(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 October 2014 (02.10.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/160235 A1

(51) International Patent Classification:
G01N 33/569 (2006.01) *G01N 33/554* (2006.01)

(21) International Application Number:
PCT/US2014/026116

(22) International Filing Date:
13 March 2014 (13.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/779,362	13 March 2013 (13.03.2013)	US
61/780,138	13 March 2013 (13.03.2013)	US
14/209,009	13 March 2014 (13.03.2014)	US

(71) Applicant: **APPLIED BIOMOLECULAR TECHNOLOGIES** [US/US]; 1381 Kinnear Road, Columbus, OH 43212 (US).

(72) Inventors: **ZUPANCIC, Thomas, J.**; 1381 Kinnear Road, Columbus, OH 43212 (US). **KITTLE, J.D.**; 1381 Kinnear Road, Columbus, OH 43212 (US). **ZENG, Lingchen**; 1381 Kinnear Road, Columbus, OH 43212 (US). **VEDAMOORTHY, Srikanth**; 1381 Kinnear Road, Columbus, OH 43212 (US). **MO, Min**; 1381 Kinnear Road, Columbus, OH 43212 (US).

(74) Agents: **MILLER, Courtney J.** et al.; McNees Wallace & Nurick, LLC, 100 Pine Street, PO Box 1166, Harrisburg, PA 17108-1166 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2014/160235 A1

(54) Title: METHOD FOR SIGNAL AMPLIFICATION IN BIOSENSOR-BASED SYSTEM FOR RAPIDLY DETECTING INFECTIOUS AGENTS

(57) Abstract: A system for detecting infectious agents in biological samples in real time that includes a sample to be tested for at least one specific infectious agent; and a biosensor, wherein the biosensor is operative to detect a specific infectious agent in the sample to be tested; and wherein the biosensor emits an amplified and detectable signal when it reacts with the specific infectious agent. Signal detection is enhanced through the release of LPS from the cells of the infectious agent.

TITLE OF THE INVENTION

METHOD FOR SIGNAL AMPLIFICATION IN BIOSENSOR-BASED SYSTEM FOR RAPIDLY DETECTING INFECTIOUS AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Applications Serial No. 61/779,362 filed on March 13, 2013 and entitled "Rapid Detection System for Infectious Agents" and Serial No. 61/780,138 filed on March 13, 2013 and entitled "Biosensor-Based Rapid Detection System for Infectious Agents, the disclosures of which are hereby incorporated by reference herein in their entirety and made part of the present U.S. utility patent application for all purposes.

BACKGROUND OF THE INVENTION

[0002] The described invention relates in general to a system for detecting contaminants in biological samples and more specifically to a system for detecting infectious agents or pathogens in food samples in real time that includes a sample to be tested for a specific infectious agent; and a biosensor, wherein the biosensor is operative to detect at least one specific infectious agent in the sample to be tested; and wherein the biosensor emits a detectable signal when it reacts with the specific infectious agent.

[0003] In generic terms, a biosensor is a system or device for the detection of an analyte that combines a sensitive biological component with a physicochemical detector component. The components of a typical biosensor system include a biological element, a transducer or detector element, and associated electronics or signal processors that display test results in a meaningful and useful manner. The biological element includes biological material such as tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, and the like that may be created by known biological engineering processes. The transducer or detector element works in a physicochemical manner (e.g., optical, piezoelectric, and/or electrochemical) that transforms the signal resulting from the interaction of the analyte with the biological element into another signal that can be more easily measured and quantified. Biosensors originated from the integration of molecular biology and information technology (e.g., microcircuits, optical fibers,

etc.) to qualify or quantify biomolecule-analyte interactions such as antibody-antigen interactions. Considering that there is great demand for rapid, sensitive, easy-to-handle, and cost-effective detection tools to detect infectious agents, pathogens or/and toxins in food (see, for example, Mead *et al.*, *Food Related Illness and Death in the United States*, Emerging Infectious Diseases; Vol. 5, No. 5, September-October 1999 (607-625), which is incorporated by reference herein, in its entirety), there is an ongoing need for the utilization of biosensors in real-time, field-portable devices and instruments for the detection and identification of infectious agents, pathogenic microorganisms, toxins, and other contaminants in foods.

SUMMARY OF THE INVENTION

[0004] The following provides a summary of certain exemplary embodiments of the present invention. This summary is not an extensive overview and is not intended to identify key or critical aspects or elements of the present invention or to delineate its scope.

[0005] In accordance with one aspect of the present invention, a first system for rapidly detecting infectious agents in biological samples is provided. This system includes at least one sample to be tested for at least one specific infectious agent, wherein individual cells of the at least one infectious agent include at least one type of biomolecule that reacts with a biosensor when brought into physical contact therewith, and wherein the at least one type of biomolecule further includes lipopolysaccharides; and at least one biosensor, wherein the biosensor is operative to detect a specific infectious agent in the sample to be tested; and wherein the biosensor emits a detectable signal when it reacts with the lipopolysaccharides.

[0006] In accordance with another aspect of the present invention, a second system for rapidly detecting infectious agents in biological samples is provided. This system includes at least one sample to be tested for at least one specific infectious agent; at least one biosensor, wherein the biosensor is operative to detect a specific infectious agent in the sample to be tested; and wherein the biosensor emits a detectable signal when it reacts with the specific infectious agent; at least one type of biomolecule on the individual cells of the at least one infectious agent that reacts with the biosensor when brought into physical contact therewith; and wherein the biomolecule is released from the individual cells of the at least one infectious agent by using a

cation chelating agent to remove divalent cations and destabilize the cellular membrane; using a selected surfactant to release components of the cellular membrane; using an oxidative or reducing agent to cleave bonds that anchor the biomolecule in the cellular membrane; using an enzyme that hydrolyzes components of the cellular membrane, thereby releasing the biomolecule; using heat to disrupt the cellular membrane; using mechanical disruption of the cellular membrane; or combinations thereof.

[0007] Additional features and aspects of the present invention will become apparent to those of ordinary skill in the art upon reading and understanding the following detailed description of the exemplary embodiments. As will be appreciated by the skilled artisan, further embodiments of the invention are possible without departing from the scope and spirit of the invention. Accordingly, the drawings and associated descriptions are to be regarded as illustrative and not restrictive in nature.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] The accompanying drawings, which are incorporated into and form a part of the specification, schematically illustrate one or more exemplary embodiments of the invention and, together with the general description given above and detailed description given below, serve to explain the principles of the invention, and wherein:

[0009] FIG. 1 is a chart illustrating the effectiveness of the system of the present invention with regard to detecting the presence of one or more infectious agents in a biological sample. The Y-axis represents the amount of light flash, and the X-axis represents the time in seconds;

[0010] FIG. 2 is a graphic illustration of the LPS release methodology used in an exemplary embodiment of the present invention;

[0011] FIG. 3 is a chart illustrating the release of LPS from pathogenic *E. coli* cells using various reagents;

[0012] FIG. 4 is a graph illustrating the release of biosensor detectable LPS antigen from pathogenic *E. coli* cells; and

[0013] FIG. 5 is a flow chart of a system and process for testing a biological sample for the presence of one or more infectious agents, in accordance with an exemplary embodiment of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0014] Exemplary embodiments of the present invention are now described with reference to the Figures. Reference numerals are used in the detailed description to refer to the various elements. Although the following detailed description contains many specifics for the purposes of illustration, a person of ordinary skill in the art will appreciate that many variations and alterations to the following details are within the scope of the invention. Accordingly, the following embodiments of the invention are set forth without any loss of generality to, and without imposing limitations upon, the claimed invention.

