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Biosensors: MICREDOX—a new biosensor technique for rapid measurement of BOD and toxicity

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The combination of electrochemical, optical, acoustical and other measurement techniques with the specificity of biological recognition systems has resulted in a range of new analytical devices known as biosensors. Biosensors are under intensive development worldwide because they have a multitude of potential applications, in particular, clinical medicine, environmental monitoring and process control of industrial processes. Their basic principles, mode of operation, performance requirements and current trends will be reviewed. A brief description of a novel biosensor developed at Lincoln Technology, the MICREDOX method, is outlined and applications for rapid measurement of biochemical oxygen demand and toxicity measurements are described. Preliminary data obtained using the MICREDOX method are reported for three standard toxic materials, zinc sulphate, 3, 5-dichlorophenol and sodium lauryl sulphate.

Keywords: biosensor, biocatalytic, redox mediator, toxin, direct toxicity assessment.

Introduction

It is now estimated that there are over 100 000 chemical substances on the market and around 200 new substances are added each year (O'Sullivan and Alcock 1999). In some cases a single effluent may contain more than 20 000 substances and the value of chemical analysis becomes questionable, if not impractical, in this situation. Increasingly biological assays are being used to measure the effect of chemicals on the environment. Swift transmission of health threatening infection or contamination through aquatic environments underpins a need for a range of analytical devices with the capability of providing rapid and reliable measurements of biological responses, at low cost, to complement the existing arsenal of chemical and biological methods. Biosensors (Rogers and Gerlach 1996) are now recognized as a technology fulfilling this need because they have the capability of spanning both chemical and biological assays. They can detect in the environment and, in real time, an individual chemical, or groups of substances, and they can monitor biological effects such as genotoxicity, immunotoxicity and endocrine responses (O'Sullivan and Alcock 1999).

Biosensors are chemical sensors that use biomolecules to interact with target chemicals and are composed of two essential elements, a biocomponent and a transducer (figure 1).

The biocomponent performs the molecular recognition and may be an enzyme, antibody, nucleic acid, bacteria or even whole tissues of higher organisms. The transducer converts the signal derived from the catalytic or binding event to one that is readily displayed, for example electrochemical, optical, piezoelectric or thermal. Compared with chemical sensors, biosensors are more specific because evolution has optimized biomolecules to operate at ambient temperatures and in dilute aqueous media at around pH 7 (Byfield and Abuknesha 1994).

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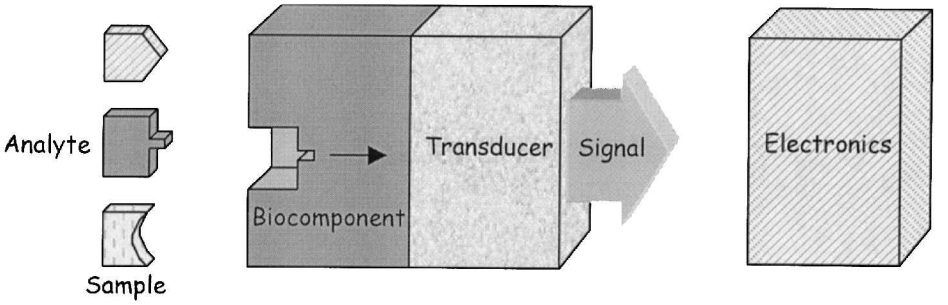

Figure 1. Biosensor schematic.

Globally the annual total sales of biosensor devices in the 1990s has surged in value from $US35M, in 1990, to around $US510M by 1996, and is projected to exceed $US890M by year-end 2000. At first glance this could indicate that biosensors are a robust and flourishing commercial industry. While this is true for the glucose sensor, it does not hold for the whole of the biosensor industry. In 1996, the blood glucose sensor accounted for $US485M of the $US510M total. The next most successful biosensor product, BIACORE™, is used more as a research tool for pharmaceutical design rather than as a chemical sensor. The success of the glucose sensor is due to a number of factors, including: a large and increasing demand for blood glucose monitoring worldwide, the stability and cheapness of glucose oxidase (Wilson and Turner 1992), and mass-manufacturability of the sensor element.

