DISSERTATION

FIBER OPTIC ENZYMATIC BIOSENSORS AND BIOSENSOR ARRAYS FOR MEASUREMENT OF CHLORINATED ETHENES

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ABSTRACT

FIBER OPTIC ENZYMATIC BIOSENSORS AND BIOSENSOR ARRAYS FOR MEASUREMENT OF CHLORINATED ETHENES

Chlorinated ethenes such as trichloroethylene (TCE), tetrachloroethylene (PCE), three isomers of dichloroethylene (DCEs) and vinyl chloride (VC) are used as solvents and cleaners in a variety of industrial and commercial areas. Chlorinated ethenes have become one of the most common environmental pollutants in groundwater contamination sites due to their widespread usage, moderate solubility compared with other organic pollutants and recalcitrance to natural attenuation. Fiber optic enzymatic biosensor was developed in this study as a continuous, real time and *in situ* measurement principle. TOM biosensor, first reported enzymatic biosensor, was initiated with toluene measurement in aqueous solution as proof-of-concept experiments. The subsequent success of TOM and TOM-Green in TCE analysis showed great potential of biosensor measurement for chlorinated ethenes, despite the ubiquitous problem for monooxygenase-based biosensor with NADH consumption overtime and after usage. In addition, epoxide toxicity also increased the difficulty of biosensor application for measurement of chlorinated ethenes, although several TOM-Green transformants could mitigate the toxicity with rapid epxoide degradation. Plasmid transformation with was introduced to manipulate the construction of new TOM and TOM-Green transformants with capability of intracellular NADH regeneration. FDH regeneration system was studied for both TOM and TOM-Green cells, while TOM+FDH showed great activity retention and regeneration ability and TOM-Green+FDH was able to retain activity over prolonged storage but failed on regeneration after repeated usage due

to the toxicity of TCE epoxide. Biosensor array was built with pH-based biosensor to measure a group of haloalkanes. The design concept of biosensor array and detection instrumentation was successful. Linear approach in array data analysis was simple and fast but lacked of accuracy, while nonlinear approach increased the complexity of data analysis to a new level with precision in sacrifice of efficiency. Multivariable chemometric approach was also introduced in array data analysis, providing a high-throughput alternative and a means of quantitatively assessing matrix effects. This project demonstrates the potential of fiber optic enzymatic biosensor and biosensor array as measurements for different analyte are described. This is also one of the first comprehensive studies in oxygen-based biosensor and its application and great potential in food, clinical, and environmental monitoring, industrial process control and other related areas.

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1.1 Project Background

1.1.1 Description

Chlorinated ethenes such as trichloroethylene (TCE), tetrachloroethylene (PCE), three isomers of dichloroethylene (DCEs) and vinyl chloride (VC) are used as solvents and cleaners in a variety of industrial and commercial areas. Chlorinated ethenes have become one of the most common environmental pollutants in groundwater contamination sites due to their widespread usage, moderate solubility compared with other organic pollutants and recalcitrance to natural attenuation.

The fate of chlorinated ethenes in groundwater has been of great interest in the past decades. The first report of complete dechlorination of PCE and TCE was published in 1989 (Freedman and Gossett 1989). The subsequent studies revealed four categories of metabolic processes were involved in the microbial biodegradation of chlorinated ethenes (Lee et al. 1998):

- Energy-yielding solvent oxidations: support microbial growth by acting as the only carbon and energy source, which usually occurred in the case of DCE and VC (Bradley and Chapelle 1996; Lovley 1997).
- Co-metabolic oxidations: partially degraded chlorinated ethenes via formation of chloroethene epoxides, not applicable for PCE though (Ensley 1991; Vogel et al. 1987).
- Energy-yield reductions (dehalorespiration): chlorinated ethenes served as electron acceptors in energy generation metabolism, particularly important in PCE and TCE biodegradation (MaymoGatell et al. 1997; Utkin et al. 1994).

- Co-metabolic reductive dehalogenation: chlorinated ethenes were reduced in a minor side-reaction in the cases of methanogens, sulfate-reducing bacteria, etc (Bagley and Gossett 1990; Corapcioglu and Hossain 1991).

Traditional techniques to detect chlorinated ethenes relied on lab-based analytical methods such as gas chromatography with mass spectrometry (GC-MS) (Delinsky et al. 2005a; Poli et al. 2005), high performance liquid chromatography (HPLC) (Chen et al. 2004; Delinsky et al. 2005a; Delinsky et al. 2005b), Ion-exchange chromatography (IC) (Dixon et al. 2004), etc (Delinsky et al. 2005a).

1.1.2 Environmental Significance

Although the demand of chlorinated ethenes have declined overtime since 1960s due to the increasing concerns about environment and public health issues (Bakke et al. 2007), large quantities of chlorinated ethenes have been, and continue to be, consumed in domestic and international markets, especially in the case of PCE and TCE. The domestic demand of PCE is around 800 million pounds and demand of TCE is around 1000 million pounds in 1998 (Morrison 2000), while the total on- and off-site releases of PCE and TCE are 4 million pounds and 11 million pounds during 1998-2001, according to the EPA Toxic Release Inventory (Moran et al. 2007). As a consequence, TCE and PCE become the most common binary mixture contaminants that have been found in the U.S. groundwater contamination sites (Jollow et al. 2009).

Chlorinated ethenes are of great concern in groundwater system, especially in drinking systems, as they could cause acute or chronic health problems including liver damage, possible kidney effects and cancer (Brown et al. 1990; Groten et al. 2001). The EPA has set the Maximum Contaminant Levels (MCLs) for all chlorinated ethenes in drinking systems at very low concentrations (5 μg/L for PCE, 5μg/L for TCE, 7μg/L for 1, 1-DCE, and 2μg/L for vinyl chloride).

While previous studies on biodegradation and bioremediation of chlorinated ethenes provided a variety of solutions for the contamination problem, environmental monitoring of chlorinated ethenes are also important since bacteria that can degrade these chlorinated compounds do not occur everywhere, and where they do occur they usually could not completely cleave the chlorines (McCarty 1997). Therefore, the contamination could last for decades, though concentrations of pollutants may only maintain at trace amounts, which could also lead to the development of environmental monitoring techniques (Moran et al. 2007).

1.1.3 Basic analytical chemistry terminology

This section provides the rigorous definitions of all the analytical chemistry terminologies that appear in the dissertation, according to the Compendium of Chemical Terminology (IUPAC 1997).

- Limit of detection (LOD): expressed as concentration, c_L, and is derived from the smallest measure, x_L, that can be detected with reasonable certainty for a given analytical procedure. The value of x_L is given by the equation: $x_L = x_{bi} + ks_{bi}$, where x_{bi} is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired.
- Limit of quantification (LOQ): is the limit at which we can reasonably tell the difference between two different values. In another word, LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy.
- Precision: the closeness of agreement between independent test results obtained by applying the experimental procedure under stipulated conditions. The smaller the random part of the experimental errors that affect the results, the more precise the procedure. A measure of precision (or imprecision) is the standard deviation.
- Accuracy: closeness of the agreement between the result of a measurement and a true value of the measured.

- Reproducibility: the closeness of agreement between independent results obtained with the same method on identical test material but under different conditions (different operators, different apparatus, different laboratories and/or after different intervals of time). The measure of reproducibility is the standard deviation qualified with the term 'reproducibility' as reproducibility standard deviation. In this study, it was studied to validate the results obtained with the same method on identical test material but different biosensors or biosensor arrays.
- Repeatability: the closeness of agreement between independent results obtained with the same method on identical test material, under the same conditions (same operator, same apparatus, same laboratory and after short intervals of time). The measure of repeatability is the standard deviation qualified with the term: `repeatability' as repeatability standard deviation.
- Selectivity: the extent to which other substances interfere with the determination of a substance according to a given procedure.

