Whole-Cell Biosensors: Research and Patents

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Abstract: In the last ten years several research articles and patents related to the obtaining and uses of whole-cell biosensors (WCB) have been published. Whole-cell sensors, whole-cell biosensors, chemical sensors, integrated sensors and bioreporters (among others) are the main terms used to define a living cell machinery adapted to detect target analytes at low concentration levels.

The methods for constructing and detection technologies for these WCB could be very diverse. Some of them include a foreign nucleic acid sequence to act as reporter, to regulate the reporter's expression or both. But some other inventors exploit the natural properties of organisms without the need of genetic engineering. Another aspect is the localization of the cell at the moment of use. Some inventors, depending on the method of detection, need cells in a special location, such as the surface of a detector, entrapped in a matrix, or in the bulk of the sample. This review intends to compile the most relevant patents related to WCB which enable to illustrate the above mentioned cases of these new types of biosensors.

Keywords: Bioreporters, chemical sensors, integrated sensors, living sensors, whole-cell sensors, whole-cell biosensors.

INTRODUCTION

As clearly stated by Nicklin *et al.* [1] a biosensor, by definition, consists of a biological material coupled with a transducer to enable the detection of various substances in different media.

During the past decades all the efforts in developing more specific biosensors were devoted to isolate and use specific biological proteins (generally enzymes) capable to detect a target analyte and send a measurable signal to a transducer. Enzyme biosensors have the advantage of displaying a fast and very specific response. However, obtaining enzyme biosensors is expensive due to the refined work needed for isolating the enzymes, which are often unstable when extracted from their native environments, the immobilization steps to fix the enzyme to a suitable support and, in addition, the issue that many enzymes require the presence of cofactors for developing their activity.

A further refinement developed in recent years for biosensors utilizes intact living cells as an alternative to immobilized enzymes, generally called Whole-Cell Biosensors (WCB). The scheme in Fig. (**1**) depicts the constituent parts and the sequential steps that define a biosensor. The first step is the development of the "biological sensing element", which will determine the type of biological signal and then constrains the transducer selection. The last step is the obtaining of a measurable response from the transducer proportional to the analyte concentration.

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The design of whole cell biosensors (WCB) is a relatively new subgroup within the main group of biosensors. In this WCB subgroup the biological sensor element should be a living cell, in most of the cases genetically engineered to produce a signal in the presence of the target analyte. This short review depicts some examples of the state of the art in WCB research, and most of the selected patents emphasize the design and obtaining procedure of the living cell which constitutes the biological sensing element within the biosensor. For this reason, among the patents chosen there may be some in which only was reached the biological sensing element design and some other more complete where the transducer element has also been developed.

WCB built with microbial cells are especially well suited for biosensor technologies; they are physically robust, withstanding under extremely harsh and widely fluctuating environmental conditions, and possessing an extensive repertoire of responses since they can be genetically engineered to generate reporter systems to any specific analyte. Furthermore, the use of live cells as biosensors provides a more vast description of the environment around the cells, giving the additional information of the bioavailability of the target molecule. Immobilization of cells has many advantages over the use of suspended cell liquid systems for use in biosensors. The chance of accidental spillage is dramatically decrease which is an important consideration when dealing with genetically modified strains. Immobilization increases the mechanical stability and allows the creation of thin films of matrix for rapid diffusion and short analysis times. The biological material can be immobilized *via* a number of known methods such as adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques, but at the same time cells used in whole cell biosensors need to be immobilized on the transducer without losing the cells´ viability and sensing activity [1,2].

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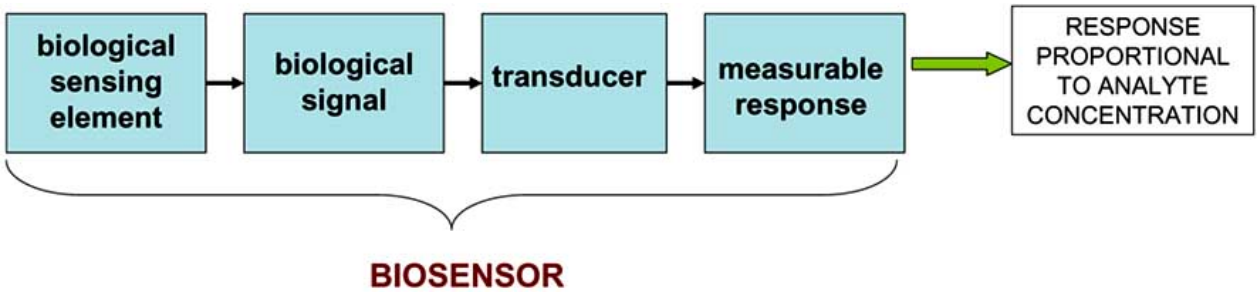

Fig. (1). The scheme shows the constituent parts and the sequential steps that define a biosensor.

