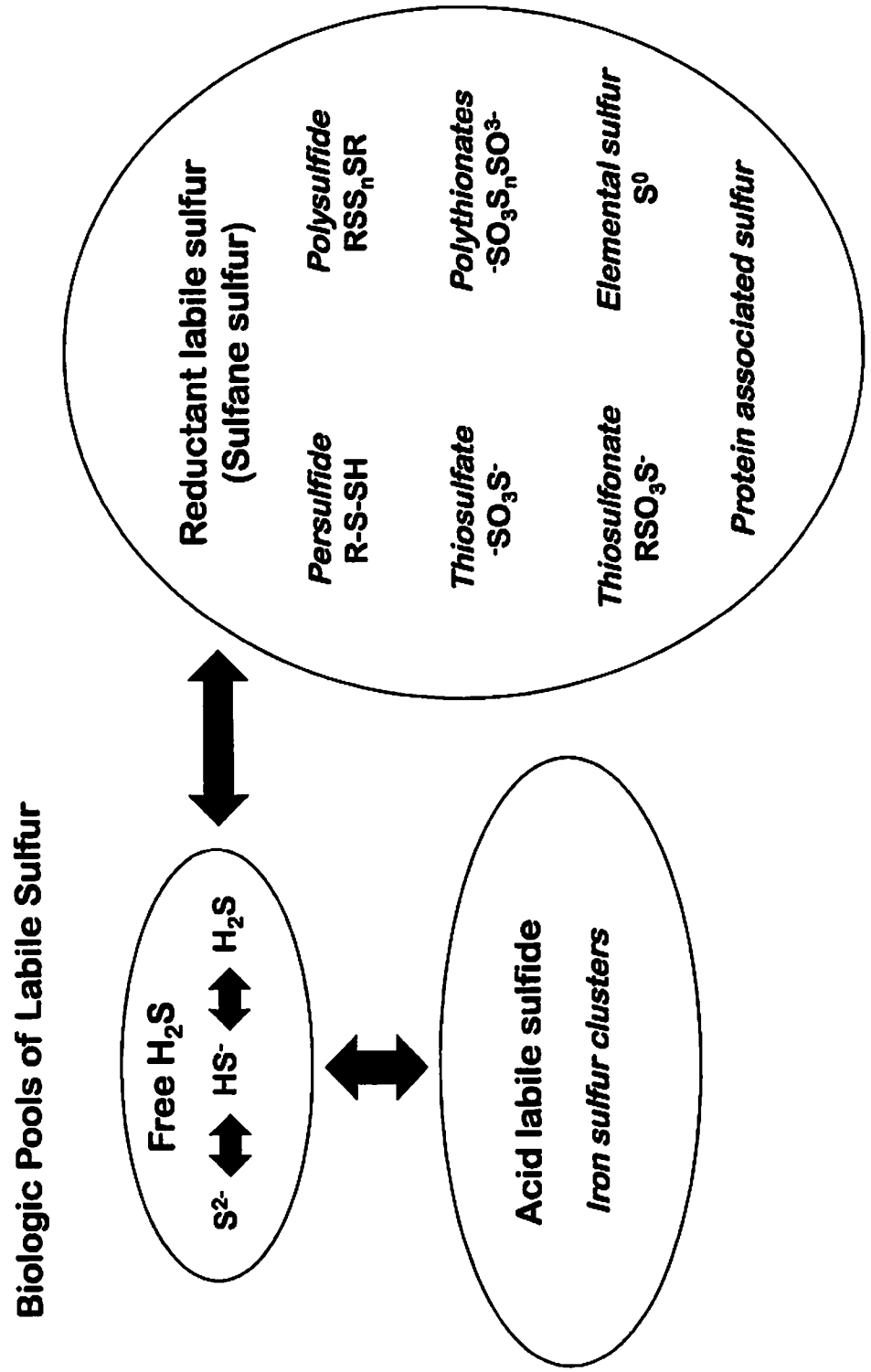

Biologic Pools of Labile Sulfur
Free H_2S
S^{2-} HS^- H_2S
Acid labile sulfide
Iron sulfur clusters
Reductant labile sulfur
(Sulfane sulfur)
Persulfide
R-S-SH
Polysulfide
RSS_nSR
Thiosulfate
$-SO_3S^-$
Polythionates
$-SO_3S_nSO^{3-}$
Thiosulfonate
RSO_3S^-
Elemental sulfur
S^0
Protein associated sulfur