1.2 Biosensors and Biosensor Arrays in environmental application

1.2.1 Description

A biosensor is a device that conjuncts a biological recognition element (biocomponent) with an electronic or optical transducer, recording information converted from the biochemical signal (D'Souza 2001). A variety of biocomponents could be selected to reflect different responses to the analyte of interest, as the main types of the biocomponents could be concluded as following:

- Whole cells: The entire cells that contain desired enzymes or even the whole metabolic systems in a protected environment. Whole cells system could carry out very complex biochemical reactions and could be modified genetically to achieve specific characteristics. Whole cells systems require less purification and separation steps while

providing a better sustainability in poor conditions because of the self-confined structure (Bousse 1996; Campbell 1998; Karube and Nakanishi 1994).

- Enzymes: Purified enzymes provide great catalytic ability with the limitation of high purification, handling and storage costs. Purified enzymes are usually used in simple and well-known biochemical reactions, since complicated and multiple-step biochemical reactions usually require a variety of different co-factors as well as intermediate metabolites (Campbell 1998; D'Souza 2001).
- Antibodies: Antibodies recognize the analyte of interest through the process of binding and have been widely used in immunoassay-based biosensors. Antibodies serve well as a highly sensitive detecting element with the concern of irreversible binding which narrows its application in continuous measurement (Campbell 1998; Kim et al. 2001; Scouten et al. 1995).
- DNA nucleotides: The DNA nucleotides have been use as biocomponent in biosensors by DNA sequence hybridization. The nature of the detecting mechanism leads to the restrains of the application in a limited area, such as genetic sequences verification or screening (Campbell 1998; Rogers 1995).

The physical tranducers deliver and translate the biological signal into a measureable signal, usually an electronic or optical signal. The most common physical transducers are listed as below:

- Optical transducers: This type of transducer can be based on light absorbance, intensity change from different luminescent or fluorescent light, phase/lifetime change of luminescence or fluorescence, e.g., surface plasmon resonance (SPR) (Campbell 1998). Optical transdcuers are usually referred to as optodes, which could be small, cost-effective and low signal losses over long distance (Pieper et al. 2008; Wolfbeis 2002).
- Electrochemical transducers: The first enzymatic biosensor – Clark's glucose biosensor in 1962, is based on an oxygen electrode coupled with glucose oxidase entrapped by dialysis membrane (Clark and Lyons 1962), which is also the first application of

electrochemical transducers. The electrochemical transducers include two major categories with different measuring principles: potentiometric and amperometric, where the latter mechanism is more favored because of its greater sensitivity, better linear detection response and ease of application (Campbell 1998).

- Others: Optical-electronic transducers such as light-addressable potentiometric sensors (LAPS) represent typical cases of biosensors based on the conjunction of optical and electronic transducers, while quartz crystal microbalances (QCMs) and surface acoustic and transverse wave sensors are common examples of biosensors established on acoustic transducers (Campbell 1998).

It is difficult to use a single biosensor to measure a group of mixed compounds since the single output could not correspond to the multiple unknown concentrations of analytes. However, a bundle of biosensor array with different performance on each analytes respectively, could become a great alternative and improve the chance to determine the concentrations of each analytes in a mixture. In addition, a biosensor array constitutes a selection of different biosensors (usually with the same measuring principle) that integrate together as a whole system to provide information collected from each individual biosensor simultaneously and resolve complex measurement problems especially in mixture analytes measurements. In fact, the idea of grouping different detecting elements has already been widely used in analytical chemistry, especially in micro-scaled system (e.g., microarray, chip arrays) (Nakamura and Karube 2003).

1.2.2 Comparison with Other Measurement Techniques

Traditional analytical lab-based measurement technologies are still the only EPA-accepted techniques for precise characterization of environmental contamination sites (Campbell 1998). Field chromatography and spectrometry methods have been developed to provide qualitative or semi-quantitative measurements for the environmental industry with purpose of savings in monitoring time and expense. Immunoassay kits and biosensors are becoming more and more

popular in environmental monitoring since they could not only provide the quantitative measurement because of the high sensitivity of antibodies or biocomponents, but also execute the monitoring tasks in a timely and cost-effective fashion (Reardon et al. 2009).

Biosensors, in particular, represent the idea of real-time, *in situ* measurement with low cost. The biochemical reactions or bindings that recognize the analyte of interest are usually considered as real-time compared with the long-waiting period of traditional analytical methods. Biosensors are generally portable and easy to deploy in various different circumstances directly, which provides the first-hand information at the actual site. This feature is extremely useful in the measurement of targeted analyte distributions at different depth in aquifers, since it is really difficult to sample water from single monitoring well at different depth without any blending. The biocomponent of biosensors are usually made of whole-cells or enzymes, which could be produced in lab through cell culture with limited expense. The transducers such as optodes or electrodes are also relatively cheap and can be easily manufactured in large-scale. As a result, biosensors could become a promising alternative to traditional analytic measurement techniques, even though the biosensors do have some disadvantages and challenges such as relative short lifetime of biocomponent, restriction on storage method, activity loss due to various reasons.

1.2.3 Scope of problem

This project was aimed at developing an environmental monitoring platform based on biosensors and biosensor arrays for measurements of chlorinated ethenes in aqueous solutions. The platform was designed to be a real-time, *in situ* and cost-effective tool in characterizations of both single analyte and complex mixture. Chlorinated ethenes contamination in groundwater and drinking water system has brought more and more attention: about one-third of municipal water supplies in U.S. contain TCE while 75% of EPA National Priority List (NPL) hazardous waste sites and Suprefund sites have TCE pollution (Jollow et al. 2009). In addition to the widespread contamination, binary chlorinated ethene mixtures or more complex mixtures are commonly

found in chlorinated ethane contamination sites since incomplete dechlorination of PCE and TCE would generate their derivatives and form complicated chlorinated mixtures.

Biosensors and biosensor arrays have been proven to be successful measurement alternatives in many cases and could provide quantitative measurement with thoughtful development and appropriate selection and integration of biocomponent and transducers (Campbell et al. 2006; D'Souza 2001; Reardon et al. 2009). In addition, different detection mechanisms could result in diverse biosensor design concepts with various combinations of biocomponent and transducers, which is another important scope in this project.

Finally, developing and optimizing chemometric approaches to solve the biosensor arrays measurement of chlorinated mixture would also become challenging since the biorecongition reactions in biosensor measurement usually involve multiple kinetic parameters and nonlinear equations.

1.3 Research Questions

The overall objective of this research has been to develop fiber optic enzymatic biosensors for measurement of chlorinated ethenes by using oxygen optode and *E. coli* whole cells expressing selected monooxygenase and to gain a better understanding of biosensor array for mixture measurement with optimal approach in calibration and characterization. Specific research questions explored in the course of this dissertation include:

- Does the new design concept of fiber optic enzymatic oxygen biosensor meet the expectation of quantitative monitoring? What are the advantages and disadvantages of this oxygen biosensor system?
- Does the new oxygen biosensor perform well in chlorinated ethenes (e.g. TCE) measurement? Does chlorinated ethene epoxide affect the measurement? How can the toxicity effect be minimized or alleviated?

- How can the NADH depletion problem caused by biosensor storage or repeated biosensor measurement be solved? Are there any other alternative besides the primary approach?
- Does the biosensor array system work well with mixture measurement (ternary mixture)? How can the system be calibrated with optimal approach with minimum measurements? Are there any other alternatives to calibrate the biosensor array system?

In order to better understand the research questions, preliminary experiments such as oxygen optode characterization and oxygen biosensor concept design were addressed in Chapter 2, together with the detailed research approaches and overall dissertation layout.