[0015] As previously discussed, the present invention provides a system for accurately and rapidly (i.e., typically within one to five minutes) detecting infectious agents in biological and non-biological samples, particularly samples derived from beef, poultry, fish, or vegetable matter, although other biological materials may be analyzed using this invention. The biosensor is operative to detect a specific infectious agent in the sample to be tested by emitting a detectable signal when it reacts with the infectious agent. This system provides very high sensitivity without the need to culture infectious agents such as bacteria obtained from samples prior to testing. In some embodiments, the system of the present invention is capable of detecting a predetermined number of cells of an infectious agent such as, for example, one thousand cells or less. Some embodiments of this invention are expected to be able to detect very small numbers of cells, down to even a single cell of a particular infectious agent. In an exemplary embodiment, the specific infectious agent is *Escherichia coli*, although other infectious agents (such as Salmonella, Listeria, and Campylobacter), toxins, and various contaminants may be detected with the present invention. *Escherichia coli O157:H7*, *O26, O45, O103, O111, O121,* and *O145*, in either separate assays or multiplexed assays, may all potentially be detected using

this invention. This invention provides a biosensor that is based on a design that simplifies the construction process and improves the performance characteristics of the biosensor cell over prior art systems.

[0016] A critical component of the adaptive immune system is the B lymphocyte, which is a type of white blood cell that begins its development in the bone marrow and as such is involved in "humoral" immunity. Upon encountering an antigen, a B lymphocyte differentiates into a plasma cell, which then secretes immunoglobulin that functions as an antibody to the antigen. B cells are distinguishable from other lymphocytes, such as T cells and natural killer cells (NK cells) by the presence of a protein on the outer surface of the B cell known as a B cell receptor (BCR). This specialized receptor protein allows a B cell to bind to a specific antigen; thus, the principal functions of B cells are to (i) make antibodies against antigens; (ii) perform the role of antigen-presenting cells (APCs); and (iii) develop into memory B cells after activation by antigen interaction. Given these characteristics, biosensor or pathogen sensor systems may utilize or incorporate B lymphocytes for the purpose of detecting specific antigens. For example, B cells lines have been engineered to express cytosolic aequorin, a calcium-sensitive bioluminescent protein from the *Aequoria victoria* jellyfish, in addition to membrane-bound antibodies specific for certain pathogens. In this system, cross-linking of the antibodies by even low levels of the appropriate pathogen elevated intercellular calcium concentrations within seconds, causing the aequorin to emit a detectable signal of light.

[0017] In accordance with this invention, an exemplary biosensor includes a human B lymphocyte engineered to express a bioluminescent protein and at least one membrane-bound antibody specific for a predetermined infectious agent. With regard to this type of biosensor, cell-based biosensor (CBB) systems that incorporate whole cells or cellular components respond in a manner that can offer insight into the physiological effect of an analyte. As will be appreciated by those skilled in the art, cell-based assays (CBA) are emerging as dependable and promising approaches for detecting the presence of pathogens in clinical, environmental, or food samples because living cells are known to be extremely sensitive to modulations or disturbances in "normal" physiological microenvironments. Therefore, CBBs have been employed to screen and monitor "external" or environmental agents capable of causing perturbations of living cells

(see, for example, Banerjee *et al.*, *Mammalian cell-based sensor system*, Adv. Biochem. Eng. Biotechnology, 117:21-55 (2010), which is incorporated herein by reference, in its entirety). Compared with traditional detection methods (e.g., immunoassays and molecular assays such as PCR), a biosensor provides distinct advantages such as: (i) speed, i.e., several seconds to less than 10 minutes; (ii) increased functionality, which is extremely important for reporting active components such as live pathogens or active toxins; and (iii) ease of scale-up for performing high-throughput screening.

[0018] An aequorin-based biosensor system is utilized with certain embodiments of the present invention. Aequorin is a 21-kDa calcium-binding photoprotein isolated from the luminous jellyfish *Aequorea victoria*. Aequorin is linked covalently to a hydrophobic prosthetic group (coelenterazine) and upon calcium (Ca2+) binding, aequorin undergoes an irreversible reaction, and emits blue light (at 469 nm). The fractional rate of aequorin consumption is proportional, in the physiological pCa range, to [Ca2+]. Application of the aequorin-Ca2+ indicator to detect *E. coli* contamination in food products was reported in 2003 (see, Rider *et al. A B cell-based sensor for rapid identification of pathogens*, Science, 301(5630):213-5 (2003), which is incorporated by reference herein, in its entirety). Engineered B lymphocytes were used to express antibodies that recognize specific bacteria and viruses and aequorin, which emits light in response to the calcium flux triggered by the binding of a cognate target to the surface-antibody receptor. The resulting biosensor cell emitted light within minutes in the presence of the targeted microbes. To create these biosensor cells, antibody heavy and light chains with variable regions were cloned and expressed in a B-lymphocyte cell line. The resulting immunoglobulins become part of a surface B-cell–receptor complex, which includes the accessory molecules immunoglobulinα (Igα, or CD79a) and immunoglobulinβ (Igβ, or CD79b). When the complex is cross-linked and clustered by polyvalent antigens, such as microbes, a set of signaling events quickly leads to changes in the intracellular calcium-ion concentration, which then causes aequorin to emit light. This mechanism essentially hijacks the B-cell's intrinsic capacity to specifically recognize the antigen presented in the *E. coli* by the B-cell membrane IgG antibody, and this binding triggers a transient Ca2+ influx to cytosol, which binds the aequorin proteins engineered in this B-cell, and subsequently emit blue light (see, Relman, *Shedding light on*

microbial detection, N England J Med, 349(22):2162-3 (2003), which is incorporated by reference herein, in its entirety).

[0019] Selection of an appropriate B cell is an important aspect of this invention and any proposed cell line should be tested to confirm that the B cell receptor signaling pathway is fully functional. Individual B cell clones having the aequorin gene should be tested to identify a particular clone with high aequorin activity as significant variation from one clone to the next is possible (see, generally, Calpe *et al.*, *ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation*, Blood, 118(16):4401-10 (2011) and Cragg *et al.*, *Analysis of the interaction of monoclonal antibodies with surface IgM on neoplastic B-cells*, Br J Cancer, 79(5/6): 850–857 (1999), both of which are incorporated by reference herein in their entirety). A high-aequorin expressing B cell is important for achieving high levels of sensitivity when using this detection system. In an exemplary embodiment, the receptor response for the biosensor was verified by using the Ramos human B cell line. Ramos cells were first transfected with the aequorin gene and the transfected cells were then selected for aequorin expression for two weeks. After that, mixed Ramos cells were charged with coelenterazine (CTZ), stimulated with anti-IgM Ab, and flash signal elicited by the reaction was captured by a luminometer. As shown in FIG. 1, anti-IgM stimulation causes an expected sizeable and prolonged flash (from 45 to 65 seconds). In FIG. 1, the Y-axis represents the amount of light flashing, and the X-axis represents the reaction time in seconds. At 30 seconds, the anti-IgM solution was injected into the Ramos-Aeq cell solution; the first spike (from 30-37 seconds) is noise signal, and the second larger and longer peak is the biological response to anti-IgM stimulation. To improve the overall signal/noise ratio, the CTZ was removed from the CTZ-charged Ramos-Aeq cells. Removal of CTZ from the cell solution decreases noise signal from around 150 to about 50 without significantly compromising the amount of the true peak signal.

[0020] In accordance with this invention, an exemplary protocol for cell handling and flash-testing includes: (i) culturing Ramos-Aeq cells with regular culture medium and keeping these cells healthy (i.e., viability >98%); (ii) charging the Ramos-Aeq cells with CTZ at a final concentration of 2 μM, the cell density being 1–2 million per milliliter; (iii) charging the cells at 37^0C with 5% CO_2 in an incubator for at least 3 hours; (iv) removing the charging medium

containing CTZ; (v) flash testing by taking 200µl cell solution plus 30µl stimulants (anti-IgM) and reading with a luminometer; and (vi) confirming CTZ and aequorin functionality by adding 30-40 uL Digitonin (770 µM).