In principle, the WCB can produce any type of signal (optical, electrochemical, etc) when a particular substance is detected and metabolized. However, most of the WCB are based on luminescent detection due to the high sensitivity of this technique. The bioluminescent-bioreporter integrated circuit consists of a genetically engineered organism (which luminesce when a targeted substance is encountered) interfaced with an integrated circuit, designed to detect this luminescence, processes the signal and communicate the result. The bioreporters are then part of a circuit as another component, analogous to a transistor or a resistor. In this way, WCB combine the advantage of having the complete machinery of a whole bioengineered cell with an enhanced response amplified by MEMS nanotechnology (Micro-Electro-Mechanical Systems), which integrates all the mechanical elements of the sensor through microfabrication processes. In this aspect many patents have their core consisting on the description of the nanotechnology involved in the assemblage of the cell to the transductor. In this work we are not going to describe in detail the transduction process, there are excellent reviews in the literature regarding these key aspects (Shacham-Diama *et al.*, 2010 [3]; Quinn and O'Kennedy, 1999 [4]), we will rather focus on the bioanalytical uses of WCBs. In some cases the signal produced by the bioreporter is not luminescence, although this is the most common technique. It can also be a photochemical process such as Fluorescence Resonance Energy Transfer (FRET), or an electrochemical signal, an electrode potential or even a smell.

To help the reader in this compilation of patents, we tried to classify them into different groups according to their main fields of use: medical & biochemical, environmental and analytical. However, the boundaries between those groups can fluctuate depending on the target molecules and each particular interpretation.

1. Medical and Biochemical WCB

The trends in emerging technologies prompt the development of biosensors in order to minimize response time and maximize reliability; even more to allow automation of the detection and diagnosis of diseases or metabolic disorders, either *in vivo* or *in vitro*.

Responding to this demand Sayler *et al*. [5] designed an implantable diagnosis device for monitoring one or more target substances, analytes, of metabolites in the human body. These implants can monitor the level of blood glucose in a diabetic or hypoglycemic patient, do on-line monitoring for enzymes associated with the occurrence of blood clots (strokes, heart attacks, etc.), make the detection and quantitation of clotting factors, hormone replacement or make continuous drug monitoring of compounds affecting mental illness, among the main examples. The bioreporters can be a plurality of eukaryotic or prokaryotic cells that produce a bioluminescent polypeptide in response to the presence of the target analyte. Prokaryotic cells such as one or more strains of bacteria, and eukaryotic cells such as mammalian cells are particularly preferred. Exemplary mammalian cells are human cells such as islet β-cells, immortal stem cells, or hepatic cells, with immortal stem cells being particularly preferred. The implantable bioelectronic device contains an integrated circuit including a transducer to generate the electrical signal in response to light incidence. Detected light is produced by genetically engineered cells containing nucleic acid segments encoding a luciferase polypeptide or a green fluorescent protein that is synthesized due to the presence of the target analyte. Cells are disposed in a polymeric matrix made of polyvinyl alcohol, poly L-lysine or alginate preventing the passage from the bioreporter cells but allowing the target molecule to reach said cells.

These versatile WCB may also be used to control or regulate the delivery of a drug or other pharmaceutical agent from an associated external or implantable drug delivery system. For example, the device may form part of an artificial pancreas to regulate insulin dosage in response to the level of glucose detected *in situ*.

Similarly, Edelberg and Christini [6] developed electrical implantable physiological or pathophysiological biosensors that comprise a tissue or cell capable of carrying out a physiological or pathophysiological function. This biosensor makes use of excitable tissue/cells such as cardiac tissue/cells and neuronal tissue/cells coupled *via* an electrical interface to an electronic measuring device or an electronic amplifying device which can be used to monitor the presence of a chemical such as blood glucose, insulin, thyroid hormone, clotting factors and components and endocrine hormone above others, or to control tissues functions like cardiac rate.

Making use of a similar technology but not for implantable WCB, Hickman *et al.* [7] employed a neuronal cell with a predefined axonal/dendritic polarity on a substrate capable of supporting cell's metabolism over a signal transducer. These cells are able to produce an action potential or an axonal wave potential in response to a bioeffecting substance that is later detected by the signal transducer. This biosensor comprises one insulating barrier to prevent direct contacts between the culture medium and the transducer. Some possi-

ble applications of this biosensor are toxicity testing, drug discovery, bioremediation monitoring, bioelectronics and understanding basic neuroscience. Kovacs *et al.* [8] designed an apparatus and method for monitoring cells and a method for monitoring changes in cells upon addition of an analyte to the cells environment. This equipment comprises a device which includes an array of microelectrodes disposed in a cell culture chamber. Upon which a portion of the cells adhere to the surface of the microelectrodes, a voltage signal is applied across each of the microelectrodes and the reference electrode. Detection and monitoring of the signal resulting from the application of the voltage signal provides information regarding the electrical characteristics of the individual cells, including impedance (combined cell membrane capacitance and conductance), action potential parameters, cell membrane capacitance, cell membrane conductance, and cell/ substrate seal resistance. Cell can be human or simian totipotent primordial stem cells, capable of differentiating into many groups of differentiated cells capable of detecting and screening a wide variety of biological and chemical agents.