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Chapter 2 Research Approaches and Dissertation Overview

2.1 Microbial Biosensor Fundamentals

Microorganisms have several advantages as a biocomponent in biosensor manufacture. They are found all around and are able to utilize a variety of chemical compounds as metabolites. Microbes have a strong ability to adapt to difficult living conditions and to evolve the ability to degrade new chemicals overtime. Microorganisms are also suitable for genetic modification via mutagenesis or recombinant DNA technology and provide an important supply of intracellular enzymes (D'Souza 2001).

Initial development of microbial biosensor in environmental monitoring was focused on assaying biochemical oxygen demand (BOD) as well as toxicity testing in wastewater (Rawson et al. 1989). A number of whole-cell biosensors were developed using different transducers (mostly electrochemical-based) with whole-cells as biocomponent (Corcoran and Rechnitz 1985; Dorward and Barisas 1984; Karube and Tamiya 1987). However, these biosensors usually provided qualitative measurement results and served often as one-time assay, which limited the application of these biosensors especially in real-time field monitoring.

Enzymes entrapped in whole-cells as biocomponent become more and more popular in microbial biosensors since the recent advances in genetic modification, which greatly increased the selection pools of enzymes through saturation mutagenesis or recombinant DNA technology. A variety of microbial biosensors were then developed for food, fermentation and allied fields, such as glucose oxidase biosensor (Lim et al. 2005; Lin et al. 2004; Wang et al. 1998), glucose dehydrogenase biosensor (Nakamura and Karube 2003; Wang 2001; Zhang et al. 2004), β-galactosidase biosensor (Liu et al. 1998; Scott et al. 1997; Svitel et al. 1998), acyl-CoA oxidase

biosensor (Schmidt et al. 1996; Ukeda et al. 1997), and tyrosine lyase biosensor (Huang and Yang 2005; Kharitonov et al. 2000; Schuck 1996). Electrochemical transducers such as oxygen electrode or carbon dioxide electrode were still the primary choice in these biosensor design concept since electrochemical transducers were cheap and easy to use in most cases.

Bioluminescence-based biosensors were another popular type of microbial biosensors and were believed as fast response, great sensitivity, large detection range and non-invasive continuous measurement with minimal manipulations required (D'Souza 2001). The bioluminescence reporter could be activated by either selective promoter gene which was controlled by temperature or other conditions (Prest et al. 1997; Webb et al. 1997; Werlen et al. 2004) or by a constitutive plasmid that was able to generate the bioluminescence reporter while growing (Li et al. 2008; Reardon et al. 2009b; Valdman et al. 2004; Willardson et al. 1998; Yoo et al. 2007). The bioluminescence biosensor was as a better alternative compared with traditional respirometry and showed greater sensitivity and reproducibility (Bousse 1996; D'Souza 2001).

2.2 Microbial Biosensors for Groundwater Monitoring

Groundwater is commonly used for irrigation, drinking water, municipal water supplies and other purpose, while it is also a vital part in the ecosystem. It is essential to monitor groundwater quality in an effective way for protection and remediation. Groundwater monitoring may cover a wild range of points of interests including biochemical oxygen demand, specific conductance, pH values, water table levels, inorganic chemical concentrations and organic chemical concentrations, etc, which usually are retrieved by quarterly or semiannually analysis of water sample collected from groundwater monitoring wells.

Although microbial biosensor is an emerging research area and a lot of studies have been addressed in the past decades (D'Souza 2001; Su et al. 2011), limited numbers of publications were found for microbial biosensor application in groundwater monitoring. Most biosensor applications in groundwater monitoring were focused on one type of contaminants while

regulators and environmental engineers usually have to monitor dozens of different parameters at a time. In addition, some groundwater monitoring sites only require being inspected quarterly or even semiannually, which make biosensor or other continuous detection principle less attractive.

Biosensor array, however, could provide alternative solution for multi-analytes mixture measurement. Although only a few studied have been reported in biosensor array measurement in groundwater (Anderson et al. 2006; Lewis et al. 2009; Sapsford et al. 2002), different types of biosensor array have been reported in mixture measurement in recent review articles, including electrochemical biosensor array (del Valle 2010; Lee et al. 2010; Polsky et al. 2008; Zeravik et al. 2009) and optical biosensor array (Deiss et al. 2010; Prieto-Simon and Campas 2009; Zhu et al. 2005), with different biorecognition elements including enzyme. In this study, a novel biosensor array with fiber optic enzymatic measurement principle and its application potential in groundwater monitoring has been discussed.

2.3 Fiber Optic Enzymatic Biosensors

All four possible chlorinated ethene metabolic pathways involve enzymes, as discussed in Chapter 1. Enzymes entrapped in whole-cells were selected as biocomponent over purified enzyme since the former choice was proved as a better alternative in various industrial and environmental monitoring processes (Bickerstaff 1997; D'Souza 1999). In addition, the chlorinated ethenes have similar structures based on the carbon-carbon double bond backbone and relative small molecule sizes compare with large biological molecules, which limit the possibility to use a binding-based biocomponent.

It is believed that chlorinated ethenes could be degraded under aerobic conditions with natural attenuation (Groten et al. 2001; McCarty 1997). In fact, trichloroethylene (TCE), one of the most common chlorined ethenes, is biodegradable to several microorganisms (Arciero et al. 1989; Little et al. 1988; Nelson et al. 1987; Nelson et al. 1986; Oldenhuis et al. 1991; Winter et al. 1989). Some recent studies suggested that oxygen and living cells were often required during

TCE mineralization (Shim et al. 2001; Sun and Wood 1996; Uchiyama et al. 1992), which allowing the possibility of detecting TCE based on oxygen consumption, shown as the following equation (Equation 2.1):

$$C_2Cl_3H + NADH + H^+ + 2O_2 \rightarrow 2CO_2 + NAD^+ + 3HCl. \qquad \textbf{Equation 2.1}$$

In this research, toluene ortho-monooxygenase (TOM) is selected as one of the enzymes of interest. TOM is from a wild-type bacterium named *Burkholderia cepacia G4*, which was recognized as a promising TCE-degrading bacterium (Canada et al. 2002; Shields et al. 1989; Shields et al. 1995). In addition, TOM-Green, a TOM mutation via saturation mutagenesis is also selected due to its activity on TCE (Canada et al. 2002; Rui et al. 2004a). Both of these two enzymes are expressed by *Escherichia coli* with the selected plasmids. TOM and TOM-Green cells were also modified with plasmid transformation to add a secondary plasmid in Chapter 4 and 5 to perform different research objectives such as toxicity mitigation and NADH regeneration.

Optical oxygen sensors (oxygen optodes) were selected as the transducer in this research, providing less signal loss and interference by the environment conditions compared with traditional electrochemical transducers or other transducers, optical transducers (Collingridge et al. 1997; Kohls and Scheper 2000; Monk and Walt 2004; Ramamoorthy et al. 2003; Wolfbeis 2002). The oxygen optodes were generally designed as shown in Figure 2.1, created from 25 cm sections of PMMA optical fiber terminated with an ST connector.

