WHOLE-CELL BASED FLUORESCENCE BIOSENSORS FOR ENVIRONMENTAL APPLICATIONS

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Abstract— Tonnes of wastes from different industries are discarded into the environment yearly, which leads to transfer of organic and inorganic pollutants into various water bodies. The detection of these pollutants profoundly relies on sophisticated equipment such as atomic absorption spectrometer (AAS), and inductively coupled plasma mass spectrometer (ICP-MS). The integration of whole-cells in optical biosensors provides a good sensitivity and accuracy in screening for pollutants in the environment. The inhibition of the metabolic activity by whole cells results in the emission of light such as fluorescence and bioluminescence. This feature is exploited for the development of whole-cell based optical biosensors.

Keywords—bioluminscence, fluorescence, heavy metals, optical biosensors.

I. INTRODUCTION

Tonnes of wastes from different industries are discarded into the environment yearly, which leads to the transfer of organic and inorganic pollutants into various water bodies such as rivers, lakes, oceans and seas. According to the United Nation World Water Development Report 2 [1,2] the emergence of these pollutants into the environment threatened the safety of drinking water of the human population. In Malaysia, Environmental Department of Malaysia studied dissolved oxygen, ammonium (NH_3-N), biochemical oxygen demand (BOD), suspended solids and pH for the evaluation of the safety of our fresh water [3]. Nonetheless, the evaluations are insufficient to reflect the presence of organic compounds such as pesticides, herbicides and inorganic compounds such as heavy metals. The detection of the pollutants profoundly relies on sophisticated equipment such as atomic absorption spectrometer (AAS), and inductively coupled plasma mass spectrometer (ICP-MS). These equipments provide high accuracy but; they are costly, laborious, tedious pre-analysis treatments, require off-site analysis and high risks of contaminations [4].

To overcome all the drawbacks, the biosensor field has become one of the most exciting researches which are reflected by the constant increase in reviews and publications. The first biosensor was designed by Clark and Lyon in 1962; an amperoteric enzyme electrode for the detection of glucose [5]. Since then, there has been a rapid growth in the development of different types of biosensors. In 1975, the first fiber-optic biosensor was developed by Lubbers and Opitz where it then became a trend in the biosensor industry [6]. Biosensors signify the product which incorporated of the fundamental knowledge in biology, chemistry, computer science and engineering to meet the demand for the applications of various fields in the industry. Hence, the term "biosensor" has multiple definitions; depending on the background and the field of the users. In environmental monitoring, a biosensor is an analytical tool that exploits a biological recognition element that interacts with a specific analyte (or any targets) and causes a chemical interaction which is translated into a measurable electrical signal by a transducer [7]. A biosensor consists of bio-recognition elements (whole-cells, enzymes, DNA, antibodies, tissues and cells, etc.) and transducers (optical, electrical, thermal, waveguide, etc). The interaction between the recognition elements and the target analytes will result in the release of chemical reactions. Such reactions are measured by the transducers and the signals released are converted and analysed by computer systems [8, 9].

Hence the purposes of this paper are to review the basic principle of optical biosensors and their components; as well as their applications in detecting environmental pollutants are reviewed. The sensitivity and applicability between the different types of optical sensors will be compared and discussed.

A. Basic Principle of Optical Biosensors

In general, optical detection detects the photons released during the interaction between the bio-molecules and the substrates. The interaction at certain concentrations leads to the emission of lights, which is transferred to the detection system through optical transmission media. Common examples of transmission media are fiber optic and waveguide [10]. The transduction process of an optical biosensor is triggered by certain changes in a phase, amplitude, polarization or the frequency of light input due to the chemical or physical interaction between the bio-recognition molecules and targets. The concentration of the analyte affects the generated optical signals produced by the recognition elements. The signals are later transmitted to a detector using optical fibers [7]. Light transmission using optical fibers works on the basis of total internal reflection (TIR). Optical fiber is more favorable in biosensors design because it can be used for remote monitoring for perilous environments

due to its ability to transfer light over a long distance with loss of signal [11]. An optical fiber is made up of two layers with different refractive index: glass index or plastic core with high refractive index surrounded by a fully-covered (cladding) material. Light is collected and transduced in the fiber by being reflected at the interface of two layers until light travels to the place where the signal is collected [12]. When combined with modern technologies such as micro-fluidics systems, optical waveguide or micro-electromechanical sensors (MEMs), optical methods are more adaptable and applicable for real environmental applications due to their miniaturize size, low risk of electrode shock during in-vivo measurements and capability to gather data with a minute amount of analytes [13].