[0021] With regard to the biosensor component, one embodiment of the present invention utilizes a hybridoma cell based biosensor. Hybridomas are immortalized cells derived from the fusion of a specific antibody-producing B cell with a myeloma (B cell cancer) fusion partner that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies (in contrast to polyclonal antibodies). As previously discussed, mammalian B cell based biosensors have previously been constructed by introducing into B cells genes encoding the heavy and light chains for a membrane bound form of IgM antibody as well as a gene encoding a detectable reporter protein. Because B cells already express the alpha and beta chains of the B cell receptor (BCR), the modified B cells that are created this way can bind to the antigen recognized by the introduced antibody and trigger the B cell receptor signal transduction pathway. Activation of this pathway leads to a transient increase in intracellular calcium concentration; thus, it is possible to generate a detectable signal in response to binding to a target antigen by using a reporter protein that is activated when the calcium concentration is increased.

[0022] The present invention provides a mammalian cell biosensor that is useful for the detection of infectious pathogens or agents in environmental samples and that is based on a design that simplifies the construction process and improves the performance characteristics of the biosensor cell over prior art systems. Rather than introducing a target antigen specific IgM antibody into a B cell expressing the B cell receptor, the method of the present invention begins by isolating a hybridoma cell that is selected because it naturally expresses an endogenous anti-target antigen specific IgM. This cell is then converted to a BCR biosensor in a single step by adding a gene encoding a detectable reporter gene.

[0023] The method of this invention eliminates a number of problems that are inherent in the traditional biosensor design. First, the addition of multiple genes into a host cell requires the

use of antibiotic resistance genes and corresponding antibiotics that are added to the culture medium in order to select for and maintain the introduced genes in the cell. If independent gene delivery vehicles are used for reporter, antibody heavy chain and antibody light chain, three different antibiotics are required, thereby adding to the cost of production and increasing the undesirable use of large amounts of antibiotics in the cell production process. Advantageously, the hybridoma approach of this invention only requires a single gene delivery vehicle/selectable marker gene/antibiotic. Additionally, B cells are relatively difficult to engineer using contemporary genetic engineering techniques. More specifically, B cells are relatively refractory to standard gene delivery techniques such as chemical transformation and electroporation. This characteristic of B cell lines complicates the task of cell engineering. Most significantly, many promoter elements required for efficient transgene expression perform poorly in B cells making it necessary to test alternative expression constructs for the engineered antibody heavy and light chains. This complicates the need to achieve an appropriate balance between heavy and light chain expression. In contrast, the hybridoma cells of the present invention express the target antigen specific using the endogenous heavy and light gene promoters and naturally achieve appropriate relative amounts of heavy and light chain protein, overcoming this problem. Finally, the hybridoma cell approach eliminates problems with cell line stability that typically exist with genetically modified cell lines. Cells that have been engineered using traditional methods are prone to deletion events over multiple generations that ultimately result in the loss of the key transgenes that are required for functionality. Furthermore, transgenes that are introduced into a host cell using traditional methods are susceptible over time and through multiple cell divisions to gene silencing, such as by DNA methylation. Since the hybridoma cells of the present invention use the endogenous antibody genes, these problems are presumably eliminated.

[0024] In accordance with an exemplary method, a hybridoma cell based biosensor for pathogenic *E. coli* strain O111 was constructed using the following approach. An anti-O111 *E. coli* IgM expressing hybridoma cell was identified by inoculating mice with the insoluble membrane containing fraction from a preparation of O111 *E. coli* cells. These mice were further boosted with purified lipopolysaccharide (LPS) that had been isolated from the outer membrane of O111 *E. coli* cells. Once the mice had generated a suitable anti-O111 specific antibody titre, B

cells were isolated from the mice and fused to a standard myeloma fusion partner following standard protocols. Individual clones were then generated from this population of hybridomas. These clones were screened to identify a particular clone that expressed and anti-O111 *E. coli* IgM antibody (clone 1E7).

[0025] Clone 1E7 was analyzed by RT PCR to demonstrate that this cell line expressed the membrane-bound form of IgM as well and the alpha and beta chains of the B cell receptor. A plasmid expression vector containing an aequorin reporter gene (on pcDNA3.1 with G418 selectable marker) was then introduced into the cells by electroporation. The cells were then subcultured in the presence of G418 antibiotic. The G418 resistant cells obtained were tested for expression of the luminescent aequorin reporter gene by charging the cells with coelentarizine followed by addition of digitonin using standard methods. The cells were then analyzed in a luminometer to demonstrate a rapid flash, indicating that active aequorin enzyme was present. Then, IgM mediated B cell receptor signaling was demonstrated by charging the cells with coelentarizine as above, followed by addition of and anti-murine IgM antibody. When analyzed in a luminometer, these cells showed an IgM-BCR mediated flash, demonstrating the key function of the biosensor cell.

[0026] One embodiment of this invention utilizes a specific signal amplification methodology. Bacteria express surface biological molecules that can be detected using various immunochemical methods such as ELISA or the biosensors described herein. As previously mentioned, one such group of biomolecules is the lipopolysaccharides (LPS), also known as lipoglycans. LPS are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond and are found in the outer membrane of Gram-negative bacteria where they act as endotoxins that elicit strong immune responses in animals. LPS acts as the prototypical endotoxin because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages and B cells. The detection of these molecules using the biosensors of the present invention relies on effective surface contact between the biosensor and the biological molecules, i.e., LPS. LPS is typically present in concentrations of 10,000,000 copies per cell. If a detection system is limited by surface contact, then the detectable signals generated by a single bacterium may be limited by

the physical accessibility of the target biomolecule throughout a heterologous test sample (i.e., the biomolecules may be present in masses or inaccessible groups or clumps on the bacteria). Therefore, to assure maximum signal detection and assay sensitivity, it is desirable to release the LPS from the surface of the bacteria (SEE FIG. X). Thus, this process of bacterial cell fragmentation or LPS release is particularly important with the biosensor system because the intensity of the signal that is generated by is dependent on the number of biosensor cells that interact with the target antigen. Given the topological constraints of the interaction between a mammalian cell sensor and a bacterial cell, it is to be expected that an individual bacterial cell can only interact with a single mammalian biosensor cell, limiting the intensity of the signal that can be generated. In contrast, by fragmenting the bacterial cell into pieces or releasing the target LPS molecules from the bacterial cell surface it is possible for the antigens from a single bacterial cell to interact with multiple biosensor cells, substantially increasing the intensity of the signal that is generated by the biosensor system.