Several inventors agree in the idea of covering the surface of the sensor with a polypeptide matrix to facilitate eukaryotic cell's attachment, trying to resemble, in this way, the extracellular matrix [9,10]. Regarding the strategy to sense the target analyte, some authors share the idea of assembling a hybrid polypeptide where one part of this molecule senses the target molecule, another one localize the peptide in a specific location into the cell depending on the presence or absence of the target molecule, and the third component acts as reporter, making the constructed polypeptide detectable and insert this construction into a cell to form a complete WCB. Liou & Meyer [11] illustrate a method to determine states of intracellular calcium stores in a eukaryotic cell and provide methods for identifying an agent that modulates intracellular calcium store levels. This method is based on the detection of a calcium biosensor polypeptide (CBP) distribution pattern in a cell. This CBP comprises a detectable domain, in this case a fluorescent polypeptide, operably linked to a stromal interaction molecule (STIM) polypeptide. Calcium binding to the STIM polypeptide modulates changes in the localization of the CBP in the cell. Furthermore, this whole cell biosensor makes use of a second calcium-intensive marker (CIM), but in this case, it has been genetically modified to have a decreased binding affinity for calcium causing CIM aggregation and using an alternative detectable domain to allow its detection in parallel with the CBP detection. When these two polypeptides (CIM and CBP) are provided in the same host cell, CIM can act as an internal control and marker of a pattern reflective of low calcium state. A punctate CBP distribution pattern is indicative of depletion of intracellular store calcium where a diffuse CBP distribution pattern is indicative of levels intracellular store calcium that are not depleted. A diffuse pattern for the CBP and a punctate pattern for the CIM reveal that the cell is in a normal calcium state, but when the cell is in a low calcium state, the first and second emission spectra are co-localized due to aggregation of the CIM and the CBP. In this way, a decrease in calcium in the cell can be determined by assessing co-localization of two different emission spectra.

Following the same argument, Delfino *et al.* [12] detected the activation of an Extracellular signal-regulated Kinase (ERK). After induction of the ERK biosensor with ponasterone A, cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) and intracellular localization of the inducible ERK biosensor was assessed using standard fluorescence microscopy techniques. In untreated cells the inducible ERK biosensor was predominately localized in the nucleus, but in treated cells it was localized in the cytoplasm, consistent with TPA induced ERK activation causing the net translocation of the inducible ERK biosensor from the nucleus to the cytoplasm.

In the same direction Jones *et al.* [13,14] developed a mitosis biosensor polypeptide (MBP) comprising a plasma membrane targeting domain, a nuclear localization signal (NLS) and a fluorescent label to monitor a mammalian cell's cycle phase. As a result, the NLS of the MBP provides for localization of the MBP at the nuclear membrane until nuclear envelope breakdown (NEB) occurs at the beginning of the mitosis phase of the cell cycle. Thus, during interphase (G1/S/G2) MBP is contained within the nucleus. After NEB, the MBP is localized at the plasma membrane, indicating the cell is in mitosis. Upon nuclear envelope reformation (NER) at the end of the mitosis phase, the MBP is localized in the nucleus once again, indicating the cell is in interphase. Coinciding in this reporting method, the patent registered by Hahn [15] describes a WCB able to detect biochemical reactions such as phosphorylation, binding processes, activation states, conformational changes and subcellular locations of selected targets, based on the signal produced by an associated dye, thorough changes in fluorescence properties like altered fluorescence intensity and modification of lifetime, excitation or emission maxima.

Using this identical strategy Barak *et al.* [16] built up a WCB for the detection of a G protein-coupled receptor (GPCR) ligand in a test sample, providing a cell comprising a GPCR and an arrestin conjugated to a Green Fluorescent Protein (GFP) , wherein the cellular distribution of the detectably labeled arrestin changes in response to activation of the GPCR. If when comparing the cellular distribution of the detectably labelled arrestin in the presence of the test sample to the cellular distribution of the same protein in absence of the test sample, the cellular distribution changes then it indicates the presence of a GPCR ligand in the test sample.

The whole cell biosensors developed by Liou & Meyer, Delfino, Jones, Hahn and Barak [11-16] utilizes Confocal Laser Scanning Microscopy (CLSM) for the determinations. Although this method at first sight appears to be problematic as a methodology to obtain a measurable signal for the sensor, there are several semi automated [17] and fully automated programs for image acquisition and processing [18,19].