B. Whole Cell as Recognition Element

Sensitive agents including naturally-present bacteria, green algae, cyanobacteria, fungi and yeast have successfully been incorporated into biosensors as the recognition element for the detection assessment of toxicants in the environment [14,15]. The microorganisms have been used to assess heavy metals, pathogens, biocides (pesticides and herbicides – both organic and non-organic) and insecticides [16,17]. Whole cells make great candidates for biosensors because they are available abundantly in the environment and are able to metabolize a broad range of compounds, possess great ability to adapt in adverse environments, able to degrade new molecules at specific time, ease of manipulation due to constant research in genetic mutation and recombinant DNA technology and economical sources of intracellular enzymes. In contrast to enzyme-based biosensors, whole-cell biosensors are more resistant to loss of activity as their many enzymes and cofactors are optimized by nature. On top of that, their co-existence results in the ability of the cells to cooperate and compromise their selectivity in detecting the different types of pollutants in the environment [18,19]. These features make them the ideal candidates for incorporation into biosensors.

II. OPTICAL BIOSENSORS: APPLICATIONS IN ENVIRONMENTAL ASSESSMENT

C. Fluorescence-based Biosensors

The emission of fluorescence is a result of electrons that have been excited at a specific wavelength and re-release radiation at a lower energy and cause the longer wavelength [26]. Excitation light absorbed by fluorescent-emitting microorganisms causes the increment of energy from ground state to a higher energy state. As the electrons return to the ground state, fluorescent light is emitted by the molecules. The excitation and emission occur at different levels of energy, hence giving out distinct fluorescent spectra of fingerprints, a feature that is significant in the application of optical biosensors [7, 23].

It is no doubt that conventional techniques such as absorption spectroscopy is a powerful tool for the detection of various harmful compounds but the lowest concentration of a molecule that it could detect is only at about 10^{-6} M under optimum conditions whilst for fluorescence detection could go as low as 10^{-15} M [24-26]. Furthermore, the fluorescence signal is specific, easy to detect and quantitate even with the presence of other molecules [27]. For these reasons, the measurements of fluorescence and luminescence signals are preferred for analysis of pollutants at low concentrations [27]. Wong *et al.*, [28] had successfully constructed a sensitive optical biosensor; by exploiting the natural cyanobacteria *Anabaena torulosa*, for the detection and quantitation of heavy metals and pesticides in polluted water samples [24]. *A. torulasa* cells were entrapped onto a cellulose membrane through a filtration process and a customized cylindrical well was used to fix and dry the cells and attached to an optical probe. An optical fiber was used to connect the probe to a fluorescence spectrometer and then to a computer [24]. The lowest limits of detection obtained in their study were ranged from 0.025-1.195 $\mu g/L$ [28]. Besides, Wong *et al.*, [28] enhanced the experiment with the addition of pHEMA (poly(2-hydroxyethyl methacrylate)) in between the immobilized layer of *A. torulosa*. The addition of the pHEMA layer had increased the limits of detection in the range of 0.017 – 1.410 $\mu g/L$ for Cu, Cd, Pb, 2-4D and chlorpyrifos respectively; as well as increasing the storage ability.

Besides cyanobacteria, *Chlorella vulgaris* was utilized in detecting herbicides where cells were entrapped on a quartz microfiber filter and placed in a five-membrane-home-made-flow cell [29]. After exposure to herbicides (specifically atrazine that inhibits PSII systems), the detection limit of atrazine was found to be 0.25 ug/L [29]. Singh et al., (2004) employed the same algal species and compared the sensitivity towards pesticides and herbicides of *C. vulgaris* without entrapment. In their study, the pollutants inhibited the alkaline phosphatase present in the cells. The inhibition was measured and the detection limits found for free cells were between 10^{-10} M – 10^{-6} M for herbicides and 10^{-10} M -10^{-7} M for pesticides [29].