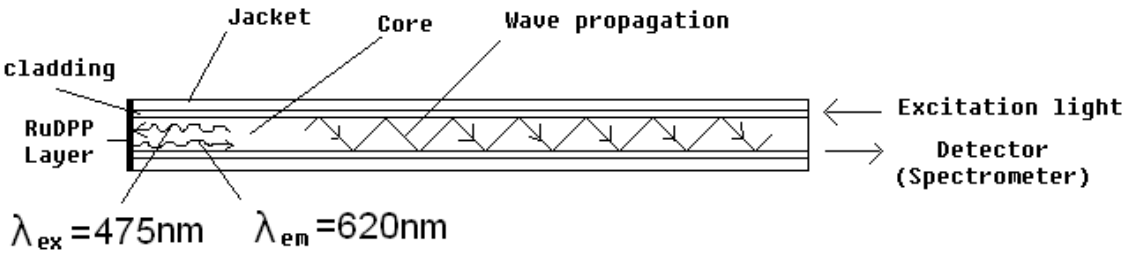

Figure 2.1 - oxygen optode schematic

The selected dye, Tris (4, 7-diphenyl-1, 10-phenanthroline) ruthenium (II) (RuDPP) (Figure 2.2) is oxygen-sensitive fluorophore, which would exhibit strong orange phosphorescence

(λ_{em}=620nm) under the excitation blue light (λ_{ex}=475nm). Moreover, this phosphorescence could be quenched with the presence of oxygen in solution (Klimant and Wolfbeis 1995; Lee et al. 2001; Pieper et al. 2008; Wolfbeis 2000). This process could be described by the dynamic Stern-Volmer relationship, where the occurring decreased oxygen concentration increased the phosphorescence intensity or decay lifetime of the ruthenium complex, which could further make intensity or decay lifetime dependent on the concentration of the analyte (Carraway et al. 1991; Pieper et al. 2008).

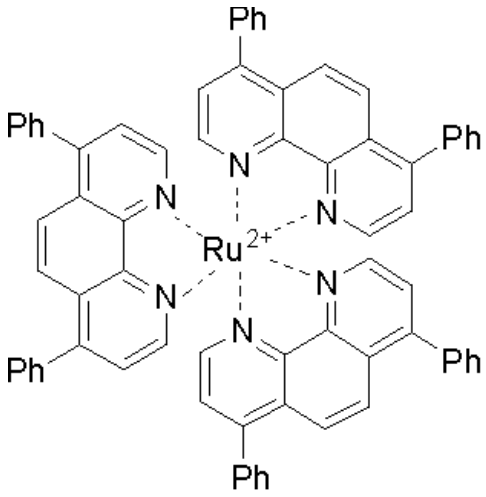

Figure 2.2 - structure of Tris (4, 7-diphenyl-1, 10-phenanthroline) ruthenium (II)

In the general form of the Stern-Volmer relationship (Equation 2.2), [Q] is the quencher concentration, *I*'s are intensities, K_{sv} is the Stern-Volmer constant, K_{eq} is the association constant for binding of the quencher to the luminescent species, and the subscript 0 denotes the value in the absence of quencher (Carraway et al. 1991).

$$I_0/I = 1 + (K_{SV} + K_{eq})[Q] + K_{eq} K_{SV}[Q]^2 \qquad \textbf{Equation 2.2}$$

Therefore, I_0/I versus quencher concentration was linear if the association constant was zero or much smaller than the Stern-Volmer constant. A number of oxygen optodes were tested with a variety range of different oxygen concentrations and the calibration curve (Figure 2.3) was collected with oxygen concentration versus the signal change as detected with an optoelectronic detector. The calibration curve indicated a linear relationship between the oxygen concentration and the signal change ΔV (signal change).

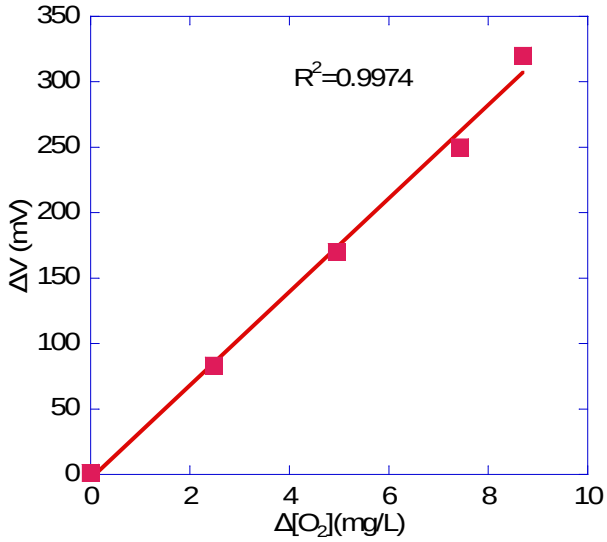

Figure 2.3 - oxygen optode calibration curve (forced to across origin)

In addition to the calibration experiments, a study of phosphorescence dye layer thickness was also performed to find the optimal amount of RuDPP dye immobilization for oxygen optodes, the RuDPP layer thickness measurement method were measured by Mr. Sean Pieper and discussed further in his publication (Pieper et al. 2008).

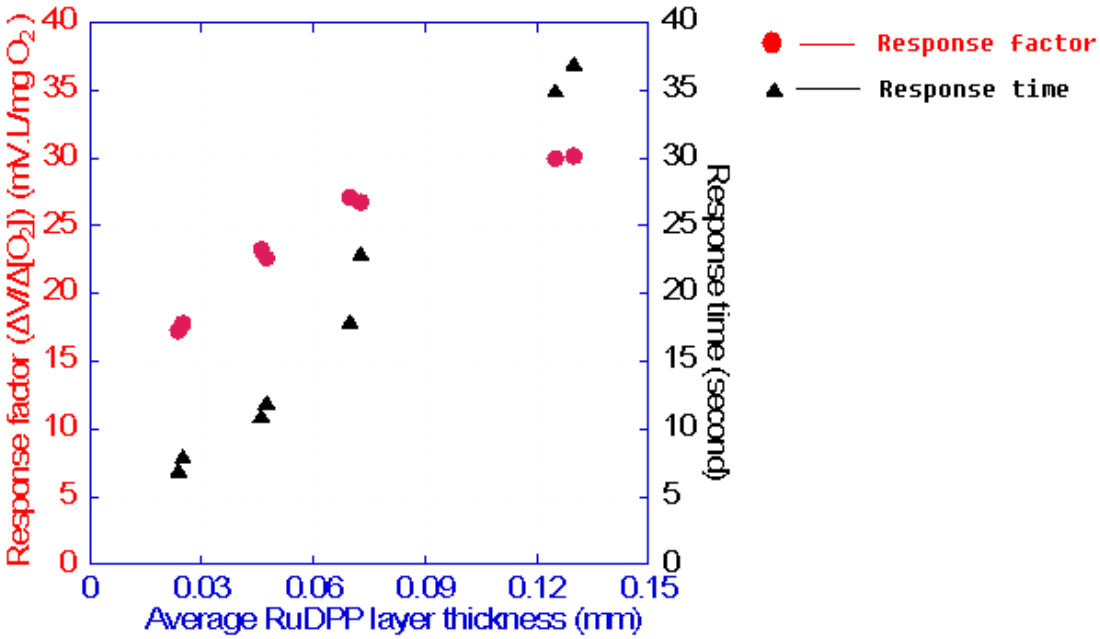

Figure 2.4 - effect of RuDPP layer thickness for an oxygen optode (Pieper et al. 2008)

A group of oxygen optodes with different amounts of dye mixture immobilized on the tips (1 μL, 2 μL, and 3 μL) were tested with the same oxygen concentration change (Figure 2.4). The results showed that both the response time and sensitivity of the oxygen optode increased monotonically with the dye layer thickness, which was also observed in a similar fluorescent dye system before (Zhujun et al. 1989).

Several pH-based biosensors were also made to build the biosensor array system. These pH-based biosensors carried with the similar design concept except with different enzyme and fluorophore in biocomponent and transducer part respectively (Campbell 1998; Campbell et al. 2006; Reardon et al. 2009a).