On the other hand, some inventors base the detection method in a more global change of cells over the surface of a sensor. This is the case of Fang *et al.* [20] who examined the function of one or more antibodies against a specific cellular target by using a resonant waveguide grating (RWG) biosensor to monitor the ligand induced dynamic mass redistribution (DMR) within the bottom-most portion of adherent cells. The DMR signal represents an integrated cellular response which resulted from a ligand-induced dynamic, directed, and directional redistribution of cellular targets or

molecular assemblies. With the same method, these authors, analyze a G protein-coupled receptor (GPCR) signalling pathway and evaluate a pathway active compound measuring for example the impact of forskolin-stimulated cells on GPCR ligand-induced biosensors [21]. Marx *et al.* [10] described a biosensor composed by a quartz crystal microbalance (QCM), a conducting element and a selective substrate film made up covalently bound polypeptides, comprising the amino acid sequence RGDY, YIGSR or both, disposed on the surface of the conducting element. This selective substrate film allows the attachment and spread over the surface of specific cells that can quickly achieve a steady state. Virtually any eukaryotic or prokaryotic cells can be used in the whole cell QCM biosensor. The QCM technique is based upon the piezoelectric effect, which is a crystal oscillation brought about by an alternating electric field applied across opposite sides of a quartz crystal. In general, quartz crystals oscillation frequency shifts if a mass is bound to the crystal surface. The mass required to create a detectable shift is only about one nanogram, illustrating the extreme mass sensitivity of the QCM technique.

Detection mediated by QCM biosensors can be related to changes in mass distribution or viscoelastic properties of cells associated with changes in the cell on the biosensor. Sensing can also be carried out *via* electrical or electrochemical detection due to changes in the cells. These changes can be related to metabolic alterations, signal transduction events, and changes of the adhesive properties of the cells. With this in mind, the possible target molecules could be very diverse showing a method with an enormous potential for drug discovery and diseases diagnostic, among other uses [22].

More specifically Marx *et al.* [23] described a biosensor employing a QCM that was used to analyze the efficacy of drug candidates, such as anticancer drug candidates. The biosensor includes an endothelical cell (EC) matrix used as biological signal transduction elements and a piezoelectric mechanism for signal transduction to access attached EC as biological elements in the QCM biosensor. The biosensor detects EC cytoskeletal alterations and can be used to screen classes of biological active drugs or biological macromolecules affecting cellular attachment, regardless of their molecular mechanism of action. Additionally this sensor can be used to analyze agents targeted for signal transduction elements such as cytoskeleton, membrane bound integrines, and the extracellular matrix.

As an alternative method to the ones presented before, Li *et al.* [24] described a method and a system comprising a microfluidic and -interdigitated array microelectrode-based impedance biosensor. The method includes contacting the starting material with red blood cells capable of binding to influenza and Newcastle viruses. Then, the red blood cells can be coupled to a magnetic nanoparticle to form a magnetic target. The magnetic target is then detected with the impedance biosensor.

Walt *et al.* [25,26] developed a biosensor array, in which an individual cell or randomly mixed populations of cells, having unique response characteristics to chemical and biological materials, are deployed in a plurality of micro wells formed at the distal end of individual fiber optic array. The biosensor array utilizes an optically askable encoding scheme for determining the identity and location of each cell type in the array and provides for simultaneous measurements of large numbers of individual cell responses to target analytes. NIH 3T3 mouse fibroblast cells were employed and the optical responses of these cells to chemical or biological stimuli are detected by coupling them with appropriate indicators that do not compromise the cell response. These indicators may be either fluorophores, chromophores, stains or a dye compound, like fluoresceins, rhodamines, naphtalimides, among others.

Summarising, medical and biochemical WCB utilizes two different major strategies, the first one based on the production of a chimeric protein that could bind or not a target molecule, has a localization domain and a reporter domain that can be sensed after the exposure to a target molecule or to monitor the course of the cell duplication process, as described before. The second tactic is based on global changes produced in a cell, such as changes in the dynamic mass redistribution (DMR) due to the contact of the cell with a target molecule. For this last purpose a resonant waveguide grating (RWG) biosensor or a quartz crystal microbalance (QCM) are used to detect global cell alterations. But there are some other approaches like by using electric biosensors that can also provide useful information about virus detection, electrical cells characteristics and response to chemical compound as drugs, as it was described above.

2. Environmental WCB

The environmental section assembles the patents of WCB capable of working directly in the field. As for medical and biochemical WCB, it is possible to group patents regarding on the detection method used to sense changes caused by the exposure of WCB to, in this case, environmental pollutants. For this reason, and for a better understanding, patents in this part of this work were included into three different groups: a major group of WCB comprising a genetic construction where a fluorescent or a luminescent reporter gene is expressed after induction of the promoter sequence that regulates the transcription of this last gene. A second group where by electrochemical methods a signal response is sensed due to the present of the analyte, and a third group where a distinguishable characteristic of a genetically unmodified cell is used as reporter molecule in response to a target agent.