[0027] It is important that releasing a biomolecule from the surface of a bacteria be done in a manner that does not damage the detectability of the biomolecule, that does not inhibit or interfere with the functionality of the detection system, and that increases the availability of the biomolecule for detection by the biosensor. It is also desirable to release LPS using a method that can be easily incorporated into a rapid and efficient sample preparation procedure. Releasing LPS or other biomolecule from bacteria in a test sample can be referred to as "conditioning" the sample, and can be done in a fairly rapid manner that does not preclude nor require lysis of the bacterial cell. Samples may be conditioned by one or more of the following methods: (i) using a cation chelating agent such as citrate or EDTA to remove divalent cations and destabilize the bacterial membrane; (ii) using a selected surfactant that releases components of the bacterial membrane; (iii) using an oxidative or reducing agent that cleaves bonds that anchor the biomolecule in the membrane; (iv) using an enzyme that hydrolyzes components of the membrane, thereby releasing the target biomolecule (e.g. a lipase or lysozyme); (v) using a heating step to disrupt the membrane; and/or (vi) using mechanical disruption such as vortex mixing or pressure shearing. The agent(s) that result in release of the biomolecule are either harmless to the biosensor or are rendered harmless by dilution, chemical/enzymatic treatment,

heating, or cooling. The released biomolecule is then brought into contact with the biosensor to facilitate the reaction that generates a detectable signal. The methodology used to release the LPS or other biomolecule from the bacterial cell is matched to the requirements of the biosensor such that neither component is damaged or inactivated and such that signal strength is amplified or boosted to make detection more reliable, particularly in complex samples and samples with low numbers of bacteria

[0028] With reference to FIG. 2, the lipopolysaccharides (LPS) and biosensor cell (hybridoma cells) are the two key components of the biosensor pathogen detection system of this embodiment. Increasing the chance of pathogen contact and/or signal strength is the key event which determines the sensitivity of the biosensor system. To achieve the goal of high sensitivity (detecting very low number of pathogens) amplifying signal strength by increasing the number of biosensor-antigen molecule contact events is therefore important. LPS molecules, which are disease causing pathogen specific antigens embedded on the cell membrane of bacteria (#/cell) may not be available for access by maximum number of biosensor cells. Releasing these molecules by chemical treatment prior to mixing the sample with the biosensor cells will presumably maximize the number of cells that contacted the target antigen. Hence, LPS release from the bacteria and testing its efficacy is imperative and achieved by the following method.

[0029] A rapid and efficient procedure to release LPS from *E. coli* cells was demonstrated using the pathogenic *E. coli* strain O111 which expresses a unique lipopolysaccharide molecule on its surface, the O111 antigen. O111 bacterial cells (1×10^9) grown for 8 hours were washed with 10 mM Tris buffer (pH 8.0) to remove adherent LPS. Washed cells were resuspended in 100 μL Tris buffer (10 mM, pH 8.0), treated with 200 μL 1% or 3% F68 and/or 5 or 10 mM EDTA and incubated at 37^0C for 20 min. Released LPS was diluted (2,000,000 times) in 10 mM Tris buffer (pH 8.0), quantified using the Toxin Sensor Chromogenic LAL Endotoxin Assay Kit (GenScript USA Inc., NJ). The results shown in FIG. 3 indicate that LPS can be released from the pathogenic *E. coli* cells by using a number of alternative rapid and simple procedures. Furthermore, the quantity of LPS released varies depending on the specific method and reagents used. EDTA at approximately 5mM effectively

induced LPS release as did the relatively hydrophobic detergent Pluronic F68. In contrast, the relatively hydrophilic detergent Triton X100 had a much smaller effect. Thus, it is useful to use EDTA and/or the detergent Pluronic F68 to induce LPS release. It will be clear to one skilled in the art that other similar reagents can also produce similar results and thus the present invention is intended to include those alternative chelating agents and relatively hydrophobic detergents.

[0030] The LPS release procedure was demonstrated using the bacterial pathogen *E. coli* O111 LPS antigen. As a detector, a hybridoma cell based biosensor was constructed by inserting the chemiluminescent enzyme aequorin into hybridoma cell line (1E7) that expresses and anti-O111 specific IgM molecule on its surface. 2 x 10^5 1E7 hybridoma cells were charged with 2 μM CTZ for 4h at 37^{0}C. Cells were resuspended in fresh phenol free DMEM one hour before the experiment. Washed O111 bacterial cells (1 x 10^9) were resuspended in 200 μL of 10 mM Tris buffer (pH 8.0). EDTA and/or Pluronic F68 were added to the solution and incubate at 37^{0}C for 30 min before adding to the 1E7 cells. 30 μL of O111 LPS released from O111 bacteria were used for experiments. Luminescence (Relative Luminescence Units, RLU) was recorded using Glomax 20/20 luminometer (Promega). The results shown in FIG. 4 demonstrate the release of biosensor detectable LPS antigen from the pathogenic *E. coli* cells. The effectiveness of the alternative methods of LPS release varied between the various treatments and the combination of 4mM EDTA and 1% Pluronic F68 resulted in the generation of a sample that induced the highest response from the 1E7 hybridoma based biosensor cells. Thus, the combination of 4mM EDTA and 1% Pluronic F68 is particularly useful for preparing a sample of released LPS for detection by a mammalian cell biosensor.

[0031] As previously indicated, in some embodiments, the bioassay described herein may be carried out in a testing subunit or test cartridge designed for use with a bench-top or portable testing system and device such as that disclosed in U.S. Patent Application No. 13/711,296, which is incorporated by reference herein, in its entirety.. The test cartridge, which may be a single-use, disposable item, receives both the sample and the biosensor, and introducing the biosensor into the test cartridge mixes the sample and the biosensor in a predictable manner. The test cartridge further includes a reaction chamber for receiving the sample and the biosensor,

wherein the reaction chamber has a predetermined internal geometry and has been further adapted to minimize or eliminate background noise for the purpose of improving the overall signal to noise ratio. A least one stabilizer may located in the reaction chamber for minimize shear force damage to the sample and biosensor during the mixing process.

[0032] In an exemplary embodiment (not shown in the Figures), the reaction chamber and fluid channels that lead to the reaction chamber within the test cartridge are designed to achieve several objectives. An inlet channel for fluid entering the reaction chamber is a tubular shape and the diameter of the tube is relatively small and tapers to become smaller at the inlet to the reaction chamber. This increases the velocity of fluid entering the reaction chamber and promotes more vigorous and homogenous mixing due to the bulk motion of the reagents within the reaction chamber. It is desirable to mix the reagents and sample in a way to promote mixing beyond molecular diffusion, in order to minimize the duration of the test by ensuring that any infectious agent present in the sample rapidly encounters the biosensor. The inlet channel may be offset from the central axis of the reaction chamber to promote a clockwise or counterclockwise rotational motion of the reagents around the central axis of the test chamber as the fluids are mixed in order to increase homogeneity of the mixture. The inlet channel is also approximately tangent to the interior surface of the reaction chamber for allowing incoming fluid to travel from the inlet channel to the reaction chamber while remaining in contact with the side surface of the reaction chamber, which allows for a minimally turbulent flow and minimal introduction of air bubbles into the mixed fluids. Bubbles are undesirable due to the unpredictable refraction of light they cause as light emitted by the reagents travels through bubbles within the mixed reagents or on the surface of the mixed reagents. The axis of the inlet channel may be angled above horizontal (e.g., about 30 degrees) to provide a partially downward direction to the incoming fluid flow to ensure that the reagent is mixed with the fluid residing at the bottom of the reaction chamber. Alternatively, the reagents may be introduced to the test chamber using alternative fluid delivery means such as a vertical channel to deliver the reagents to the bottom of the reaction chamber, or delivering the fluid directly on the central axis of the test chamber in order to create a column of reagent flowing into the test chamber thereby promoting mixing through entrainment.

[0033] The shape (i.e., predetermined geometry) of the reaction chamber may be a revolved section facilitating clockwise or counterclockwise motion of the mixing fluids around the central axis of the reaction chamber. Alternatively, if desired, a reaction chamber shape other than a revolved section such as a rectangular or irregular shape may be utilized. In one embodiment, the revolved section used to form the reaction chamber is a portion of an ellipse for facilitating the collection of stray light emitted by the reagents and reflecting this light toward the surface of the detector, which may be a photomultiplier tube (PMT) (Hamamatsu). The surface of the reaction chamber may be reflective, in order to enhance the light collection properties of the elliptical shape. In some embodiments, the maximum diameter of the surface of the PMT is limited to achieve a maximum signal to noise ratio of the output of the system. The diameter of the reaction chamber may be designed to approximately match the diameter of the PMT, which influences the elliptical shape that can be achieved in a reaction chamber designed to hold a specific volume of fluids. Due to the constrained elliptical shape, the reaction chamber surface color may be a partially diffusing white due to the additional light collection that occurs when light that would not otherwise be reflected directly to the PMT surface is partially diffused by the white surface and a fraction of this is directed toward the PMT surface. Alternatively, other surface finishes and materials such as a near-mirror finish aluminum, or a transparent material could be used if desired. Further, it is desirable for the reaction chamber material to be minimally phosphorescent, in order to prevent light emitted from the reaction chamber itself from eclipsing any emitted light from the reagents and preventing detection. Although white polymeric materials such as acrylonitrile butadiene styrene or other such polymeric materials have been found to exhibit a low level of phosphorescence, the additional light collection provided by the combination of light reflection and diffusion has been found to be a benefit to the signal to noise ratio of the light sensing circuit output.