Recombinant technology allows researchers to fuse a reporter gene that encodes for fluorescent protein (i.e. green fluorescent protein) into non-fluorescent-emitting hosts such as *Escherichia coli*, *Staphylococcus* sp. and *Bacillus* sp. to induce fluorescent under environmental stress [31-33]. The sensitivity and stability of green fluorescent protein (GFP) have made GFP as the best gene reporter; hence it is commonly applied in various environmental applications for the fabrication in whole-cell based optical biosensors [21, 34]. In the detection of toluene and their respective compounds, the feasibility of GFP as a reporter for pollutants was addressed by Stiner & Halverson (2002). In their study, *Pseudomonas fluorescens* strain A506 (pTS)

was modified with a transcriptional activator and its promoter, and a promoter-less gfp gene on a vector. A flow cytometry and a spectrofluorometer were used to compare the detection of the gfp-based sensor. The results corresponded with each other with apparent K (Kapp) values of 0.5±0.7 to 3.3±1.8 µM with a maximal fluorescence increase of 14 to 15-fold. The fluorescence response of A506(pTS) cultures exposed to 0.022 µM toluene was statistically greater ($P < 0.05$) than that of the no-effector control, indicating that the biosensor's limit of toluene detection is at least 0.022 µM [25]. Recombinant technology has also allowed the modification of other microorganisms into producing the green fluorescent protein – such examples include the modifications of a common bacterium species such as *Pseudomonas aeruginosa* and an eukaryotic species such as S*accharomyces cerevisiae* [35,36]. The results presented by the studies lucratively outlined the flexibility, sensitivity, rapidness of induction times and predictability of this type of biosensor.

III. BIOLUMINESCENT-BASED BIOSENSORS

The idea of luminescent light derives from Robert Boyle's early experiments in the mid 1600's showing the oxygen removal resulted in light cessation from microorganisms that able to release luminescent light [37]. Later in 1880's, it was found in beetles that bioluminescence required a luciferase and luciferin to function [37]. Fluorescence and bioluminescence are similar at atomic level however the only thing that differentiates the two is the triggering mechanism. In bioluminescence, luciferin/luciferase complex must be present whereas in fluoresence, it is the by-product of pigments that absorb external source of light [38].

To date, luciferase, an enzyme that catalyzes luminescence reactions is frequently utilized due to its sensitivity and the linear range of response is superior to some conventional reporters such as chloramphenicol, β-galactosidase, green fluorescent protein (GFP) and β-glucuronidase. The addition of luciferin and some co-factors, hence triggering the emission of bioluminescence; making it the more suitable reporter enzyme for the measurement of gene expression quantitatively [39]. In the mechanism of bioluminescence production, the approach taken to control the expression of lux gene can be grouped into two manners – constitutive and inducible expressions [25]. Constitutive manner is when the bioluminescent light was caused by lux-gene-coded luciferase that exists constitutively as long as the organism remains active. The density of bioluminescence produced is greatly affected by the presence of other compounds such as toxicants; therefore luminescence makes a great parameter for the determination of the additional compounds. On the other hand, the inducible luminescent occurs when the luciferase gene is fused with a promoter and is regulated by the analytes concentration [25].