Fig. 1

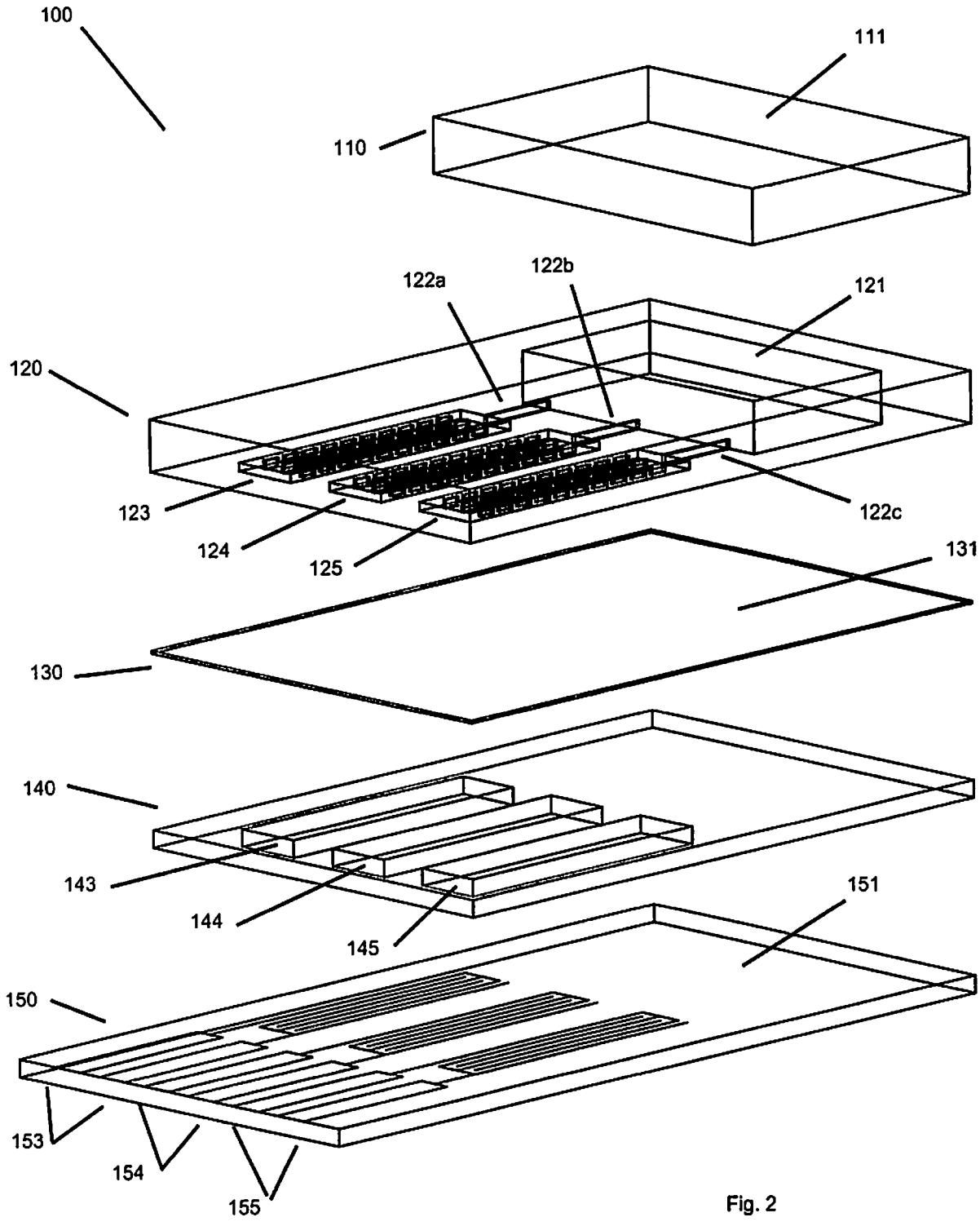

100
111
110
122a
122b
121
120
123
122c
124
125
131
130
140
143
144
145
151
150
153
154
155