The commercial success of any new analytical device hinges on its ability to be either superior or cheaper (Weetall 1996). In general, biosensor technology does not offer superior performance, particularly in terms of sensitivity, selectivity, and reproducibility. Applications favouring the use of biosensors are, therefore, those demanding portable, easy to use, real-time monitoring devices. Examples include clinical medicine (point-of-care testing), screening for genetic diseases, areas of public health (detection of pathogenic organisms), and environmental monitoring. These applications, and those where measurement is inaccessible without biosensor technology, accept a premium for the provision of on-site and rapid results.

The Lincoln Ventures Biosensor programme has concentrated on developing biosensor technology applicable to the New Zealand context, by providing sensors for environmental monitoring and to improve the efficiency of primary production. Given the low transferal of biosensor research to the commercial sector, our efforts have been constrained to areas favouring biosensors or where the use of biomaterials is obligatory. In the environmental monitoring area we are targeting biochemical oxygen demand (BOD) and toxicity monitoring. Currently for each of these assays, there are a number of methods available, indicative of a lack of satisfaction and there remains a demand for the development of tests that are low-cost, rapid and reliable. Both BOD and toxicity applications are essentially biological assays and therefore amenable biosensor technology.

MICREDOX—a method for quantifying microbial substrate conversion

The enormous diversity of microbial species available makes whole cells potentially valuable biocatalysts for biosensors with environmental applications

(Rawson *et al*. 1989). To date, the majority of techniques for quantifying substrate catabolism utilizing microbial respiration focus on measuring oxygen uptake. However, the availability of oxygen has been shown to be a limiting factor for microbial catabolism and is a significant factor contributing to the 5-day requirement of the traditional BOD_5 test (Reshetilov *et al*. 1998). Accordingly, we have devised a method in which the natural co-substrate, oxygen, is substituted by a synthetic co-substrate. This substitution confers two significant advantages. First, contrary to oxygen, the reduced form of the synthetic co-substrate has an initial background concentration of zero, thereby increasing the accuracy when measuring substrate conversion. Second, replacing oxygen with a synthetic co-substrate of higher solubility, a higher number of bacteria can be used and incubation time is reduced. Keeping the concentration of co-substrate in excess, the number of bacterial cells can be manipulated to increase the rate of substrate conversion. One application of the method is to rapidly determine soluble BOD in wastewater (Pasco *et al*. 1999). We have demonstrated that the MICREDOX substrate conversion efficiency from a 1 h test is comparable to that achieved in a traditional 5-day BOD test. High concentrations of co-substrate and microbial cells, the initial conditions provided by the MICREDOX method (Pasco *et al*. 1999), create an environment where substrate is the rate-limiting reagent and, therefore, reaction time to an equivalent extent is condensed 100-fold. Another application permits quantitative measurement of the effect of toxic substances on microbial catabolism. By incorporating the initial conditions of the MICREDOX process, measurement of inhibition of bacterial respiration by toxins should receive similar benefit.

Laboratory-oriented toxicity studies tend to focus on determining direct toxic effects, while field-oriented studies are more directed toward changes in communities and populations. Bacteria offer a number of advantages for laboratory-based toxicity testing. In addition to being widely distributed in aquatic environments, they are economical and fast to grow, resulting in tests that are rapid and easy to perform. They possess many of the biochemical pathways of higher organisms and show a high degree of membrane structural organisation (Berkowitz 1979). Statistical advantages are also derived from the large number of bacteria relative to the small number of organisms used in non-bacterial assays. Moreover, the respiration inhibition test using activated sludge, is an accepted standard OECD guideline for the testing of chemicals (OECD 1981).

Comparison among toxicity testing procedures

In a previous study four systems were evaluated using: Microtox®, *Spirillum volutans* motility inhibition, *Pseudomonas fluorescens* growth inhibition, and inhibition of respiration in a synthetic activated sludge (Liu and Dutka 1984). A summary of the results for three of 13 chemical substances included in their study is shown in table 1. LC_{50} is the lethal concentration at which 50% of the organisms die. EC_{50} is the effective concentration at which 50% of the organisms are affected in terms of loss of mobility, failure of metabolic activity, growth inhibition, etc. IC_{50} is the inhibition concentration that decreases the respiration rate by 50%.