Employing natural luminescent-producing microorganisms still an option for many researchers because these organisms are available in abundant, stable, well-studied and well-adapted to harsh environment. For example, marine bacterium *Aliivibrio fischeri* was entrapped in alginate microspheres where it was exposed to toxic metals such as Cu^{2+}, Cd^{2+}, Pb^{2+}, Cr^{6+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Ag^{+}, and Fe^{3+} [40]. Immobilized *A. fishceri* responded to the changes in the toxicity through the loss of bioluminescence [40]. Calcium alginate beads were used to immobilize *A. fisheri*. This prolonged the cells metabolic activity up to 6 weeks due to encapsulation in alginate biopolymer without any noticeable diminishes in the response of the bioluminescence response. With respect to this form of optical biosensor, the experiments were repeated by using an atomic absorption spectroscopy (AAS) and the outcomes corresponded with each other. A naturally-producing bioluminescent bacterium *Pseudomonas fluorescens* was modified at transcriptional level to carry naphthalene and salicylate catabolic reporter (nahG-lucCDABE) [41]. In the presence of naphthalene and salicylate, *P. fluorescencs* would emit the bioluminescence which was used as the indicator. The response time of P. fluorescence was found to be dependent on the concentrations of naphthalene and salicylate. This biosensor was introduced to real pollutants such as JP-4 jet fuel or an aqueous leachate from a manufactured-gas plant soil (where naphthalene is present in both pollutant mixtures), this sensor showed a specific hike in the bioluminescence detection [41]. Hence, this makes this type of biosensor is reproducible, sensitive and applicable for real pollutants detection [41].

Marine green alga *Ostreococcus tauri* was also employed as a novel luminescent biosensor for toxicity of biocides such as diuron and irgarol that are commonly found in coaster waters [42]. Cyclin-dependent kinase (CDKA), a protein involved in the cell cycle, was fused to a luciferase and the result was encouraging wherein an accurate determination of the 50% effective concentration (EC50) just after 2 days of exposure [42]. With these findings, the same approach can be applied to various microorganisms and not just limited to natural luminescent-producing bacteria. Other recombinant microorganisms that have shown similar potential and widely implemented in optical biosensors include *Escherichia coli* and *Pseudomonas putida* for the detections of mercury and toluene respectively [42,43].

IV. ADVANTAGES AND LIMITATIONS

Fluorescence and luminescence are commonly applied method in optical biosensors and particularly significant in optical sensing due to its specificity and sensitivity. Luminescence sensitivity is about 1000 times greater than that of most spectrophotometric methods and it can detect analyte of interest at trace

concentration ($\mu g/L$) in polluted water. Measuring the intensity of molecular emission is also at the top of the game as the instrumentation required is rather simple and cost-effective [44]. The measurements of fluorescence are relatively fast; where individual determination can be made in less than a micro-send. The utilization of UV-visible transmitting fiber optic cable to couple with the sensing element with the transducer permits measurements over long distances and multiplexing of sensors from numerous report locations [45]. For further development considerations, naturally present marine and fresh water microalgae offer a flexible solution in constructing novel biosensors *in-situ.* The photosynthetic pigments in these algae are very responsive towards changes in nature and allowing the exploitation of such feature for the detection of pollutants [46]. Besides that, the specificity of GFP as bioreporters has allowed the sensors to specifically detect target of interest with the presence of other compounds in the contaminated environment. For example, chromium ions can exist in various oxidation states in contaminated water, in which they are not all bioavailable. With GFP-based bacterial reporter systems, they can provide information about the bioavailability of this metal, which is the most relevant information in environmental assessment and the potential biological impact of a contaminant [47]. GFP also provides stability. The amount of GFP in a cell reflects the number of copies of the gene, expression duration and the strength of transcriptional promoter as well as determining the amount of toxic compounds present. A study shows that there is a direct relationship between the duration of expression and GFP when there is exposure to pollutants. This feature provides a practical implication where it permits flexibility in monitoring protocols and allows the removal of the cells from the pollutant samples, hence reducing detrimental effects on pollutant toxicity towards biosensor response for longer exposure to pollutants. This also allows the sensing system to maintain its sensitivity [25].

In terms of precision and accuracy, luminescence intensity-based sensors are highly affected by the flux in the intensity of the light sources, sensitivity of detector, inner filter effects, indicator concentrations, samples turbidity and thickness of sensing layer [48]. Fortunately, these issues can be overcome by measuring luminescence lifetimes instead of intensities. However, the applications of luminescence lifetime have some drawbacks such as expensive instrumentations and limited number of indicator dyes available that indicate the significant changes of analyte in the lifetime [48]. Despite the great advantages of fusing the GFP into host cells, they also have other limitations include unstable plasmids (from hosts), interference by other fluorescent particles or bacteria and low expression of GFP (or none) under anaerobic conditions [49,50].