Patents using genetically modified cells are widely employed. In this regard, Yin *et al.* [27] developed a method to detect the first gaseous intermediate in denitrification processes, nitric oxide (NO). This was accomplished by fusing the promoter of the gene *nnrS* from *Rhodobacter sphaeroides* 2.4.3, whose expression requires nitric oxide, with the gene encoding green fluorescent protein, GFP. The presence of the fusion in *R. sphaeroides* 2.4.3 resulted in a significant increase in fluorescent intensity of the cells in the presence of exogenously produced nitric oxide. With an optical response measurable by fluorescence imaging, this green fluorescent protein-based assay provides a useful method responsive to the NO levels produced by denitrifiers; is easily utilizable and is not subject to false-positives resulting from production of other nitrogen oxides. The utility of the GFP-

based reporter in screening NO at low levels is potentially useful to detect the presence of denitrifiers.

On the other hand, Hillson *et al.* [28] centred the WCB development on the detection of heavy metals, taking advantage of previous studies that reflect the feasibility of detecting this kind of compounds by the use of WCB [29]. In this biosensor, *Caulobacter sp* was the organism genetically engineered to express a fluorescent reporter in response to the presence of heavy metals such as cadmium, chromate, dichromate, plutonium and uranium. An alternative proposal for the simultaneous detection of any of these heavy metals was the alternative of including distinguishable fluorescent reporter genes that could be discriminated from the others by choosing a different wavelength of emitted light, under the regulation of diverse specific promoters for each heavy metal.

Water in areas receiving fallouts from roaster off-gas emissions coming from mining activities can become contaminated with arsenic and antimony compounds. For that reason, detection of arsenic is of major concern in analytical chemistry. Accurate determination of arsenic compounds is usually done by atomic fluorescence spectroscopy or by atomic adsorption spectroscopy, but both techniques require substantial investments and are not available in rural and poorer areas. Chemical fields tests exist as well, but this are not accurate in the range of the present drinking water standards (10 to 50 micrograms per litre) and themselves give rise to pollution with heavy metals like mercury and zinc. Most available tests use hydrochloric acid and zinc to produce arsine-gas, which is then reacting with mercuric bromide on a paper producing a brown colour. However this test is still unreliable at concentrations below 150μg/l. Van der Meer and Roelof [30] developed a method for the detection of arsenic ions in water samples, consisting in a WCB in a test kit suitable for field tests, comprising a colorimetric paper strip containing the genetically engineered bacteria, immobilized and dried in a biocompatible matrix. The microorganisms sense the pollutants activating the expression of one or more genes, whose extension serves as a measure of the available ("sensed") concentration of the compound. The arsenite resistance regulatory gene is derived from the naturally occurring *E. coli* plasmid R773, cloned into any suitable *E. coli K12* plasmid like pBR322, pUC18, pACYC184 and RSF1010. The promoter sequences are then fused to reporter genes like those coding for bacterial luciferase, β-galactosidase or green fluorescent protein (GFP). The obtained WCB is remarkable sensitive and detect arsenite at concentrations of 5μg/l, showing high selectivity for arsenite and antimonite. Other anions like phosphate, carbonate, nitrate and sulphate do not induce the system.

Daunert *et al.* [31] searched for two WCB, one to detect arsenic and the other to sense zinc, with the particular characteristic that both strains used for this purpose were able to sporulate. For the detection of arsenic the *Bacillus subtilis* strain *ars-23* was used, the reporter gene was *lac-Z* and it was regulated by the *ars* operon. The activity of the β-galactosidase enzyme was monitored by using a chemiluminescent substrate, a 1,2-dioxetane β-D-galactopyranoside derivative. In order to develop a bacterial sensing system for zinc, plasmid pSD202 was designed to incorporate the gene for the reporter protein GFP under transcriptional control of the promoter and regulatory protein SmtB of the *smt* operon, and was transformed into *Bacillus megaterium*. It was observed that the biosensor also responded to Cd^{+2}, Ni^{+2} and Co^{+2} although the response was less than that to Zn^{+2} ions. Both WCB were exposed to sporulation and then to germination conditions, observing that the biosensors were still active. This innovative method is very suitable for transport and storage of these kinds of detection systems.