[0034] In some embodiments of the invention, the stabilizer included in the test chamber is Pluronic F68, which is used in cell culture as a stabilizer of cell membranes by protecting from membrane shearing and additionally as an anti-foaming agent. Certain embodiments of this invention also include at least one additive located in the test chamber for minimizing the formation of bubbles in the test chamber during mixing of the sample and the biosensor. This

additive may further include a surfactant. Some embodiments also include a device for disrupting individual cells of the infectious agent prior to mixing the sample with the biosensor for purposes of amplifying the light signal generated by the biosensor reacting with the infectious agent. An example of such a device would be a sonicator. The detector for detecting and amplifying the signal generated by the biosensor may be a photomultiplier tube (Hamamatsu) having an active surface, and wherein the size (i.e., diameter) of the active surface has been optimized to reduce background noise and increase the signal to noise ratio of the detected signal. The system of the preset invention may also include at least one data processing component in communication with the testing unit for receiving information therefrom. The at least one data processing component further includes both computer hardware and software for organizing, interpreting, and storing the information received from the testing unit and for presenting the information in a useful and meaningful manner.

[0035] FIG. 5 provides a flow chart of a system and process for testing a biological sample for the presence of one or more infectious agents, in accordance with an exemplary embodiment of the present invention, including: (i) growing appropiate engineered B cells at process step 10; (ii) charging the cells with coelenterazine at process step 12; (iii) removing excess coelenterazine at process step 14; (iv) adding a cell stabilizer (e.g. pluronic F68) at process step 16; (v) adding a cryopreservative (e.g., dimethyl sulfoxide (DMSO) at process step 18; (vi) loading biosensor cells into a disposable test cartridge at process step 20; (vii) loading positive control samples into the disposable cartridge (anti-IgM, digitonin, etc.) at process step 22; (viii) freezing and storing the frozen cartridge (below -40^{o} C) at process step 24; (ix) thawing the cartridge at process step 26; (x) inserting the cartridge into a detection device at process step 28; (xi) preparing a test sample having an infectious agent therein at process step 30 by fragmenting the infectious agent (using sonication, pressure gradient, and/or enzyme treatment, for example); (xii) injecting the sample into the reaction chamber at process step 32; (xiii) activating the detection device analyze sample at process step 34; and (xiv) performing a positive control at process step 36.

[0036] As previously discussed, proper operation of the system of the present invention includes reducing background noise to a minimum. This may be accomplished by (i) removing

CTZ after charging the B cell (CTZ spontaneously oxidizes and creates background photons); (ii) shielding the PMT from electromagnetic radiation by, for example, positioning the PMT at an appropriate distance from sources of background EM radiation; (iii) using a plastic material with minimal fluorescence (plastic glows in the dark and the appropriate selection/coating of the plastic reduces this noise; and (iv) using a chamber surface coating the blocks photons from the plastic and that does not emit photons itself. Other important aspects include: (i) adjusting the cell concentration to an optimal level to balance the need for many cells to detect rare events with the fact that cells themselves absorb light and interfere with transmission of the signal to the detector; and (ii) incorporating an automatic positive control into the test process using, for example, an anti-IgM antibody or a generic calcium influx inducer such as digitonin. In a two-step process, step one includes mixing the biosensor cells with the sample to detect infectious agents, and step two includes adding the positive control inducer to validate that the biosensor cells well fully active. Further biosensor signal amplification may be achieved by fragmenting the individual cells of the infectious agent, using for example: (i) an enzyme such as lipase to release O antigens from the cell surface (part of LPS); (ii) sonication to fragment the cells; (iii) a French Press or equivalent to fragment the cells; or (iv) a chemical treatment to release LPS from the cells.

[0037] While the present invention has been illustrated by the description of exemplary embodiments thereof, and while the embodiments have been described in certain detail, it is not the intention of the Applicant to restrict or in any way limit the scope of the appended claims to such detail. Additional advantages and modifications will readily appear to those skilled in the art. Therefore, the invention in its broader aspects is not limited to any of the specific details, representative devices and methods, and/or illustrative examples shown and described. Accordingly, departures may be made from such details without departing from the spirit or scope of the applicant's general inventive concept.

CLAIMS

What is claimed:

(1) A system for rapidly detecting infectious agents in biological samples, comprising:

(a) at least one sample to be tested for at least one specific infectious agent, wherein individual cells of the at least one infectious agent include at least one type of biomolecule that reacts with a biosensor when brought into physical contact therewith, and wherein the at least one type of biomolecule further includes lipopolysaccharides; and

(b) at least one biosensor, wherein the biosensor is operative to detect a specific infectious agent in the sample to be tested; and wherein the biosensor emits a detectable signal when it reacts with the lipopolysaccharides.

(2) The system of claim 1, further comprising:

(a) a test cartridge for containing the sample and the biosensor, wherein introducing the biosensor into the test cartridge mixes the sample and the biosensor, and wherein the test cartridge further includes:

(i) a reaction chamber for receiving the sample and the biosensor, wherein the reaction chamber has a predetermined internal geometry, and wherein the reaction chamber has been adapted to maximize the detectability of the signal while simultaneously reducing background noise;

(ii) optionally, at least one stabilizer located in the reaction chamber, wherein the stabilizer is operative to minimize shear force damage to the sample and biosensor during mixing of the sample and the biosensor; and

(b) a testing unit adapted to receive the test cartridge, wherein the testing unit further includes a detector for detecting and amplifying the signal generated by the biosensor and wherein detection of the amplified signal is indicative of the presence of the infectious agent; and

(c) wherein detection of the specific infectious agent in the sample occurs in real time.

(3) The system of claim 1, wherein the lipopolysaccharides are released from the individual cells of the at least one infectious agent by using a cation chelating agent to remove divalent cations and destabilize the cellular membrane; using a selected surfactant to release components of the cellular membrane; using an oxidative or reducing agent to cleave bonds that anchor the biomolecule in the cellular membrane; using an enzyme that hydrolyzes components of the cellular membrane, thereby releasing the biomolecule; using heat to disrupt the cellular membrane; using mechanical disruption of the cellular membrane; or combinations thereof.

(4) The system of claim 1, wherein the biosensor further includes B lymphocytes that have been engineered to express cytosolic aequorin and at least one membrane-bound antibody specific for a predetermined infectious agent.

(5) The system of claim 1, wherein the biosensor further includes a hybridoma, and wherein the hybridoma further includes:

(a) a B lymphocyte that has been engineered to express cytosolic aequorin and at least one membrane-bound antibody specific for a predetermined infectious agent; and

(b) a myeloma fusion partner.

(6) The system of claim 1, wherein the biosensor is pre-charged with coelenterazine, and wherein any excess coelenterazine is removed from the biosensor prior to reacting the biosensor with the sample to be tested.

(7) The system of claim 1, further comprising a means for disrupting individual cells of the infectious agent prior to mixing the sample with the biosensor.

(8) The system of claim 7, wherein the means for disrupting individual cells of the infectious agent prior to mixing the sample with the biosensor further includes at least one of an enzyme operative to release O antigens from the cell surface, a sonicator operative to fragment the cells, a French Press operative to fragment the cells, and a chemical treatment operative to release LPS from the cells of the infectious agent.