2.4 Research Objectives

The research objectives of this study have been focused on developing fiber optic oxygen-based enzymatic biosensor system for measurement of chlorinated ethenes, sequential improvement and modification, and further development of biosensor array system for measurement of mixture analysis and characterization. Specific research objectives discussed in this dissertation include:

- Development and characterization of fiber optic oxygen-based enzymatic biosensor for measurement of toluene. Provide a proof-of-concept for the biosensor design and comparison with other microbial biosensor system.
- Development of a fiber optic oxygen-based enzymatic biosensor for measurement of trichloroethylene. Identify the critical concern of TCE-epoxide and provide solutions or alternatives for the problems.
- Modification and improvement of the fiber optic oxygen-based enzymatic biosensor system to address the NADH-depletion problem and evaluate the alternative approach by plasmid transformation of a secondary NADH-regeneration expressing gene in whole cells.

- Development of fiber optic enzymatic biosensor array system for measurement of a 3x3 system (three biosensors with different enzymes for measurement of three analyte of interests). Study and design the optimal approach for characterization of the biosensor array system.

2.5 Dissertation Overview

The initial study (Chapter 3) focused on the proof-of-concept experiments of the biosensor design in this project. Toluene ortho-monooxygenase (TOM) and toluene were selected as enzyme and analyte of interest combination in the study since a number of previous studies showed positive evidence that whole-cells expressing TOM enzyme could partially catalyze toluene with presence of oxygen and NADH (Canada et al. 2002; Rui et al. 2004a; Shields et al. 1995). The experiments were successful and further characterization suggested that these biosensors had better performance in calibration and limits of detection (LOD) compared with other microbial biosensors for toluene measurements. However, the NADH-depletion problem limited the biosensor storage time and prevented multiple measurements with the same biosensor over time.

The further study (Chapter 4) focused on the TCE measurement with the current biosensor design. TOM-Green, a mutation of the TOM enzyme, was selected to match for the measurement of TCE since it has the capability to biodegrade TCE with the presence of oxygen and NADH (Canada et al. 2002; Rui et al. 2004b). The results were encouraging and further measurements provided a variety of characterization parameters for these biosensors. The TCE epoxide toxicity was addressed but the NADH-depletion problem remained unsolved. Two different plasmids were introduced in the TOM cells separately with different approach to mitigate the TCE epoxide toxicity (Rui et al. 2004b), and sequential experiments suggested that both approaches improved the performance of biosensors, while comparison between two approaches were also addressed.

NADH-depletion problem was discussed in Chapter 5, with a primary solution provided via plasmid transformation. The proof-of-concept experiments were performed with TOM enzyme plus formate dehydrogenase (FDH) for measurement of toluene and the results showed successful regeneration of biosensor signals after repeated measurements or over time in storage. The TOM+FDH system was then discussed in the activity retention experiments compared with the TOM enzyme only system. Other alternative choices for NADH regeneration were also discussed such as PTDH system. The success of biosensor regeneration showed great potential of this biosensor system and increased its capacity of applications in environmental monitoring and other related areas.

A ternary biosensor array system was developed as the proof-of-concept of multiple analytes measurement (Chapter 6). Both biosensor array system design and optoelectronic hardware were introduced in this chapter, while measurements of a three-analyte mixture with three different enzymes were studied. Linear approaches were discussed with the assumption of ideal enzymatic reaction and competitive inhibitions. Optimal approaches for biosensor array calibration with minimal measurements required were addressed while further alternatives and approaches were also discussed. The proof-of-concept experiments provided a strong evidence of competitive inhibition under the circumstance of mixture measurement when these mixtures had similar chemical structure and properties. Additional characterization of the biosensor array system were also based the competitive inhibitions approach.

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Chapter 3 Fiber optic monooxygenase biosensor for toluene concentration measurement in aqueous samples

Zhong Z, M, Fritzsche, S. B. Pieper, T. K. Wood, K. L. Lear, D. S. Dandy, K. F. Reardon. (2010). *Biosensors and Bioelectronics* 26(5): 2407-2412

3.1 Abstract

Measurements of pollutants such as toluene are critical for the characterization of contaminated sites and for the monitoring of remediation processes and wastewater treatment effluents. Fiber optic enzymatic biosensors have the potential to provide cost-effective, real time, continuous, in situ measurements. In this study, a fiber optic enzymatic biosensor was constructed and characterized for the measurement of toluene concentrations in aqueous solutions. The biological recognition element was toluene orthomonooxygenase (TOM), expressed by Escherichia coli TG1 carrying pBS(Kan)TOM, while an optical fiber coated with an oxygen-sensitive ruthenium-based phosphorescent dye served as the transducer. Toluene was detected based on the enzymatic reaction catalyzed by TOM, which resulted in the consumption of oxygen and changes in the phosphorescence intensity. The biosensor was found to have a limit of detection of 3 μM, a linear signal range up to 100 μM, and a response time of 1 h. The performance was reproducible with different biosensors (RSD = 7.4%, n = 8). The biosensor activity declined with each measurement and with storage time, particularly at elevated temperatures. This activity loss could be partially reversed by exposure to formate, suggesting that NADH consumption was the primary factor limiting lifetime. This is the first report of an enzymatic toluene sensor and of an oxygenase-based biosensor. Since many oxygenases have

been reported, the design concept of this oxygenase-based biosensor has the potential to broaden biosensor applications in environmental monitoring.

3.2 Introduction

The large-scale consumption of gasoline, diesel, and other petroleum-derived fuels has led to soil and groundwater contamination by spills and leakage from fuel tanks and pipelines. Due to its moderate solubility in water, toluene is one of the fuel hydrocarbons of particular concern. Toluene causes kidney and liver toxicity and damage to the central nervous system (Hartley and Englande 1992). Developing a sensitive, reliable, cost-effective, and *in situ* method for toluene detection is thus of great importance for monitoring aquifers, surface waters, and water treatment systems.

Analytical methods for toluene measurement based on gas chromatography (GC) are well established. US EPA methods have excellent limits of detection (LOD): 0.002 µM with EPA method 602 for purgeable aromatics, 0.06 µM with EPA method 624 for purgeable organics, and 0.001 µM with EPA method 8260b for volatile organic compounds. However, these laboratory-based methods are time-consuming, expensive, and dependent on the quality of sample collection and storage.

Biosensors are measurement devices that combine a biological recognition element (biocomponent) with a transducer that is typically optical or electronic (D'Souza 2001; Reardon et al. 2009). Enzymes are often chosen as the biocomponents since they result in biosensors with high sensitivity and good specificity (D'Souza 2001; Ivnitski et al. 1999; Mulchandani et al. 1998; Rainina et al. 1996). Optical transduction, especially with optical fibers, has potential advantages for environmental monitoring since no reference signal is required and signal losses over long distances can be low (Campbell et al. 2006; Ivask et al. 2007; Monk and Walt 2004; Wolfbeis 2002). Many biosensors are reagentless and can thus provide continuous, *in situ* measurements.

The goal of this study was to develop a fiber optic biosensor based on toluene *ortho*-monooxygenase (TOM) from *Burkholderia cepacia* G4, which initiates toluene catabolism by *ortho*-hydroxylation (Shields et al. 1995). Toluene measurements with this biosensor relied on the detection of oxygen consumption by TOM during the hydroxylation reaction, which requires both oxygen and NADH (Shields et al. 1989). Whole cells containing TOM were immobilized in an alginate gel on a fiber optic oxygen sensor (oxygen optode). The oxygen optode was based on a phosphorescent indicator chemical that exhibits reduced light emission by molecular oxygen via dynamic quenching. In the presence of toluene, the enzymatic reaction caused a decrease in oxygen concentration within the alginate layer, detected as an increase in phosphorescence.