Table 1 shows that each microbial screening procedure has its own sensitivity pattern, demonstrating the hazards of assessing the impact of toxins on the basis of a single species or organism.

Table 1. Sensitivity comparison of four toxicity screenings to selected chemicals.

Chemical (ppm)	Microtox EC_{50} 15 min	*S. volutans* MEC_{90} 120 min	*P. fluorescens* EC_{50} 18 h	Syn act sludge IC_{50} 3 h
$ZnSO_4$	3.5	11.6	367.0	1.2
3, 5-dichlorophenol	2.9	5.0	3.2	30.2
Sodium lauryl sulphate	1.8	43.0	1650	480

MICREDOX toxicity method

The MICREDOX toxicity test quantifies the effect of toxic substances by comparing the response of the toxin with a control, in a 60 min incubation. The method is similar to traditional respirometric techniques except that the co-substrate oxygen, which acts as the terminal electron acceptor for microbial respiration, is replaced by a more soluble synthetic co-substrate. By keeping the concentration of the co-substrate, potassium hexacyanoferrate(III), high and in excess, the number of bacterial cells can be manipulated to give the required rate of substrate consumption, reducing the incubation time. After a 1 h incubation of the microorganisms with a fixed quantity of organic substrate, potassium hexacyanoferrate(III) and toxin, the amount of ferricyanide reduced by the micro-organisms is determined coulometrically by measuring the charge required for its re-oxidation in a bulk electrolysis cell. The charge, therefore, is a direct measure of the quantity of a known substrate oxidized by the micro-organisms and the ratio of the coulombs, recorded in the presence of toxin relative to that recorded in the absence of toxin, provides a ready index of the toxins inhibition (see inhibition quotient, equation 1). Non-electrochemical methods may also be used to measure the concentration of co-substrate.

Materials and methods

Organism and culture conditions

Escherichia coli cultures were maintained on Trypticase Soy Agar plates at 4°C. Bacterial cultures were grown aerobically at 35°C for 16 h in a Davis minimal media broth (Atlas 1997) on an orbital shaker (150 rpm). Cells were harvested by centrifugation at 8670 × *g* for 10 min at 4°C. The cell pellets were washed twice in phosphate buffer (0.05 M KH_2PO_4/K_2HPO_4, pH 7) and resuspended in phosphate buffered saline solution (0.05 M KH_2PO_4/K_2HPO_4, pH7, 0.1 M KCl). Uniform concentrations of cells were achieved by adjusting the absorbance to the desired value using a Unicam 8625 spectrophotometer operated at 600 nm and pathlength 1 cm.

Bacterial reduction of mediator

Each toxicant was examined at three concentrations corresponding to 0.1, 1, and 10 times the *S. volutans* MEC_{90} values (table 1). Replicate samples containing 3 ml *E. coli* (OD_{600} of 25), 3.6 ml 0.25 M potassium hexacyanoferrate(III), toxicant at the appropriate level and 9.7 ml of BOD_5 100 standard solution (75 mg l^{-1} glucose, glutamic acid, respectively) were incubated for 60 min at 35°C under anaerobic conditions. Positive control measurements substituted distilled water for toxicant. Negative controls contain no toxin or BOD_5 standard substrate. Microbial action was terminated by centrifugation at 1500 × *g* for 10 min and the supernatant was filtered through a 0.45 um filter to remove any residual bacteria. Samples were stored under nitrogen at 4°C until analysed by bulk electrolysis.

Bulk electrolysis

Reduced mediator was quantitatively re-oxidized using a bulk electrolysis cell coupled to a Bioanalytical Systems 100B/W electrochemical analyser operating in coulometric mode. Electrolysis

was performed at 35°C and was terminated when the current diminished to 1% of its initial value. Cylindrical platinum gauze electrodes were used for both the anode and the cathode. A Ag/AgCl reference electrode was positioned within the anolyte compartment and the potential of the anode was set at +600 mV relative to the reference electrode during bulk electrolysis. Coulombic outputs were computed by integrating the area under current versus time curves.