Fig. 2

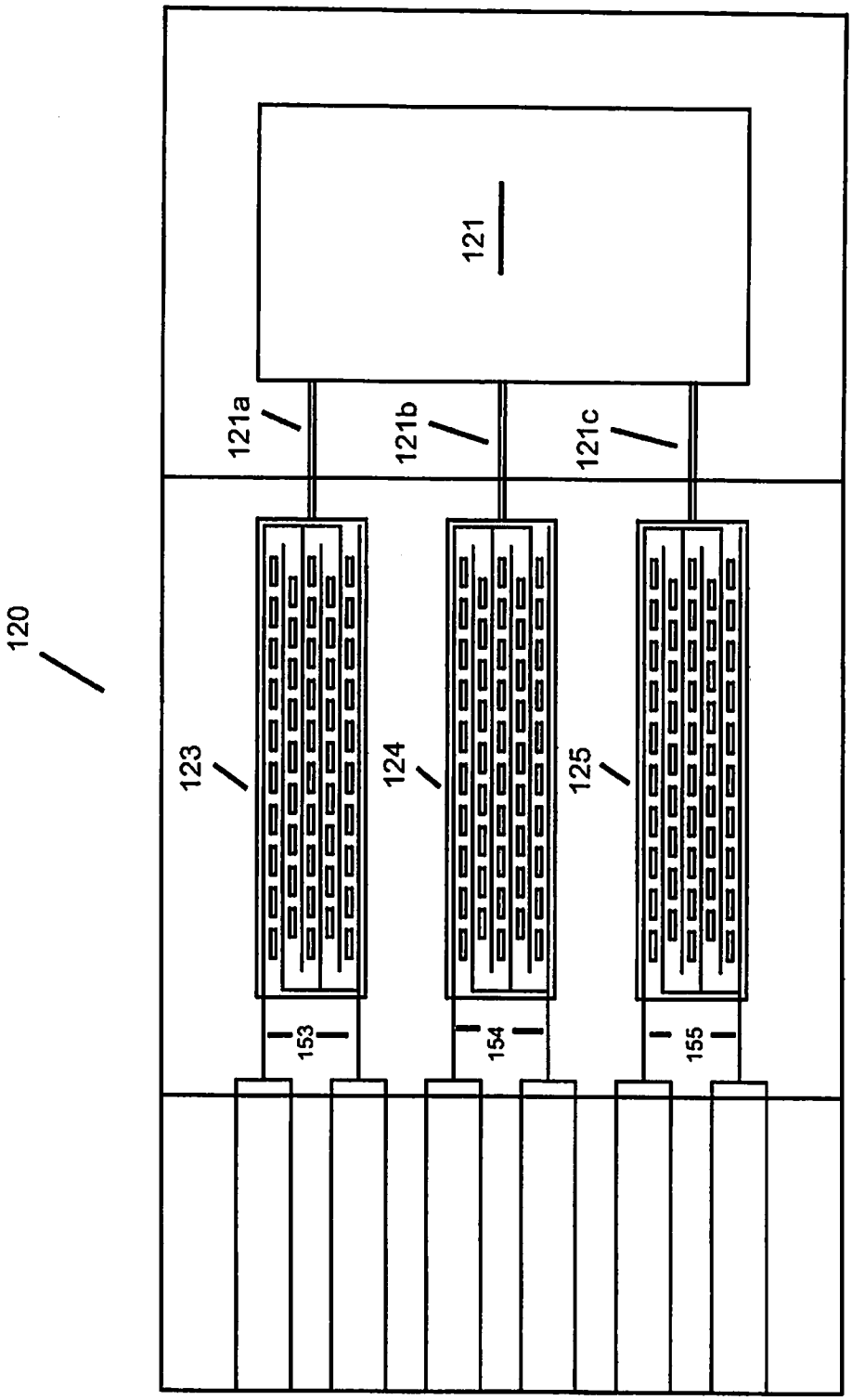