(9) The system of claim 1, wherein the sample to be tested is derived from beef, poultry, fish, or vegetable matter.

(10) The system of claim 1, wherein the specific infectious agent is *Escherichia coli*.

(11) The system of claim 1, wherein the detectable signal further includes light.

(12) The system of claim 2, wherein the at least one stabilizer further includes Pluronic F68.

(13) The system of claim 2, wherein real time is within about one to five minutes from creation of the sample to be tested.

(14) A system for rapidly detecting infectious agents in biological samples, comprising:
(a) at least one sample to be tested for at least one specific infectious agent;
(b) at least one biosensor, wherein the biosensor is operative to detect a specific infectious agent in the sample to be tested; and wherein the biosensor emits a detectable signal when it reacts with the specific infectious agent;
(c) at least one type of biomolecule on the individual cells of the at least one infectious agent that reacts with the biosensor when brought into physical contact therewith; and
(d) wherein the biomolecule is released from the individual cells of the at least one infectious agent by using a cation chelating agent to remove divalent cations and destabilize the cellular membrane; using a selected surfactant to release components of the cellular membrane; using an oxidative or reducing agent to cleave bonds that anchor the biomolecule in the cellular membrane; using an enzyme that hydrolyzes components of the cellular membrane, thereby releasing the biomolecule; using heat to disrupt the cellular membrane; using mechanical disruption of the cellular membrane; or combinations thereof.

(15) The system of claim 14, further comprising:

(a) a test cartridge for containing the sample and the biosensor, wherein introducing the biosensor into the test cartridge mixes the sample and the biosensor, and wherein the test cartridge further includes:

(i) a reaction chamber for receiving the sample and the biosensor, wherein the reaction chamber has a predetermined internal geometry, and wherein the reaction chamber has been adapted to maximize the detectability of the signal while simultaneously reducing background noise;

(ii) optionally, at least one stabilizer located in the reaction chamber, wherein the stabilizer is operative to minimize shear force damage to the sample and biosensor during mixing of the sample and the biosensor; and

(b) a testing unit adapted to receive the test cartridge, wherein the testing unit further includes a detector for detecting and amplifying the signal generated by the biosensor and wherein detection of the amplified signal is indicative of the presence of the infectious agent; and

(c) wherein detection of the specific infectious agent in the sample occurs in real time.

(16) The system of claim 14, wherein the at least one biomolecule further includes lipopolysaccharides.

(17) The system of claim 14, wherein the biosensor further includes B lymphocytes that have been engineered to express cytosolic aequorin and at least one membrane-bound antibody specific for a predetermined infectious agent.

(18) The system of claim 14, wherein the biosensor further includes hybridomas, and wherein the hybridomas further include:

(a) B lymphocytes that have been engineered to express cytosolic aequorin and at least one membrane-bound antibody specific for a predetermined infectious agent; and

(b) a myeloma fusion partner.

(19) The system of claim 14, wherein the biosensor is pre-charged with coelenterazine, and wherein any excess coelenterazine is removed from the biosensor prior to reacting the biosensor with the sample to be tested.

(20) The system of claim 14, further comprising a means for disrupting individual cells of the infectious agent prior to mixing the sample with the biosensor.

(21) The system of claim 20 wherein the means for disrupting individual cells of the infectious agent prior to mixing the sample with the biosensor further includes at least one of an enzyme operative to release O antigens from the cell surface, a sonicator operative to fragment the cells, a French Press operative to fragment the cells, and a chemical treatment operative to release LPS from the cells of the infectious agent.

(22) The system of claim 14, wherein the sample to be tested is derived from beef, poultry, fish, or vegetable matter.

(23) The system of claim 14, wherein the specific infectious agent is *Escherichia coli*.

(24) The system of claim 14, wherein the detectable signal further includes light.

(25) The system of claim 14, wherein the at least one stabilizer further includes Pluronic F68.

(26) The system of claim 14, wherein real time is within about one to five minutes from creation of the sample to be tested.

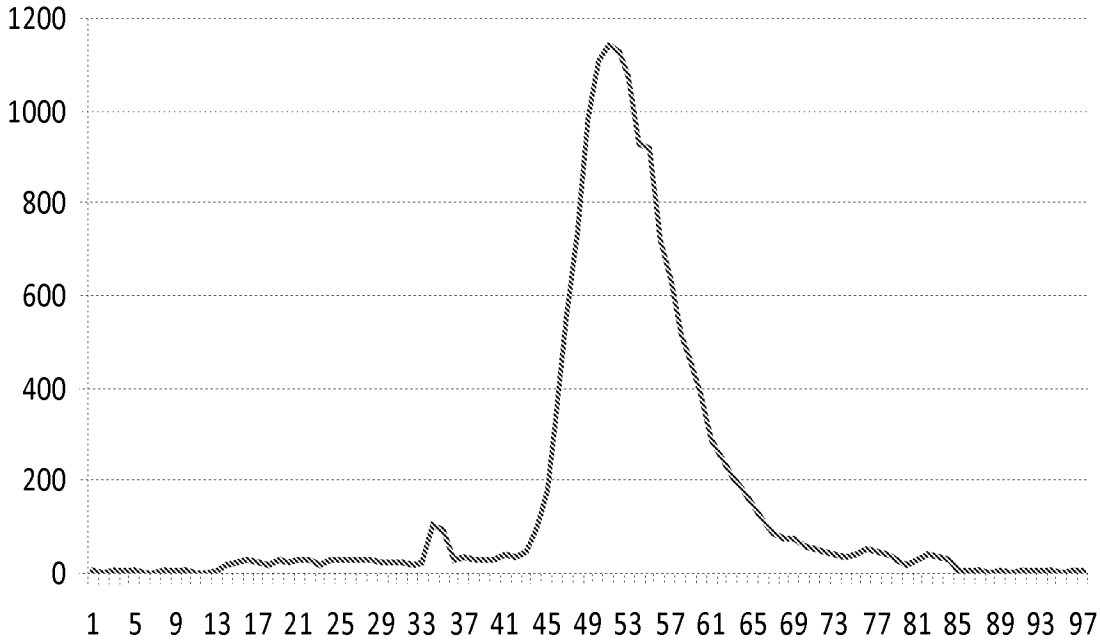
1200
1000
800
600
400
200
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97