Table 1 shows the comparison among different elements utilized for the development whole-cell based optical biosensors. From the data, it is possible to determine that not only do different platform of biosensor types perform dissimilarly, but the differences can be quantified and explored as well [51].

TABLE 1 : A TABLE COMPARING THE SENSITIVITY OF THE DIFFERENT TYPE OF AVAILABLE OPTICAL BIOSENSORS

Transducer	BRE[a]	Details	LDL[b]	Ref.
Fluorescence	*Chlorella vulgaris*	**Herbicide:** Atrazine	0.25 ug/L	[25]
GFP	Recombinant *Escherichia coli*	**Heavy metal:** Arsenic	7.5 $\mu g/L$	[22]
GFP	*Pseudomonas aerugenosa*	**Heavy metal:** Cadmium	10^{-8} mol/L	[35]
GFP	*Recombinant Saccharomyces cerevisiae*	**Heavy Metal:** Copper	5×10^{-7} M	[52]
Luminescence	*Ostreococcus tauri*	**Antifouling biocides:** Diuron Irgarol	5.65±0.44 µg/L 0.76±0.10 µg/L	[4]
Bioluminscence	*Aliivibrio fischeri*	**Heavy metals:** Cu(II) Cd(II) Pb(II) Zn(II) Cr(VI)	6.40 µg/L 1.56×10^{2} µg/L 0.47×10^{2} µg/L 0.32×10^{3} µg/L 0.10×10^{4} µg/L	[37]

a. BRE – Bio-recognition Element

b. LDL – Lowest limit of detection

V. COMMERCIALIZED OPTICAL BIOSENSORS

In the current biosensor market, the number of commercialized whole-cell based optical biosensors is limited however bbe Moldaenke has successfully commercialized a few different online bio-monitoring equipments that utilize biological entities such as algae and bacteria as the recognition elements for the monitoring of water quality; with automated systems [53,54]. Daphnia Toximeter, Fish Toximeter and Algae Toximeter are the instruments designed to scrutinize various water streams. The Fish and Daphnia Toximeters are monitored by motion analysis software designed specifically for these toximeters but for Algal Toximeter, the changes in the fluorescence emission are recorded using software specifically for this toximeter. The responses from the organisms are recorded and interpreted by those softwares – in which alarms are triggered to indicate the presence of deleterious pollutants in the samples. Besides Algae Toximeter, bbe Moldaenke has also developed AlgaeOnlineAnalyser an online monitoring system to measure chlorophyll fluorescence, class of algae and photosynthetic activity of natural algae accumulation in the suspected water streams. In a long run,

AlgaeOnlineAnalyser is convenient in monitoring any changes in the algae community in the sample water streams due to pollutions [53].

Other examples include a bacterial biosensor - BIOMET™, a specific biosensor kit used to test the bioavailability of various heavy metals – which is proven to be sensitive towards cadmium, zinc, nickel, copper, chromium, mercury or lead; in which *Ralstonia metallidurans* glows (producing bioluminescent) when exposed to those heavy metals [54]. Another kit that adopts the same concept – arsenic-specific test kit was developed by Aboatox is inexpensive and rapid (able to assess over 100 samples per day) [55].

CONCLUSIONS

Over the years, luminescence and fluorescence have definitely gained their spots in the biosensing field especially in exploiting these features for the environmental assessment. The question that is yet to be answered is, which one is better? Is it luminescence or fluorescence? It can be said that bioluminesce genes (*luc*) have played a prominent role as the reporting elements in various whole-cell based biosensors. Since the past few years, many researchers have shown their interest in versatile fluorescent protein genes (GFP) to the extent where they are available for general use; hence increasing their demand as the reporter gene for the applications of whole-cell biosensors. When real-time monitoring of the developing signal is impractical, fluorescence might prove to be more reliable. The sensitivity will be significantly improved if sufficient time is allowed for signal.

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