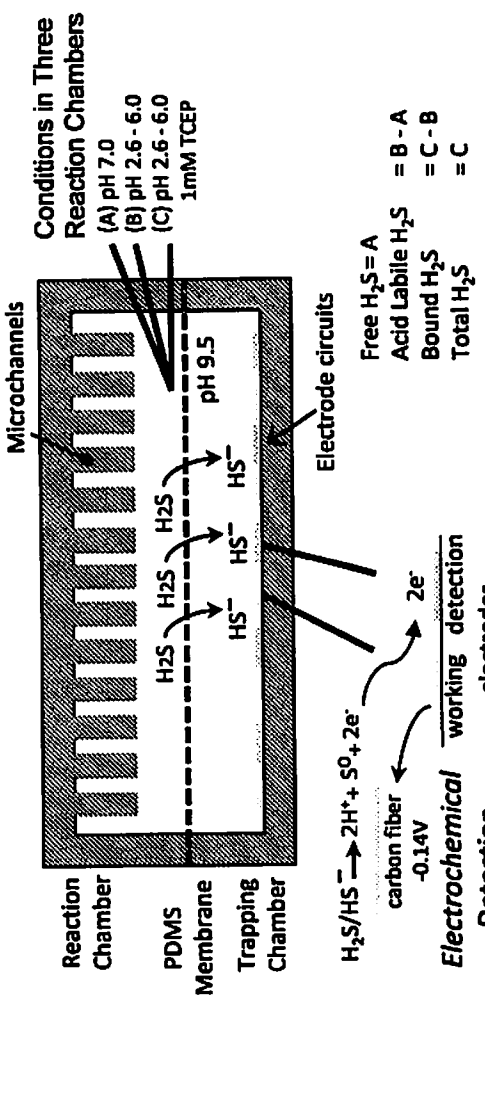
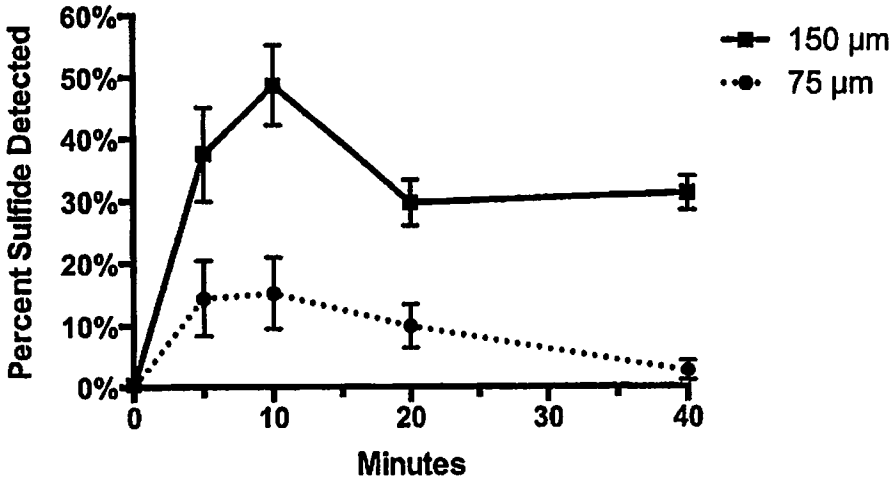