FIG. 1

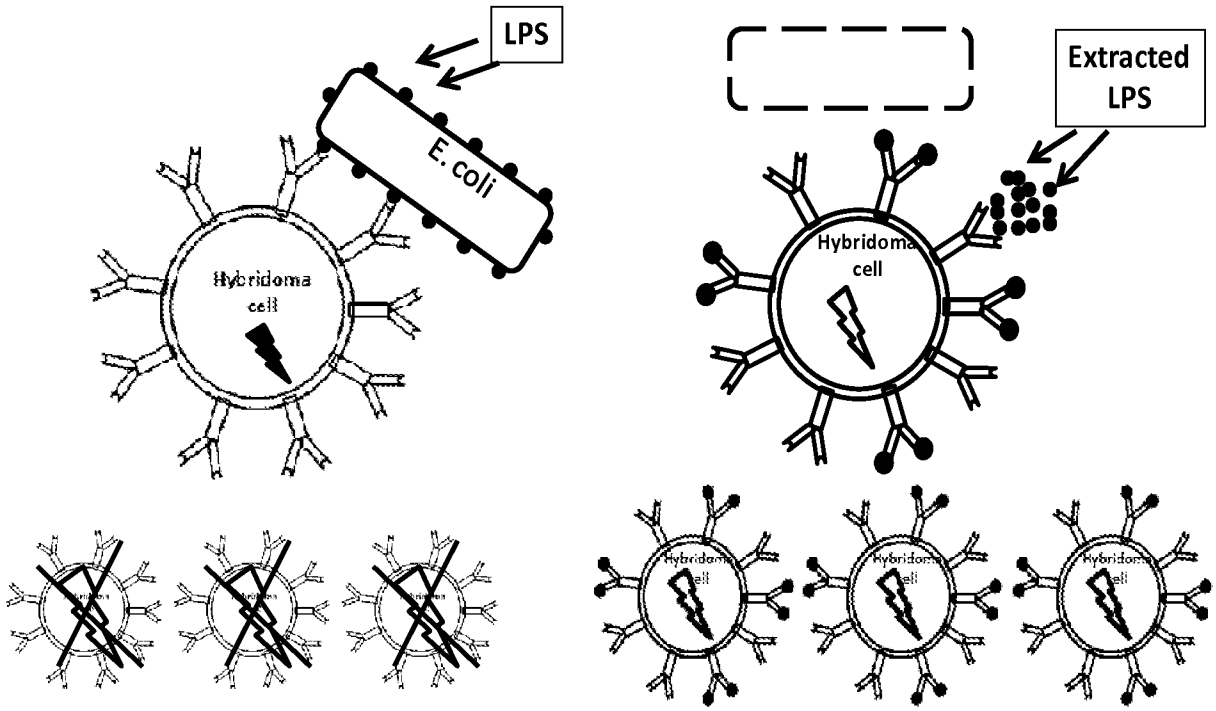

FIG. 2

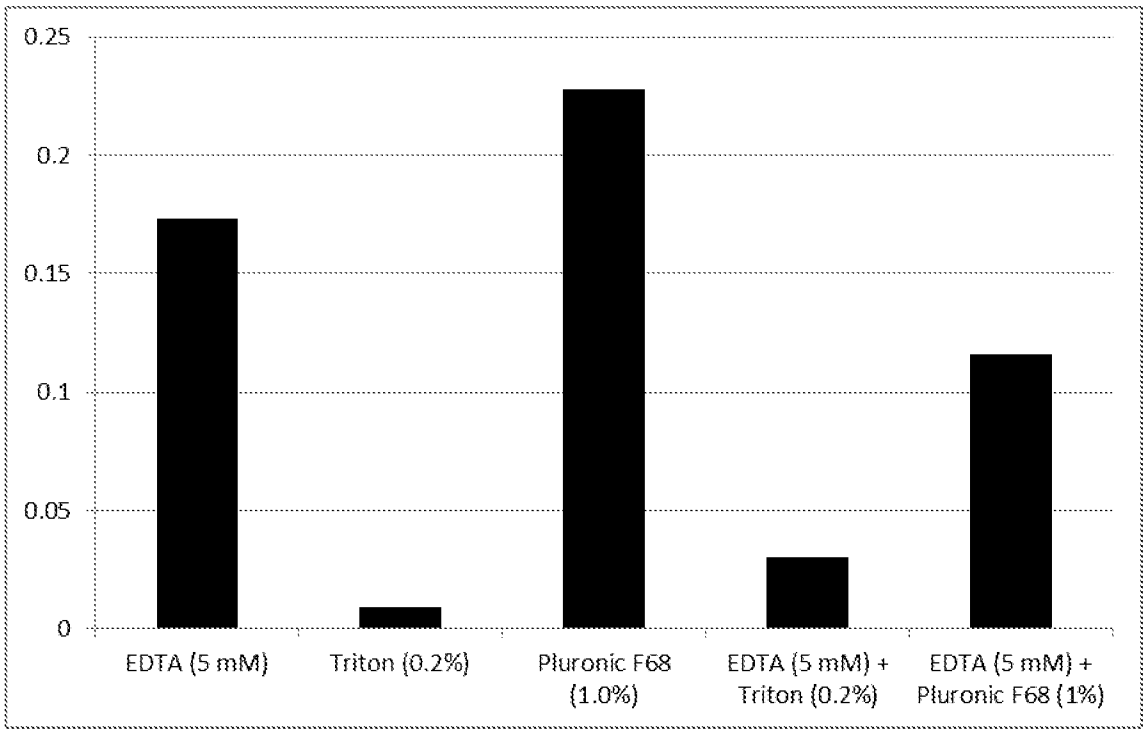
0.25
0.2
0.15
0.1
0.05
0
EDTA (5 mM)
Triton (0.2%)
Pluronic F68 (1.0%)
EDTA (5 mM) + Triton (0.2%)
EDTA (5 mM) + Pluronic F68 (1%)

FIG. 3

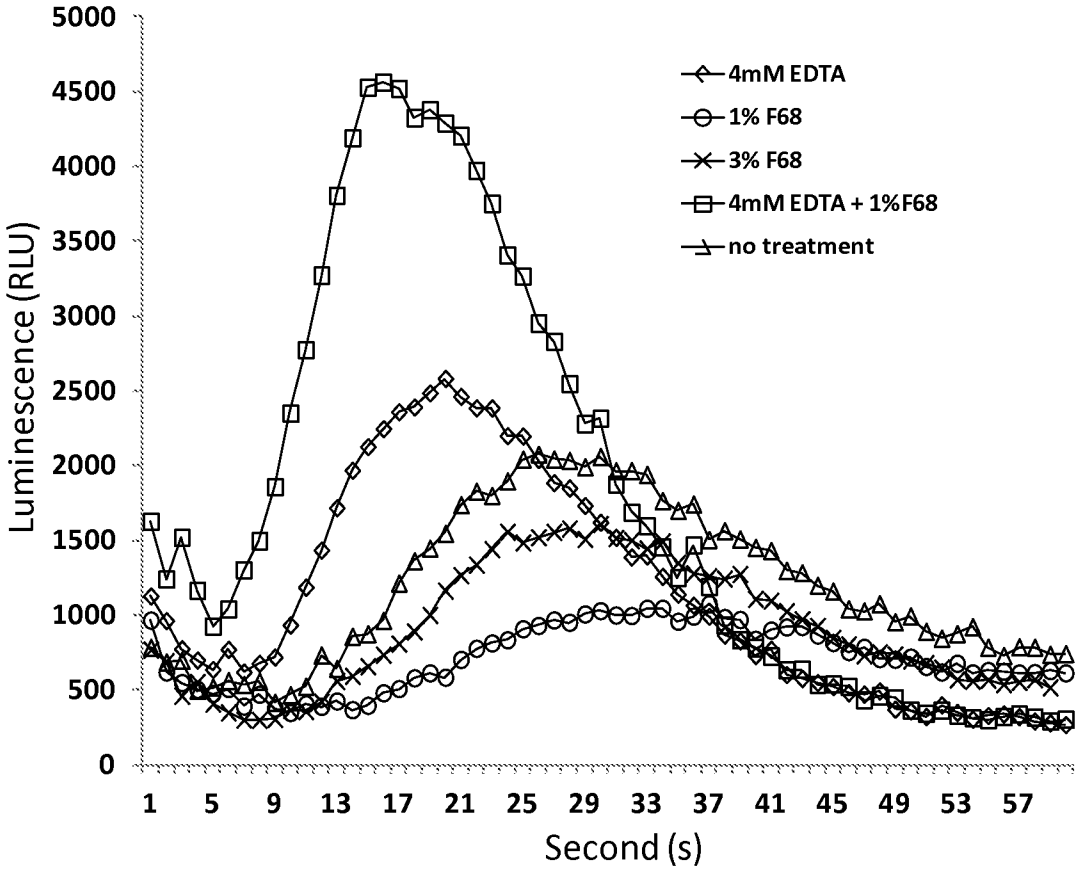
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Luminescence (RLU)
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
Second (s)
4mM EDTA
1% F68
3% F68
4mM EDTA + 1%F68
no treatment