Inspired by the wide variety of organic compounds available at the present time in natural water courses, especially aromatic molecules, and their negative effects on the environment [32], Lau *et al.* [33] produced a WCB to detect aromatic pollutants. This method consists in genetically engineered *Pseudomonas putida* strain called *Pseudomonas putida* F1G4 (PpF1G4), where *sep-lux* transcriptional fusion was inserted in the chromosome of the bacteria. In this construction the *sep* operator/promoter regulates the expression of the *lux* gene that acts as reporter gene. The *sep* operator is blocked during the absence of aromatic solvents such as benzene, toluene, ethylbenzene and xylenes (BTEX), naphthalene and complex mixtures of hydrocarbons containing trichloroethylene and or limonene, but when any of these compounds is present then the regulator of the *sep* gene stops blocking the transcription of the genes located downstream of the promoter, that in this case is the reporter gene *lux* allowing the detection and quantification of this aromatic pollutants. In this same direction, Huang *et al.* [34] provided a method to sense toluene or xylene using a biosensor generated by inserting *xylR* and *pu-luxCDABE* into the chromosome of *Acinetobacter baylyi ADP1*. Moreover Schofield *et al.* [35,36] reported the use of yeast expressing fluorescent reporters in response to organophosphate or organophosphate hydrolytic products.

Alternatively, Glover *et al.* [37,38] and Abd-el-haleem *et al.* [39] centred the development of their respective WCB on the effect produced by the pollutants on the viability of the cell. Glover *et al.* designed a biosensor using yeasts, where the emission of light by the reporter protein was diminished if the viability of the cell was affected because of the presence of a toxin. Abd-el-haleem *et al.* employed this technique too, but the WCB was also able to detect antibiotics such as tetracycline, neomycin and rifamycin by the use of recombinant *Acinetobacter* bacterium DDGG genetically modified with lux reporter gene, designated as DF4/PUTK2 [40].

Continuing with the natural water courses pollution determination but changing the way of determining or detecting these compounds, Chovelon *et al.* [41] designed a WCB based on the enzymatic activity of unicellular algae and conductimetric or potentiometric measurements for the detection of water dissolved pollutants. The invention comprises a conductimetric or potentiometric sensor, with a reference electrode and a measuring electrode whereon living unicellular algae are immobilized in a proportion of about 2.10^3 algaes/mm^2. The algae have its native pool of membrane-bound enzymes capable of detecting pollutants dissolved in the aqueous media. The main algae utilized are *scenedesmus subspicatus, pseudokirchneriella subcapitata* and *chlorella vulgaris*. The procedure is based on the measurements of the

enzyme activity which is inhibited by the action of a pollutant, producing a change in solution conductance in comparison with control samples, due to the decrease of substrate cleavage. Some of the enzymes involved are alkaline phosphatases, inhibited by heavy metals such as Cd, Zn and Pb; estearases, inhibited by organophosphates (pesticide pollutants) and nitrate reductases, inhibited by ammonium.

Another approach for using electrochemical sensors was done by Hashimoto and Karube [42]. They patented a methodology to evaluate the ability of target soil microorganisms to adapt to various field soil environments. They could examine the growth potential of general soil microorganisms and pathogenic microorganisms in ecosystems, monitoring the balance in the soil and assessing the risk of disease occurrence and the bio-controlling effect of general soil microorganisms. The invention use two electrodes, one with a "reference" constituted by general soil microorganisms and the other electrode containing pathogenic microorganisms. Depending on the signal ratio general/pathogenic microorganisms, an index is obtained which allows inferring the global state of microorganism population in the soil. As a particular example, the SN3B strain of *Fusarium oxysporum f. sp. Lactucum* was used as representative phytopathogenic microorganism and the K12N strain of *Bacillus cereus* was used as representative antagonistic microorganism to make the respective electrodes. Fig. (**2**) shows a scheme of the patented device.

When the electrode response ratio is high, the growth of general soil microorganisms exceeds that of pathogenic ones, and the balance in the soil ecosystem is therefore predicted to shift toward a state where there are more general soil microorganisms than pathogenic microorganisms. Further, the risk of disease occurrence or spread is predicted to be low, and the biocontrol effect of the general soil microorganisms in the soil is predicted to be high.

One more example of this type of environmental WCB is the one developed by Wei-Chun and Soonjo [43] using mammalian respiratory epithelial cells grown on a porous support, placing the porous support in contact with a cell nutrient medium on one side and the air-liquid interface on the other side. In this way the device is able to sample the air atmosphere so that the respiratory epithelial cells placed can monitor and detect harmful agents. Airway ciliated cells and goblet cells can be stimulated by various factors such as pH, cigarette smoke, air pollutants, organic solvents and toxins. The major response for ciliated cells is the change of ciliary beating frequency (CBF), while goblet cells respond by the secretion of mucins, normally detected by fluorescence due to the accumulation of a fluorescent dye called Quinacrine by the acidic secretory granule of airway goblet cells.