This monooxygenase-based biosensor is different than previously reported oxidase-based biosensors such as those for glucose (Gouda et al. 2002; Lim et al. 2005; Lin et al. 2004; Liu et al. 2005; Svitel et al. 1998); ethanol (Mitsubayashi et al. 1994); and *L*-amino acid (Endo et al. 2008; Setford et al. 2002), since one oxygen atom is transferred into the substrate (toluene) with monooxygenases while oxygen is the electron acceptor in

$$C_7H_8 + O_2 + NADH + H^+ \rightarrow C_7H_7OH + NAD^+ + H_2O$$

oxidase-catalyzed reactions (Ellis et al. 2006). Additionally, there are only a few oxidases available for biosensor applications (Azevedo et al. 2005; Komathi et al. 2009), while the oxygenase family is diverse and may be used to construct biosensors for a wide range of analytes (Park 2007; van Beilen and Funhoff 2005).

3.3 Materials and methods

3.3.1 Chemicals

Toluene (99%. v/v), alginic acid (low viscosity, sodium salt) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. Tris (4, 7-diphenyl-1, 10-phenanthroline)-ruthenium (II) complex (RuDPP) was synthesized at the University of Hannover

(Kohls 1995). Toluene standard solution (2 mM in methanol) was purchased from Sigma-Aldrich and diluted sequentially to prepare calibration standards.

3.3.2 Bacterial strain and growth conditions

The biocomponent of the biosensors, toluene *ortho*-monooxygenase, was expressed in *Escherichia coli* strain TG1 harboring the plasmid pBS(Kan)TOM with the six *tom* genes from *B. cepacia* G4 (Canada et al. 2002). *E. coli* cultures were maintained aerobically on agar plates made from Luria-Bertani (LB) medium with 20 g/L Bacto-agar (Difco) and 100 mg/L kanamycin at 30 °C for 48 h. A culture tube containing 2 mL LB medium supplemented with 100 mg/L kanamycin was inoculated from a single colony on an agar plate and shaken overnight at 30 °C and 200 rpm. The culture was then transferred to a flask containing 200 mL of the same LB-Kan medium and shaken at 30 °C and 200 rpm. Cell concentration was measured as culture absorbance at 600 nm (optical density at 600 nm, OD600) with a spectrophotometer (Spectronic® 20 Genesys™, Thermo Electron Corporation). IPTG solution was prepared with deionized water and added to the culture with a final concentration of 1 mM to induce TOM biosynthesis in the early exponential growth phase (OD600 of 0.6). The culture was harvested 4 h after IPTG was added, centrifuged, and resuspended in 20 mL of a solution containing 10 mM phosphate buffered saline at pH 7.4 and stored at 4 °C until further use.

3.3.3 Biosensor tip construction

Each biosensor tip consisted of a layer of immobilized TOM cells over an optical oxygen sensor (oxygen optode). Each oxygen optode was created from a 25-cm section of polymethylmethacrylate (PMMA) optical fiber terminated with a straight tip (ST) connector. The fiber jacket was removed from 1 mm of the distal end (non-connector terminated), and then the fiber was polished with 2000-grit and 3 μm polishing film (IF-TK4-RP2, Industrial Fiber Optics)

to reduce potential light loss due to scattering. One mg of the oxygen-sensitive phosphorophore RuDPP was dissolved into 1 mL chloroform and mixed with 200 mg silicone gel (clear RTV silicone, Permatex, Inc.). A 1-µL aliquot of this mixture was then added to the polished fiber tip. The RuDPP gel layer was affixed to the optical fiber end once the chloroform evaporated.

We note that RuDPP is referred to variously as fluorescent, phosphorescent, or luminescent in the literature. Here, we use the criteria from Lakowicz, who classifies metal-ligand complexes such as RuDPP as phosphorophores because of the nearly forbidden transitions that allow the molecule to maintain the excited state for much longer times before relaxing via photon emission (decay lifetime longer than 10 ns) (Lakowicz 2006).

Previously stored *E. coli* TG1 pBS(Kan)TOM whole cells were centrifuged and mixed with sodium alginate solution (2.5%) in a cell-to-alginate ratio (wet cell mass : alginate solution) of 1:1 (w/w) unless otherwise specified. Biosensors were constructed by placing 2 µL of the cell-alginate mixture on the tip of an oxygen optode and then immersing the optode in 0.47 M calcium chloride solution for 30 min at 0 °C. All biosensors were stored at 0 °C in a solution of 0.15 M NaCl and 0.025 M $CaCl_2$ at pH 7.0 (hereafter referred to as "measurement solution").

3.3.4 Biosensor instrumentation

The biosensor instrumentation included two separate optoelectronic modules: an excitation light source containing a 470-nm LED and a 450/60 nm optical bandpass filter (Chroma Technologies), and a detection system consisting of a computer-controlled Ocean Optics USB4000-FL spectrometer with 10 nm resolution. The 470-nm excitation light was transferred through one leg of a bifurcated optical fiber assembly that has two 1-mm fibers side-by-side in the common end (Ocean Optics, Inc.), which was connected with the biosensor via an ST connector. The phosphorescent emission light (peak at 620 nm) from the biosensor was directed back into the detector through the other leg of the bifurcated optical fiber and measured by the spectrometer (sensitivity of approximately 60 photons/count at 600 nm). The spectrometer output

from 615 nm to 625 nm was integrated over 200 ms and five such values were averaged to yield one measurement value per second. The change in the intensity of the emission light over time correlates to the oxygen concentration change in the RuDPP layer of the biosensor.

3.3.5 Biosensor measurement protocols

All biosensor experiments were performed in glass vials (5 mL) containing 4 mL of measurement solution saturated with air at room temperature. A small magnetic stir bar was used to agitate the solution thoroughly. The biosensor tip was immersed in this solution, sealed in the glass vial with a rubber septum, and shielded from external light sources. Aliquots (0.1 mL) of a toluene solution (0.11 – 4.7 mM) were added to the measurement solution after the sensor had produced a steady output, defined as the time when the variation in the output was no larger than the peak-to-peak noise for a period of at least 5 min. All measurements were performed at room temperature unless otherwise specified. Each measurement was performed with a fresh biosensor to distinguish the effect in question (e.g., temperature, pH, cell/alginate mass ratio). Biosensors were not reused unless otherwise specified.

3.3.6 Toluene concentration measurement by gas chromatography

To assess the accuracy of the toluene concentration data obtained from the biosensors, GC analysis was performed via a modification of EPA Method 8260b. After a biosensor measurement, 0.75 mL of aqueous solution was collected from the measurement vial and transferred into a 2-mL glass screw-top GC vial containing 0.75 mL of chloroform. The GC vial was then capped with a Telfon-coated septum and mixed on a rotating wheel for 15 min. One microliter of the chloroform phase was injected into a Hewlett Packard 5890 gas chromatograph equipped with a HP model 5971A mass spectrometric (MS) detector. A calibration curve of the GC-MS total ion count peak area vs. the toluene concentration in solution was obtained using dilutions of the 2

mM toluene standard solution. The GC calibration curve was linear over the range of toluene concentrations from 1 to 500 µM (R^2 = 0.998).

3.4 Results and discussion

3.4.1 Demonstration of the measurement principle of the oxygenase-based biosensor

The initial experiments with the toluene biosensor were performed as proof-of-concept for fiber optic biosensors based on oxygenase-catalyzed reactions. A 0.1 mL aliquot of 4 mM aqueous toluene solution was injected into 4.0 mL of measurement solution in which the biosensor was immersed. The proposed detection principle begins with catalysis of the reaction with toluene and oxygen by the intracellular TOM enzyme on the biosensor tip, resulting in consumption of oxygen in the solution as well as NADH inside the cells (Shields et al. 1995). The decrease of oxygen within the alginate layer would then cause an increase in the phosphorescence intensity of the immobilized RuDPP (owing to reduced quenching by oxygen). The measured phosphorescence intensity at a single condition (e.g., no analyte, 5 mg/L dissolved oxygen) is termed the biosensor reading, and the difference between the readings before and after addition of toluene is referred to as the signal.