Fig. 3



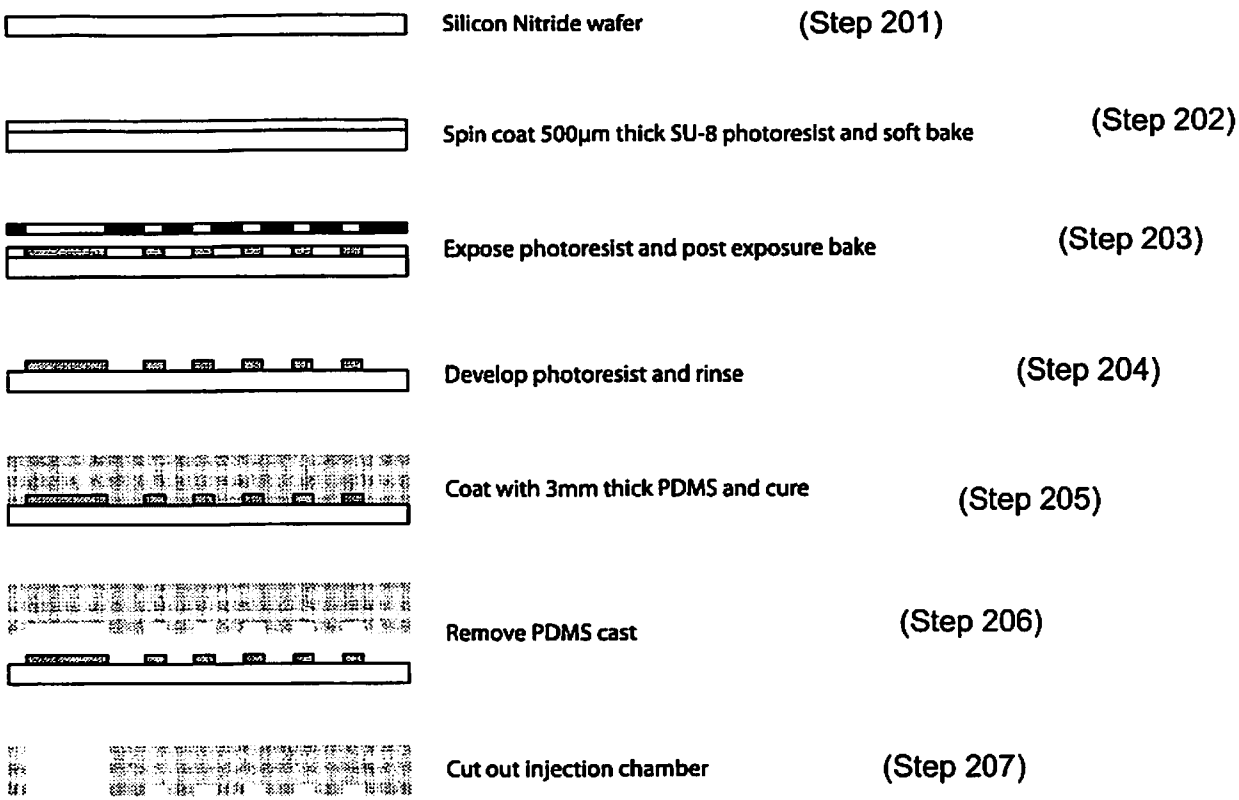
Representative working chamber conditions and electrochemical detection

Fig. 4



Diffusion of H_2S across different thickness PDMS and fluorescence detection by HPLC, showing transfer efficiency of H_2S gas transfer from the acid reaction chamber into the trapping chamber as measured using MBB detector.

