BIOLUMINESCENT BIOREPORTER INTEGRATED CIRCUITS (BBICs): WHOLE-CELL CHEMICAL BIOSENSORS¹

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ABSTRACT

We report a chemical sensing technology composed of engineered bioluminescent bacteria placed on an optical application specific integrated circuit (OASIC). The bacteria have been engineered to luminesce when the targeted compound is metabolized, while the OASIC detects, processes, and then reports the magnitude of this optical signal. In this work we report a toluene sensing device that uses the bioreporter *P. putida* TVA8, and a naphthalene sensing device that uses the bioreporter *P. fluorescens* HK44. These sensors are capable of detecting low parts per billion concentrations of the targeted substance.

INTRODUCTION

Biosensors are hybrid devices combining a biological component with an analytical measuring element. The biological component typically reacts and/or interacts with an analyte of interest producing a response that can be quantified by an electronic, optical, or mechanical transducer. The most common configuration uses immobilized biomolecules such as enzymes or antibodies as the biological component providing the needed selectivity. Another less utilized approach uses living microorganisms or sections of organs or tissues as the biological element. Originally these biosensors, sometimes referred to as whole-cell biosensors [1], used electrochemical transducers to detect the activity of growing cells [2]. Whole-cell biosensors have functioned in controlled environments but are not widely applicable, largely because of interference caused by growth on nutrients other than the target analyte.

Alternatively, molecular biological techniques have been used to produce cells or bioreporter strains that have much greater selectivity. Typically, DNA sequences that code for a specific promoter sequence are fused with gene(s) coding for reporter enzyme(s) and introduced into a host cell. When the target molecule is present, the reporter genes are expressed to produce the enzyme(s) responsible for the production of the measured signal. Thus, gene regulation and not consequences of microbial activity provides selectivity.

Some commonly used reporter enzymes are beta-galactosidase (*lacZ*) and catechol 2,3-dioxygenase (*xylE*). Both of these systems use colorimetric detection methods, requiring cell destruction to produce the signal. In the past decade, bioluminescent bioreporters have become popular since the bioluminescent response is easily detected and the assay need not be destructive to the cells. Thus, the bioreporter can be continuously monitored in real time. Genetically engineered

bioluminescent bioreporters using both eukaryotic and prokaryotic bioluminescence enzymes have been developed for detecting toxins and pollutants in water and soil and to assess the bioavailability and functional processes of pollutant biodegradation.

Bioluminescence of bioreporters has been detected by a number of different types of optical transducers, including photomultiplier tubes, photodiodes, microchannel plates, photographic films, and charge-coupled devices. In many of these applications, light is collected and transferred to the transducer using lenses, fiber optic cables, or liquid light guides. However, applications requiring small volumes, remote detection, or multiple parallel sensing necessitate a new type of instrumentation that is small and portable, yet maintains a high degree of sensitivity.

We report here a novel approach that eliminates the need for bulky and expensive optical transfer and detection devices. A bioluminescent bioreporter integrated circuit (BBIC) is formed by placing whole-cell bioreporters engineered to bioluminesce in the presence of a particular substance on an integrated circuit (IC) designed to detect light, process this signal, and then report the results. In this concept, the bioreporters can be thought of as another component (analogous to a transistor, resistor, or capacitor) available to the IC designer. The chief advantage to this approach is that the entire sensor, including all signal processing and communication functions, can be realized in a single-chip, low-power, rugged, inexpensive device. While other transducers only detect the light and require other components to make a complete system, the IC provides the analog, digital, and radio frequency (RF) circuit capabilities required to make a complete stand-alone sensor. Additionally, as there are inexpensive, prototype quantity, IC fabrication facilities, BBICs can be individually crafted to fit particular applications. Thus, a feature such as global position sensing could be included in a BBIC designed for a distributed sensing application.

BIOLUMINESCENT BIOREPORTERS

In prokaryotes, the system, designated *lux*, consists of a luciferase composed of two different subunits coded by the genes *luxA* and *luxB* that oxidize a long chain fatty aldehyde to the corresponding fatty acid resulting in a blue-green light emission near 490 nm [3-5]. The system also contains a multienzyme fatty acid reductase consisting of three proteins (a reductase encoded by *luxC*, a transferase encoded by *luxD*, and a synthetase encoded by *luxE*) which both initially converts and recycles the fatty acid to the aldehyde substrate. Thus, no exogenous addition of substrate is required to induce luminescence. The genes are contained on a

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single continuous operon. This genetic configuration allows the cloning of the complete *lux* gene cassette (reporter genes) downstream from different promoters for the utilization of bioluminescence to monitor gene expression.