The signal of a biosensor with whole cells of *E. coli* TG1 pBS(Kan)TOM resulting from an increase in toluene concentration from 0 to 92 µM was 1000 counts with a response time of 1 h (Figure 3.1). At the point at which the biosensor reading reached a steady value (variation less than or equal to the system noise), the remaining toluene concentration in the vial was found to be 90±2 µM using GC-MS. This indicates that toluene detection inside the biosensor system relies on achievement of a steady-state balance between diffusion and reaction of toluene and oxygen in the biosensor tip region rather than the depletion of toluene in the sample. A given toluene concentration results in the establishment of corresponding rates of enzymatic reaction, toluene diffusion rate, and oxygen diffusion rate, and thus determines a steady-state oxygen concentration on the biosensor tip.

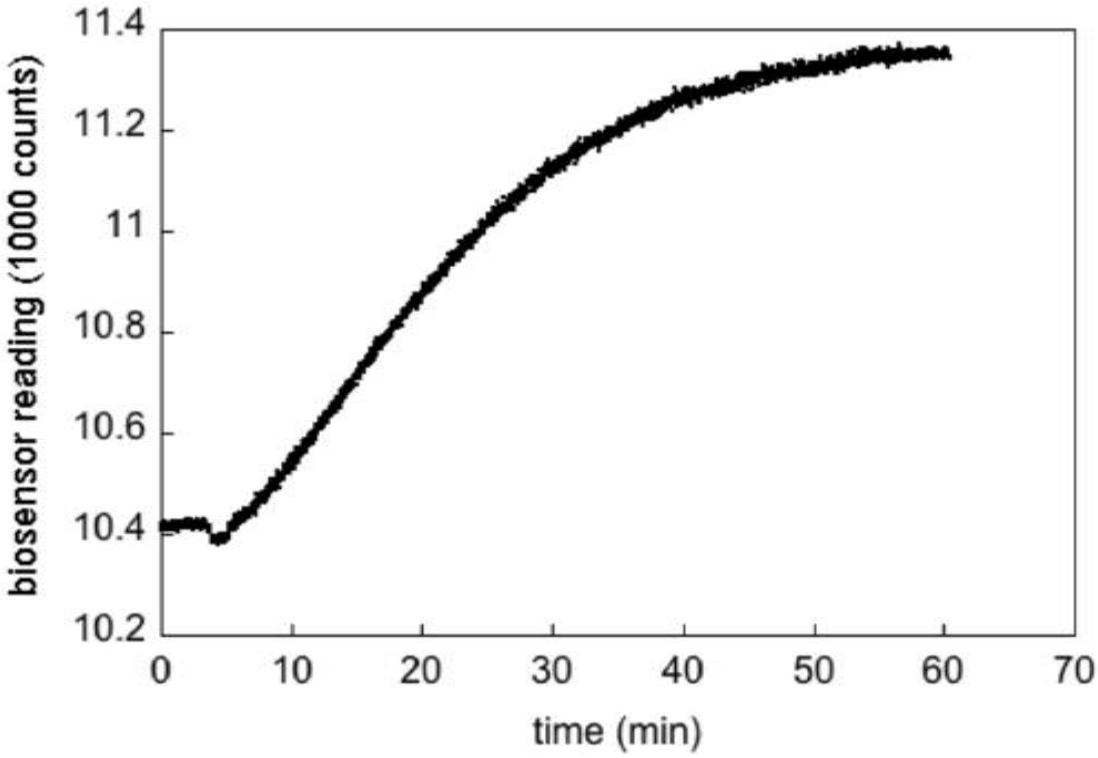

Figure 3.1 - Time course of a TOM biosensor response to the addition of 92 μM toluene.

Two sets of control experiments were performed to further test the proposed biosensing principle (Table 3.1). In the first, biosensors constructed with E. coli TG1 cells containing a "blank" plasmid – one without the gene encoding the TOM enzyme – were used to measure toluene concentrations from 3 to 93 μM. As expected, the signals from these control biosensors were not significant. The purpose of the second control experiment was to establish whether or not the biosensors would respond non-specifically to organic chemicals that might be present in natural waters. As shown in Table 1, no significant response to 1mMacetate was detected with biosensors constructed with E. coli containing the blank plasmid, and signals from biosensors constructed with E. coli cells expressing TOM were unaffected by the presence of 1mM acetate.

Sample	Toluene concentration (μM)	Acetate concentration (μM)	TG1/pBS(Kan)-TOM biosensor signal (counts)	TG1/pBS(Kan) ("blank") biosensor signal (counts)
High	**92.8**	**0**	**1056 ± 57**	**7 ± 12**
		1.0	**1040 ± 114**	**6 ± 7**
Medium	**13.3**	**0**	**217 ± 15**	**0 ± 10**
		1.0	**213 ± 21**	**3 ± 15**
Low	**2.7**	**0**	**53 ± 12**	**3 ± 6**
		1.0	**37 ± 15**	**−3 ± 6**

Table 3.1 - Results of control experiments comparing biosensors constructed with cells with the TOM enzyme vs. those without, as well as control experiments to evaluate the impact of background organic chemicals (acetate) on the biosensor response.

3.4.2 Analytical characteristics

3.4.2.1 Reproducibility

Biosensors within a group that were made at the same time under identical conditions were tested with 92 μM toluene solutions in order to quantify reproducibility. The consistency of the biosensor signal within this group was good (RSD=7.4% for n=8) and was comparable to the reproducibility reported for two induction-based toluene biosensors, RSD=10.7% for n=3 (Willardson et al. 1998) and RSD=9.5% for n=3 (Stiner and Halverson 2002). Batch-to-batch variation was also tested by comparing the signals from five sets of three biosensors. Each set of biosensors was made from a different culture of E. coli TG1 pBS(Kan) TOM cells. The RSD for this set of 15 biosensors, tested with 92 μM toluene, was 6.0%.

3.4.2.2 Effect of cell concentration

E. coli TG1 pBS(Kan) TOM cells were immobilized at different concentrations in calcium alginate to evaluate the effect of enzyme concentration on biosensor performance. Biosensors were made using three different cell-to-alginate (w/w) ratios (3:1, 2:1, and 1:1), each in triplicate, for each set. When these biosensors were tested with 92 μM toluene, no significant differences in the signal were observed ($p < 0.001$). This result suggests that the oxygen concentration gradient

from the RuDPP layer to the bulk solution is not dependent on cell concentration in the range studied, and indicates that mass transfer limitations may dominate the biosensor signal.

Similarly, the biosensor response time was unaffected by the cell concentration on the tip. A typical measurement with the TOM-based biosensor requires 1 h, which is faster than the 2 to 4 h required by induction-based biosensors, (Stiner and Halverson 2002; Willardson et al. 1998). In the conceptual model of the TOM-based biosensor, the time required for a full response corresponds to the transition from the pre-test steady state oxygen level to a new steady state. Since the biosensor response time was not a function of the immobilized cell (TOM) concentration, it is likely that one or more mass transfer processes are the primary determinants of the response time. A mathematical modeling study is underway to further investigate this issue.

3.4.2.3 Calibration curve and limit of detection

A series of toluene solutions were analyzed with TOM-based biosensors. Each biosensor was used only once, and each concentration point was measured in triplicate. The biosensor signal was linear over the range from 3 to 100 µM toluene with R^2=0.996 (Figure 3.2). The limit of detection (LOD), calculated as three times the standard deviation of the noise obtained from control experiments, was equal to 3 µM, less than the EPA Maximum Contaminant Level Goal for toluene (11 µM) in National Primary Drinking Water Regulations. Although the LOD of the TOM biosensor for toluene is higher than the 0.02 µM reported for an immunoassay-based biosensor (Eremin et al. 2005) or the 0.001 µM obtained with EPA method 8260a (GC/MS), it is comparable to the LOD of some induction-based biosensors (e.g., 11 µM by (Willardson et al. 1998) and 7.5 µM by (Li et al. 2008)), while providing a much broader linear detection range compared to the induction-based biosensors, e.g., 11 to 22 µM (Willardson et al. 1998). Furthermore, the current LOD of the TOM-based biosensor could be improved by increasing sensitivity of the optoelectronic instrumentation, or by replacing the TOM enzyme with another

oxygenase that has higher activity at low toluene concentrations or by increasing the reproducibility of the measurements.