Regarding bacterial sensing using WCB, Sayler *et al.* [44] described a very creative method and device for detection of bacteria based on recognition and infection of one or more selected strains of bacteria with a genetically modified bacteriophage. The invention utilizes two elements, a biosensor and a bioreporter, the biosensor has two purposes the first one is detecting the presence of a target cell such as a bacterium and attaching to it. The second one is transferring DNA into the target cell. An exemplary biosensor is a *luxI* integrated bacteriophage that specifically infects a particular pathogenic strain of bacteria. Infection of the bacterial target with the biosensor DNA causes the target cell to produce gene products encoded by the biosensor DNA. In the case of *luxI* gene product it is an acyl homoserin lactone (AHL) synthetase, with ultimate production of AHL. A second element of the bacteriophage/bioreporter system is the bioreporter cell. The bioreporter also has two functions; the first one is to respond to the signal it receives from the biosensor. The second one is to amplify that signal so that multiple bioreporters are in turn responsive to the signal initiated by the infected target cell line. This cell line has been genetically engineered to produce light upon stimulation by the target cell signal.

3. Analytical WCB

In recent years, the detection of chemical compounds by genetically engineered microorganisms prompting the ex-

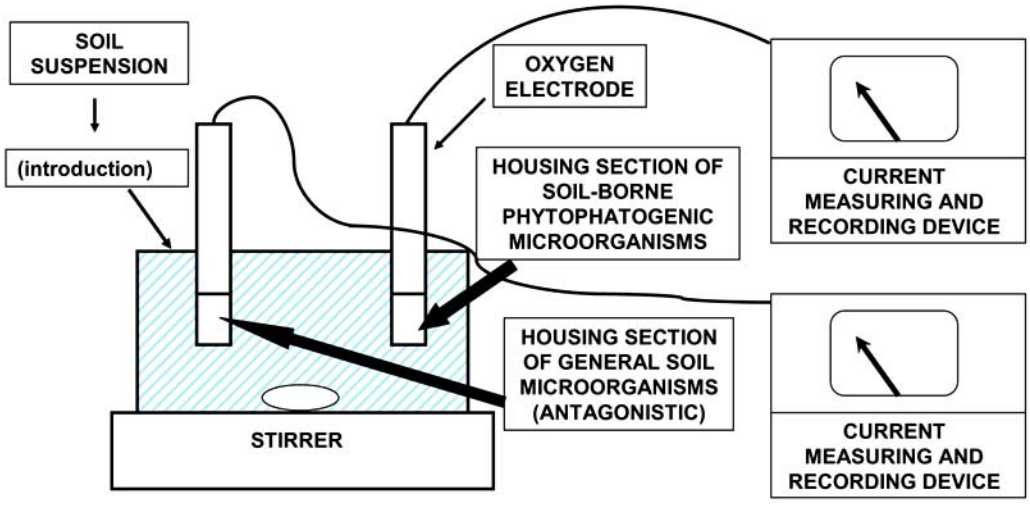

Fig. (2). Device scheme showing the assemblage of the electrodes sensing general and pathogenic soil microorganisms' activity, as described by Hashimoto and Karube in their patent: "Biosensor having soil microorganism housed therein and use thereof." (EP1700919A1 (2006)) [42].

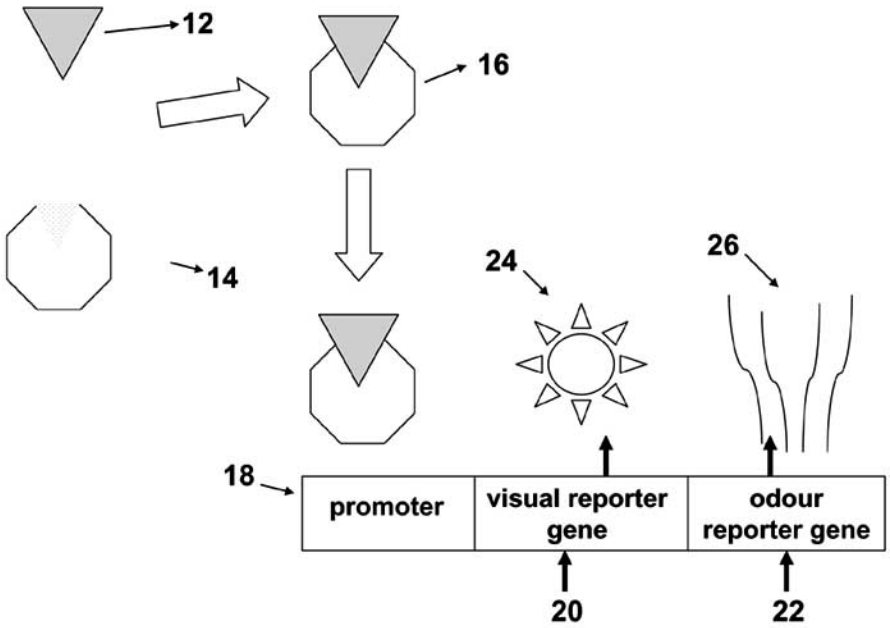

Fig. (3). Schematic representation of the recognition process to detect a target molecule with the expression of odorous and visual signals is shown here. The analyte (12) binds to a recognition protein (14). This binding event activates the recognition protein (14) and the complex (16) thus made then binds to a promoter region (18). This binding triggers transcription of the reporter genes (20), (22) for the visual (24) and the odorous (26) signals, respectively. Adapted from: Nicklin *et al.* Whole cell biosensor using the release of a volatile substance as reporter. WO2007083137A1 (2007) [1].

pression of spectroscopically active reporter proteins has become a very useful alternative and an excellent complement to traditional methods of chemical analysis.