Implicit in the use of a bioreporter strain for a BBIC is that the bioluminescent signal generated is directly related to the concentration of the target substance, most desirably in a selective and quantitative manner. In general, the *lux* reporter genes are placed under the regulatory control of inducible operons maintained in native plasmids, broad host range plasmids, or chromosomally integrated into the host strain. In these genetic systems, the target analyte or its degradation products act as the inducer of the bioluminescence genes, and are responsible for selectivity and the resultant response. For example, *P. fluorescens* HK44 is a bioreporter that produces light in the presence of naphthalene. This strain has two genetic operons positively regulated by a LysR protein. One of the operons contains the *lux* bioluminescence genes and the other the genes responsible for the degradation of naphthalene to salicylate, the metabolic intermediate of naphthalene degradation. Both operons are induced when salicylate interacts with the regulatory protein NahR. Therefore, exposure of HK44 to either naphthalene or salicylate results in increased naphthalene gene expression and increased bioluminescence.

Initial studies in continuous cultures of *P. fluorescens* HK44 have demonstrated that the magnitude of the bioluminescence response correlated with the aqueous phase concentration of naphthalene under dynamic pulsed perturbation conditions [6]. Reproducible bioluminescence was observed not only in aqueous naphthalene samples but also in soil slurry samples which were spiked with naphthalene, complex soil leachates, and the water soluble components of jet fuel [7]. *P. fluorescens* HK44 can be applied in environmental use for either quantitative analysis of contaminant presence or bioavailability. However, for such applications both the chemical complexity of the environment and the physiological conditions of the organisms must be considered in interpreting the bioluminescence response.

Many types of bioluminescent (*lux*) transcriptional gene fusions have been used to develop light emitting bioreporter bacterial strains to sense the presence, bioavailability, and biodegradation of other pollutants including toluene [8], and isopropylbenzene [9]. Analogous genetic approaches have also been reported for inducible heavy metal detoxification and resistance systems including mercury [10], as well as the heat shock response [11] and response to oxidative stress [12]. In addition, genetically engineered Gram positive bioreporters have been used to examine the efficacy of antimicrobial agents (decreased light equates to greater efficacy) [13,14]. Eukaryotic bioreporters have also been generated to detect toxic compounds [15,16], oxygen [17], ultraviolet light [18], and estrogenic and antiestrogenic compounds [19]. Environmental applications involving bioluminescence measurements have been reviewed [20].

PROTOTYPE BBIC AND EXPERIMENTAL RESULTS

A prototype BBIC was constructed by placing the toluene sensitive bioreporter, *P. putida* TVA8, above a custom optical application specific integrated circuit (OASIC) shown in Fig. 1. This device employed the p-diffusion (source and drain diffusions of p-channel MOSFETs) as a photodiode. The shallow p-diffusion has a strong response to the 490-nm bioluminescent signal [21], and the p-diffusion/n-well junction can be operated

Figure 1. The OASIC used in the BBIC prototype. This device measures 2.2 mm X 2.2 mm and was fabricated in a standard CMOS IC process.

with a zero reverse bias without being set to either power supply rail. Thus, the leakage and noise of the photodiode can be minimized.

The front-end signal conditioning circuit (Fig. 2) was a current-to-frequency converter (CFC). When compared to conventional electrometer circuits, this signal conditioning circuit provided lower noise (no resistor in the feedback path), faster recovery from overloads (no long time constants), and larger dynamic range. A digital signal proportional to the sum of the leakage and photo- current was generated by counting pulses from the CFC for a specified time (i.e. the integration time). The entire OASIC, including photodetector and CFC measured 2.2 mm X 2.2 mm, and was fabricated in a standard 1.2- μ m, n-well, CMOS process. Fig. 3 shows the BBIC (including the bioreporter enclosure) as used in the characterization experiments.

With no luminescent signal coming from the cells, multiple measurements were taken with the integration time set to 1-minute. Leakage currents produced a signal of ~ 6 counts/minute with a standard deviation (σ) of 0.22 counts/minute. As expected, the σ decreased with the square root of the integration time. Longer integration times were produced off-line by summing 1-minute measurements.

Bioluminescence was induced in the BBIC cells and a control sample of cells by exposure to toluene vapor. From the control sample measurements, we estimate that the toluene concentration was no more than 1 ppm. A signal of 12 counts/minute (6 counts/minute above background) was measured. From previous measurements, *P. putida* TVA8 is known to have a linear response to toluene concentration until saturating when the concentration reaches a level of approximately 10 ppm [8]. Assuming that a minimum detectable signal is 2σ above background, then the minimum detectable concentration (MDC) of toluene for this BBIC (in parts per billion (ppb)) is given by

$$MDC = \frac{20,000\sigma}{\sqrt{T_{\text{int}} S_{\text{sat}}}} \quad (1)$$

Figure 2. Front-end signal processing circuit for the prototype BBIC.

where S_{sat} is the signal in counts/minute at a concentration of 10 ppm, and T_{int} is the integration time in minutes. The minimum detectable toluene concentration for this BBIC as a function of integration time is shown in Fig. 4. In general, the minimum detectable concentration is also a function of the number of bioreporter cells and the area of the photodiode.