FIG. 4

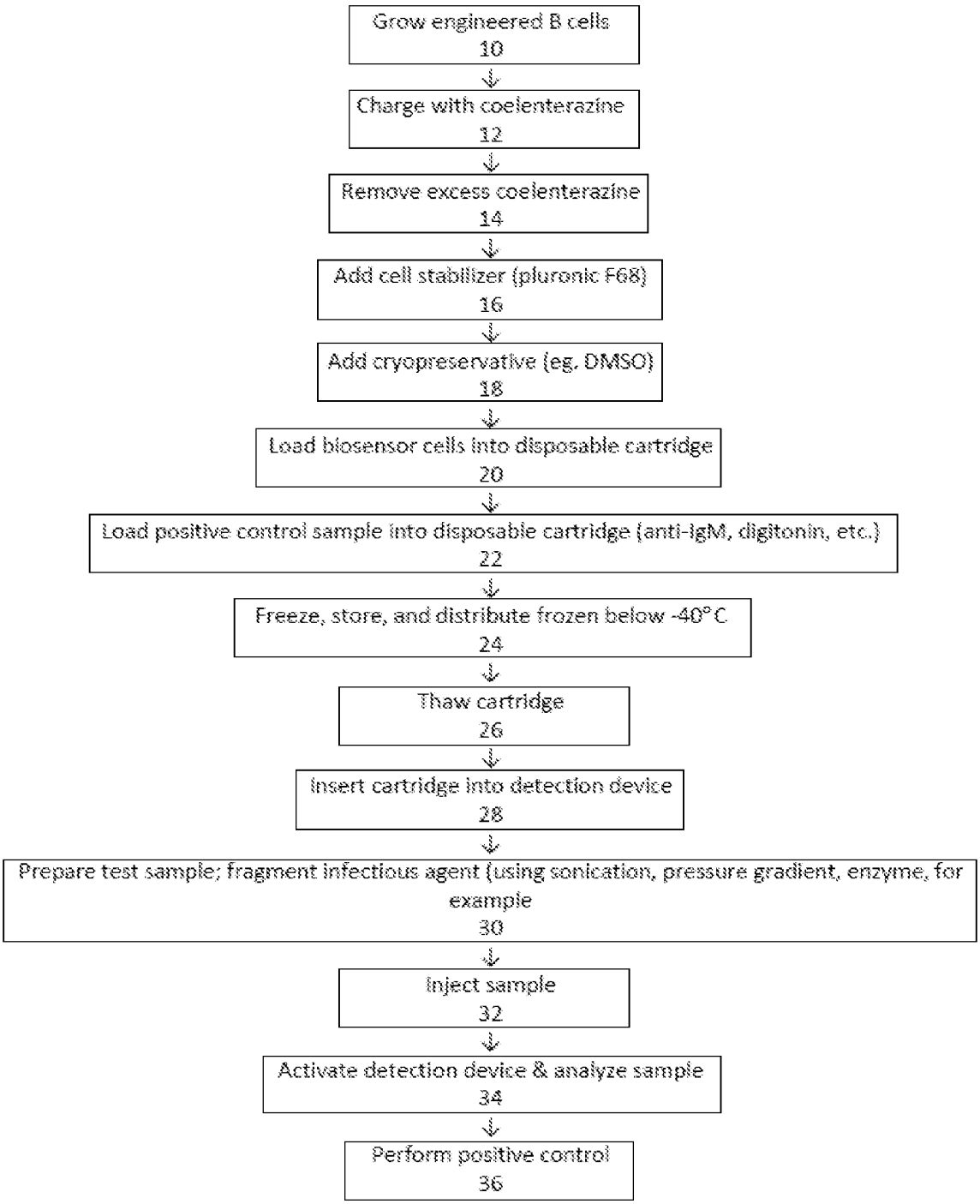
Grow engineered B cells
10
Charge with coelenterazine
12
Remove excess coelenterazine
14
Add cell stabilizer (pluronic F68)
16
Add cryopreservative (eg. DMSO)
18
Load biosensor cells into disposable cartridge
20
Load positive control sample into disposable cartridge (anti-IgM, digitonin, etc.)
22
Freeze, store, and distribute frozen below -40°C
24
Thaw cartridge
26
Insert cartridge into detection device
28
Prepare test sample; fragment infectious agent (using sonication, pressure gradient, enzyme, for example
30
Inject sample
32
Activate detection device & analyze sample
34
Perform positive control
36

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/026116

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569 G01N33/554
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, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RIDER T H ET AL: "A B CELL-BASED SENSOR FOR RAPID IDENTIFICATION OF PATHOGENS", SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 301, no. 5630, 11 July 2003 (2003-07-11), pages 213-215, XP001179643, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.1084920	1,3,4,6, 9-11,14, 16,17, 19, 22-24,26
Y	figure 2	2,5,7,8, 12,13, 15,18, 20,21,25
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2014	29/08/2014

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	Schwachtgen, J

2

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.
	& Todd H Rider ET AL: "A B-Cell Based Sensor for Rapid Identification of Pathogens: Supporting Online Material, Science Online", , 11 July 2003 (2003-07-11), XP055133964, Retrieved from the Internet: URL:http://www.sciencemag.org/content/suppl/2003/07/09/301.5630.213.DC1/Rider-SOMRevised.pdf [retrieved on 2014-08-08] page 3, paragraph 3	
Y	WO 2008/048300 A2 (MASSACHUSETTS INST TECHNOLOGY [US]; SCHWOEBEL ERIC [US]; HARPER JAMES) 24 April 2008 (2008-04-24) page 108, line 8 - page 109, line 27 claims 15-17 page 33, line 15 - page 34, line 6 page 36, line 24 - page 37, line 12	2,5,7,8, 12,13, 15,18, 20,21,25
T	CLARK: "Further characterisation of a monoclonal antibody reactive with Escherichia coli O157:H7", JOURNAL OF MEDICAL MICROBIOLOGY, vol. 43, no. 4, 1 January 1995 (1995-01-01), pages 262-269, XP055133975, ISSN: 0022-2615 abstract	1-26
T	WO 92/02820 A1 (WISCONSIN ALUMNI RES FOUND [US]) 20 February 1992 (1992-02-20) page 10, paragraphs 2,3	1-26
X,P	WO 2013/090394 A1 (FUNDAMENTAL SOLUTIONS CORP [US]) 20 June 2013 (2013-06-20) figures 1-27 paragraph [0134]	1-4, 6-11, 13-17, 19-24,26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2014/026116

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008048300 A2	24-04-2008	AU 2006350038 A1	24-04-2008
		BR PI0619234 A2	08-11-2011
		CA 2631615 A1	24-04-2007
		CN 101495869 A	29-07-2009
		CN 103278647 A	04-09-2013
		EP 1974209 A2	01-10-2008
		EP 2410335 A1	25-01-2012
		HK 1119245 A1	21-03-2014
		JP 5352237 B2	27-11-2013
		JP 2009517696 A	30-04-2009
		JP 2012168199 A	06-09-2012
		JP 2013208123 A	10-10-2013
		WO 2008048300 A2	24-04-2008
WO 9202820 A1	20-02-1992	AT 161288 T	15-01-1998
		AU 652172 B2	18-08-1994
		AU 8306291 A	02-03-1992
		CA 2066777 A1	28-01-1992
		DE 69128463 D1	29-01-1998
		DE 69128463 T2	16-07-1998
		EP 0494293 A1	15-07-1992
		JP H05502588 A	13-05-1993
		KR 165691 B1	01-05-1999
		US 5168063 A	01-12-1992
		US 5354661 A	11-10-1994
		WO 9202820 A1	20-02-1992
WO 2013090394 A1	20-06-2013	AU 2012352379 A1	03-07-2014
		CA 2857332 A1	20-06-2013
		US 2013149775 A1	13-06-2013
		WO 2013090394 A1	20-06-2013