Many review articles have shown the potential of WCB to complement both laboratory-based and on-field analytical methods for the detection of stress conditions, cyto- and genotoxic compounds, organic xenobiotics and metals (Daunert *et al.*, 2000 [45]; Carrascosa *et al.*, 2006 [46]).

For example Fluorescence Flow Cytometry used altogether with WCB response enabled rapid and efficient characterization on a single-cell basis. This method also allows the use of multivariate analysis in order to assess intrapopulation response variability to obtain higher analytical sensitivity and precision. The approach is highly efficient computationally and can be implemented on a real-time basis, thus having potential for future development of high-throughput screening applications, [47] (Busam *et al*, 2007).

Pharmaceutical, food and chemistry industry above others are always looking for new, trustable, easy using and cheap methodologies to measure quantities of a product of interest. In this way, different mono or polysaccharide molecules are very interesting target molecules to be sensed using WCB as it has previously done by Shrestha *et al.* [48]. As well as these two compounds, trehalose do not escape from this aim since this disaccharide is commonly used as cryoprotectant for cell storing, to protect tissues, proteins and even used as organ protector solution for organ transplant [49].

The main methods which were developed to quantify trehalose rely on the assessment of natural or artificial ^{13}C abundance by NMR analysis, or high performance chromatography separation and detection of carbohydrates, although also enzymatic methods were developed. To improve trehalose detection, Sarniguet and Frey-Klett [50] designed a WCB that detects qualitatively and quantitatively trehalose with engineered *Pseudomonas fluorescens* strains. These strains comprise a reporter gene such as β-galactosidase or GFP inserted in *treA* gene of operon trehalose, and in which *treA* expression is specifically induced by trehalose. Thanks to its simplicity and sensitivity, the method allows analysis of small and numerous samples. This method also provides a technique to evaluate trehalose producing organisms performances, starting from a trehalose extract of said organism and to identify culture conditions where the selected organism is able to produce trehalose in a more efficient way. Another WCB that could be used for monitoring cell culture conditions is the one designed by Ostermann *et al.* [51] that employs immobilized yeast in a xerogel matrix to determine bioavailability of nitrogen, phosphorous and sulphur.

But industries do not only care about the optimization of the product's manufacture to get as much benefits as possible. Intermediate processes are also controlled carefully to waste as less as possible and not to contaminate the final product with undesired chemicals. In this direction, Howbrook *et al.* [52] developed two WCB using *E. coli*, one encoding *lux CDABE* gene and the other *GFP*, both reporter genes were fused to a selectively inducible promoter, the *katG* promoter, and tested using hydrogen peroxide. The *lux* construction was more sensitive at lower concentrations of hydrogen peroxide and the response time to inducing agents was shorter when compared with GFP construction and was also responsive to redox cycling agents and organic peroxides, whereas the construction generating GFP as reporter molecule was sensitive over a wider concentration range.

Dhanasekaran [53,54] reported the development of a WCB to detect explosives using yeasts with at least one exogenous olfactory signalling pathway combined with a nucleotide sequence encoding GFP. A few years later, using alternative reporter molecules, Nicklin *et al.* [1] developed a whole cell biosensor that produces an odour as signalling mechanism, but also generates a luminescent or a fluorescent signal. A scheme of the construction and detection mechanism is shown in Fig. (**3**). In this construction, the synthesis of the odour is mediated by the reporter gene METase to produce of methanethiol, an easily detectable scent, green fluorescent protein for a fluorescent signal and bacterial luciferase for luminescent signal. This WCB was developed with the aim to detect and identify explosives and other dangerous or illegal substances, including detection of terrorist improvised explosive devices. This technology will not only facilitate the detection of this dangerous compounds, but also a wide range of low volatile compounds by providing a secondary vapour trail chosen specifically for ease of detection.

CURRENT AND FUTURE DEVELOPMENTS

As a biosensor depends greatly on its sensitivity and bioselectivity it is crucial for the future to develop best elements of recognition. In this respect, biotechnology and genetic engineering offer the possibility of selectively amplify the response elicited by the living cell, building highly specific recognition entities for a wide spectrum of target molecules.

From the analytical point of view two key aspects in WCB design must be addressed yet: the long-term viability and reliability-reproducibility among batches. From the technological point of view, these devices tend to industrialization, miniaturization and mass production thanks to the continuous advances in nanotechnology, bioengineering and microelectronics. It is expected that all these aspects will enable to obtain in few years a biosensors market much more available at a relative low cost.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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