A naphthalene-sensitive BBIC was produced using the OASIC described above and the bioreporter *P. fluorescens* 5RL. Using the same experimental procedure described above, this BBIC was exposed to naphthalene vapor with a concentration of

approximately 10 ppm. A signal of 240 counts/minute was recorded. The minimum detectable concentration of naphthalene as a function of integration time for this BBIC is shown in Fig. 5.

CONCLUSIONS

This paper presents early results from efforts to produce chemical sensors by combining engineered bioluminescent bioreporters and integrated circuit technology. The experimental results we have presented show that very good sensitivity can be

Figure 3. The prototype BBIC showing: (a) the OASIC mounted in a 40-pin ceramic chip carrier and the enclosure for the cells; (b) the enclosure mounted on the chip as configured for experiments; and (c) the enclosure with bioreporters on an agar plug. The o-ring is used to make a light-tight seal between the ceramic chip carrier and the bioreporter enclosure.

REFERENCES

Figure 4. Minimum detectable concentration of toluene as a function of integration time for the prototype BBIC employing the bioreporter *Pseudomonas putida* TVA8.

Figure 5. Minimum detectable concentration of naphthalene as a function of integration time for the prototype BBIC employing the bioreporter *P. fluorescens* 5RL.

obtained using the BBIC concept. In addition, the bioreporters can provide exceptional specificity. However, to this point, our experiments have been conducted in well-controlled laboratory environments.

At present we have on-going work to develop bioreporter entrapment techniques and bioresistant/biocompatible thin-film deposition techniques that promise to produce rugged, long-lived BBICs that can be deployed in useful sensing applications [22]. In addition, we are adding signal processing and communications functionality to the OASIC to increase the flexibility of the BBIC. With the successful development of these technologies, we envision BBICs being used in applications such as environmental sensing/assessment, medical diagnostics, and drug discovery.

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1. Bousse, L. (1996) *Sens. Actuat.* 34, 270-275
2. Buerk, D. G. (1993) in *Biosensors, Theory and Applications* pp. 1179-1193, Technomic Publications, Lancaster, PA, USA
3. Tu, S. and Mager, H. I. X. (1995) *Photochem. Photobiol.* 62, 615-624
4. Meighen, E. A. (1991) *Microbiol. Rev.* 55, 123-142
5. O'Kane, D. J. and Prasher, D. C. (1992) *Mol. Microbiol.* 6, 443-449
6. King, J. M. H. (1990) *et al. Science* 249, 778-781
7. Heitzer, A. *et al.* (1992) *Appl. Environ. Microbiol.* 60, 1487-1494
8. Applegate, B. *et al.* (1997) *J. Ind. Microbiol.* 18, 4-9
9. Selifonova, O. V. and Eaton, R. W. (1996) *Appl. Environ. Microbiol.* 62, 778-783
10. Selifonova, O., Burlage, R. S. and Barkay, T. (1993) *Appl. Environ. Microbiol.* 59, 3083-3090
11. Van Dyk *et al.* (1994) *Appl. Environ. Microbiol.* 60, 1414-1420
12. Belkin, S. *et al.* (1996) *Appl. Environ. Microbiol.* 62, 2252-2256
13. Andrew, P. W. and Roberts, I. S. (1993) *J. Clin. Microbiol.* 31, 2251-2254
14. Cooksey, R. C., Crawford, J. T., Jacobs, W. R. Jr. and Shinnick, T. M. (1993) *Antimicrob. Agents Chemother.* 37, 1348-1352
15. Anderson, J. W., Rossi, S. S., Tukey, R. H. and Quattrocchi, L. C. (1995) *Environ. Toxicol. Chem.* 14, 1159-1169
16. Gupta, M. and Goldwasser, E. (1996) *Nucleic Acids Res.* 24, 4768-4774
17. Filatov, D., Björklund, S., Johansson, E. and Thelander, L. (1996) *J. Biol. Chem.* 271, 23698-23704
18. Biberger, C. and Angerer, E. V. (1996) *J. Steroid Biochem. Mol. Biol.* 58, 31-43
19. Pons, M., Gagne, D., Nicolas, J. C. and Mehtali, M. (1990) *BioTechniques* 9, 450-459
20. Steinberg, S. M. and Poziomek, E. J. (1995) *Chemosphere* 11, 2155-2197
21. Simpson, M. L. *et al.* (1997) *Rev. of Sci. Instr.* 69, No. 2, Feb. 1998, pp. 377-383
22. Simpson, M. L., *et al.* (1998) *Trends in Biotech.*, to be published