Fig. 5



Manufacturing process for chip and associated reaction chambers

Manufacturing process 200 for chip and associated reaction chambers

Fig. 6

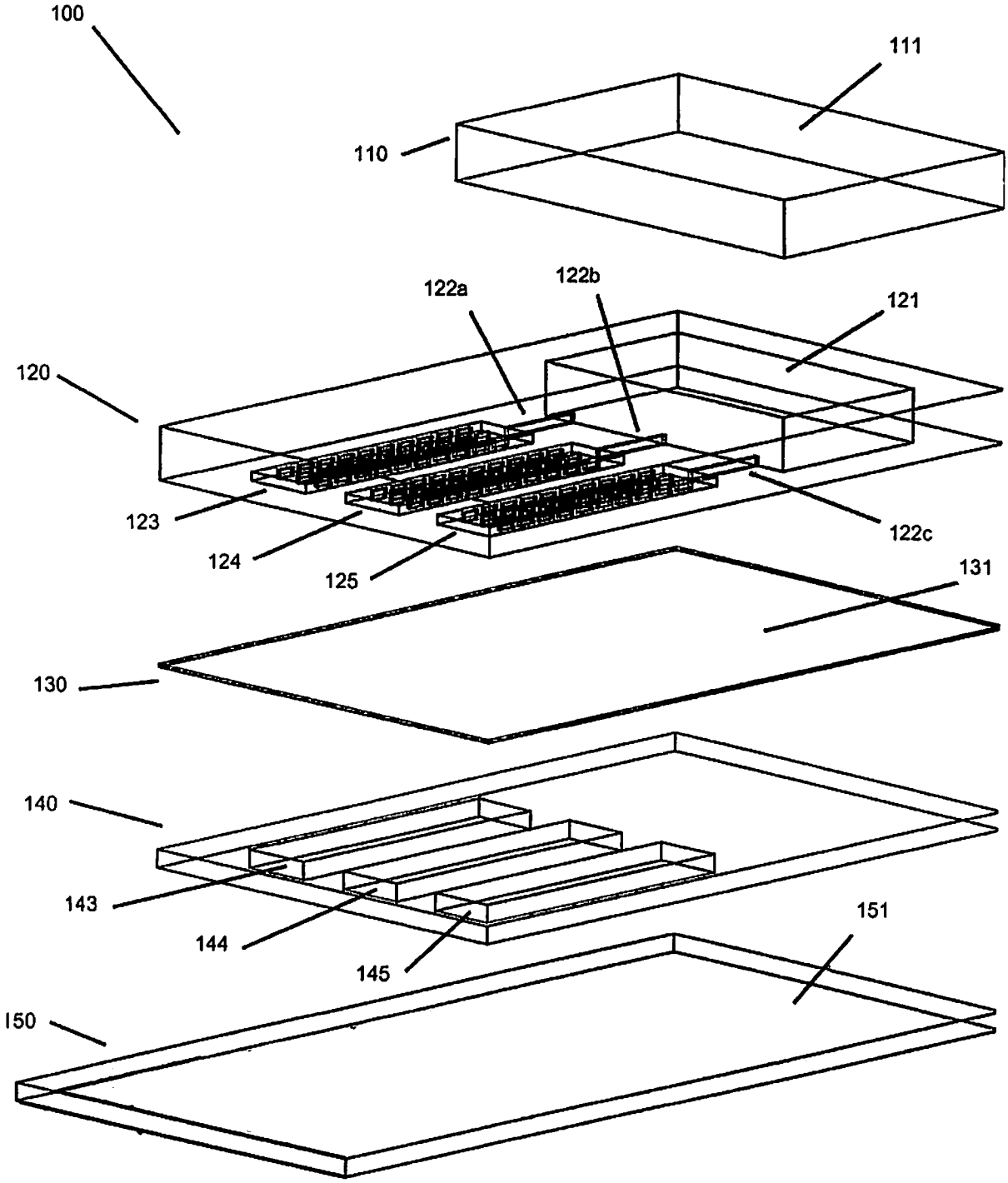

100
111
110
122a
122b
121
120
123
124
125
122c
131
130
140
143
144
145
151
150

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/032173

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 31/00 (2014.01)
USPC - 205/786.5
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)
IPC(8) - G01N 27/40, 31/00 (2014.01)
USPC - 205/786.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - G01N 27/4045 (2014.06)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar, Pubmed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/0073988 A1 (ZHANG et al) 29 March 2012 (29.03.2012) entire document.	1-20
Y	US 8,268,146 B2 (JIANG et al) 18 September 2012 (18.09.2012) entire document.	1-16, 18-20
Y	US 7,923,037 B2 (TOMASELLI et al) 12 April 2011 (12.04.2011) entire document.	4-19
Y	US 7,959,864 B2 (JIANG et al) 14 June 2011 (14.06.2011) entire document.	14
Y	US 7,282,081 B2 (VERSCHAREN) 16 October 2007 (16.10.2007) entire document.	15
Y	EP 0 584 568 A1 (MARRESE et al) 02 March 1994 (02.03.1994) entire document.	16

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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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
24 July 2014	18 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774