Results

Reduced co-substrate, potasssium hexacyanoferrate(II), is a product of the microbial incubation stage. The amount of charge, in coulombs, required for the re-oxidation of the microbially reduced co-substrate following a 60 min incubation is shown in table 2.

C^-, the negative control, is a measure of the endogenous interaction of *E.coli* with the co-substrate, potassium hexacyanoferrate(III), in the absence of both toxin and exogenous substrate (table 2) and is a measure of *E. coli*'s basal respiration activity. The rate of respiration is enhanced by the addition of exogenous substrate and is measured by difference between the positive control, C^+, and C^-. The positive and negative controls provide an upper and lower signal for the measured responses in the absence of a toxin. Each toxin was examined at three levels of concentration based on the *S .volutans* MEC_{90} concentration (table 1).

From table 2 it is possible to calculate the inhibition quotient at each concentration level by the following formula:

Inhibition Quotient:
$$IQ = 100 - \left(\frac{Q_t - C^-}{C^+ - C^-}\right) \times 100$$

where

Q_t = coulombic output at toxin concentration
C^- = negative control coulombic output
C^+ = positive control coulombic output

The inhibition quotient for each toxin at the levels tested is shown in table 3.

Table 2. Effect of toxin concentration on the coulombic output. Toxin concentrations relative to MEC_{90} values reported in table 1.

Toxin	C^+/C	C^-/C	$0.1 \times MEC_{90}/C$	MEC_{90}/C	$10 \times MEC_{90}/C$
$ZnSO_4$	8.6	2.1	7.2	8.4	0.8
3, 5-dichlorophenol	8.5	1.9	8.8	7.4	3.3
Sodium lauryl sulphate	9.1	2.4	9.0	9.1	9.6

Table 3. Variation of Inhibition Quotients with toxin concentration. Toxin concentrations relative to MEC_{90} values reported in table 1.

Toxin	$0.1 \times EC_{90}$	EC_{90}	$10 \times EC_{90}$
$ZnSO_4$	23	5	100
3, 5-dichlorophenol	0	16	80
Sodium lauryl sulphate	4	7	0

Discussion

While the BOD application of MICREDOX has been subjected to rigorous testing, these measurements are the first made using the MICREDOX method in toxicity mode. More extensive testing and data sets are required before we can make any substantive claims in relation to the utility of this method for rapid toxicity testing. However, these initial results and their potential for application in rapid toxicity testing encourage us. Measurements to fully characterize the toxicity of complex effluent usually require a suite of tests involving a range of different organisms from different phyla and further trials, using selected eukaryote and prokaryote cells, are planned.

Our initial results show that for the toxins, zinc sulphate and 1,3 dichlorophenol, the method shows good discrimination over two decades of toxin concentration. The IQs for zinc sulphate seem slightly irregular as they decrease with a concentration increase from $10^{-1} \times MEC_{90}$ to $1 \times MEC_{90}$. However, this could be a result of a sharp zinc sulphate response that is not activated until a threshold concentration, indicating a need to resolve this response curve in smaller steps. The sodium lauryl sulphate results do not correlate with table 1. At the concentrations tested the coulombic output increased relative to the positive control, possibly because the detergent increases the permeability of the cell membrane to co-substrate, increasing the diffusion across the membrane. Increased coulombic output was also observed in a supplementary measurement at sodium lauryl sulphate concentration of 4300 ppm. Although there must be some threshold at which the concentration of sodium lauryl sulphate disrupts the cell membrane and impedes cell function, the concentrations we tested over 60 min showed no evidence of toxic effect by sodium lauryl sulphate.

Since the coulombic output recorded after the 60 min incubation is equivalent to a 5-day incubation when oxygen is used as co-substrate (Pasco *et al*. 1999), we contend that the MICREDOX method is a platform for compressing the time for substrate conversion 100-fold without compression of the reaction extent. Furthermore the method provides a large integrated quantitative signal as output and there is minimal statistical uncertainty in analysing the results.

Future work will include different microbial cells, singly as we have done for *E. coli*, and in combinations. Also under investigation are various methods for immobilization of the microbial cells, avoiding the need to culture fresh cells prior to each trial. We also expect the coefficient of variation will improve when using immobilized cells. Later versions of this method will include multiple immobilized cells as the biocomponent.

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