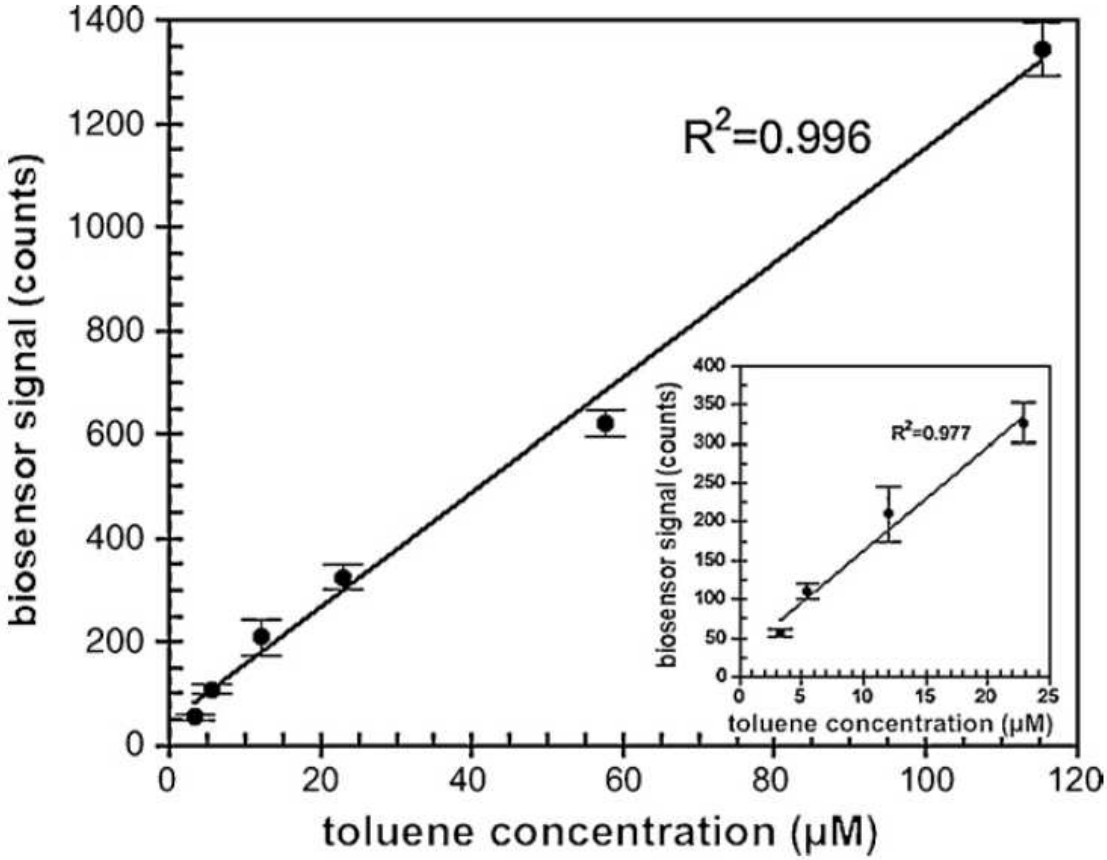

Figure 3.2 - TOM biosensor signal as a function of toluene concentration. Inset: biosensor signals in the low range of toluene concentrations (0–25 μM). Error bars represent ± 1 standard deviation. Relative standard deviations varied (e.g., 10% at 3.3 μM toluene and 4% at 115 μM toluene).

3.4.2.4 Accuracy

Toluene was spiked into water samples from two local lakes (Horsetooth Reservoir and City Park Lake, Fort Collins, CO) to assess the biosensor performance in real environmental matrices. In each case, three different toluene concentrations were used, spanning most of the linear

working range of the biosensor. The comparison between the concentrations of toluene determined by the TOM biosensor and the GC/MS method is reported in Table 3.2. The differences between GC/MS measurement values and biosensor measurement values were 0.2± 0.5 µM (95% CI, n=18), indicating that the TOM biosensor is accurate and reliable for toluene measurement in these aqueous matrices.

Sample	Toluene concentration (µM)	
	TOM biosensor	GS-MS
Spiked in Horsetooth Reservoir water		
High	**92.3±4.5**	**92.9±2.7**
Medium	**13.8±1.2**	**13.5±1.1**
Low	**2.6±0.4**	**2.4±0.3**
Spiked in City Park Lake water		
High	**89.9±5.4**	**88.4±5.5**
Medium	**13.0±1.4**	**12.3±1.0**
Low	**1.2±0.8**	**2.4±0.2**

Table 3.2 - Comparison of toluene measurements in spiked water samples. Three biosensors were used for each measurement.

3.4.2.5 Selectivity

TOM has been reported to catalyze the hydroxylation of several chlorinated and aromatic chemicals in addition to toluene (Canada et al. 2002). Hence, toluene, benzene, and trichloroethene (TCE) were chosen to evaluate the selectivity of the TOM-based biosensor. All of these analytes were measured at a concentration of 11 µM. The biosensor signal was largest for toluene (210±30 counts), followed by TCE (110±20 counts), and then benzene (40±20 counts). This trend is consistent with data from a previous study (Canada et al. 2002), in which the pseudo first-order rate constant for toluene oxidation by TOM was found to be higher than the rate constant for TCE oxidation. The response of the TOM-based biosensor to analytes other than

toluene is not due to the use of whole cells because E. coli does not transform toluene, benzene, or TCE (Table 3.1 for toluene control) but rather to the inherent substrate range of TOM.

One potential problem for the TOM biosensor, and for induction-based biosensors (Stiner and Halverson 2002; Willardson et al. 1998), is that the selectivity of a single biosensor is usually limited when detecting a group of analytes with similar chemical structures. A general strategy to overcome this selectivity issues is to use an array consisting of a group of biosensors, each with a different biocomponent, to detect a mixed group of analytes (Tsai and Doong 2005; Wadkins et al. 1998).

3.4.3 Effects of temperature and pH on biosensor signal

pH and temperature are two important factors in environmental monitoring. These affect not only the TOM component of the biosensor reported here — enzymes have optimal pH and temperature values — but also the mass transfer rates of toluene and oxygen. The phosphorescence properties of RuDPP are also temperature dependent. To evaluate the effect of pH on the TOM-based biosensor signal, sets of three biosensors were tested in measurement solutions buffered at pH 5.0, 6.0, or 7.0, spanning a common pH range in typical ground and surface waters. The signals corresponding to 92 μM toluene at different pH values were 1010 ± 160 counts (pH=5), 1020 ± 110 counts (pH=6) and 1020 ± 100 (pH=7), indicating that the measurements of the TOM-based biosensor were independent of pH in this range.

Similarly, the signals of a set of three biosensors to 92 μM toluene at three temperatures were investigated. Relative to the biosensor signal at 22 °C, the signal was 30% higher at 30 °C and 50% lower at 15 °C. The degrees to which the enzymatic reaction rate, mass transfer rates, and RuDPP phosphorescence contribute to this temperature-dependent behavior are not known and are perhaps best explored in a mathematical simulation.

3.4.4 Activity retention and regeneration

The retention of activity with use or storage is an important characteristic for any biosensor. This is a particular concern for this oxygenase-based biosensor because of the consumption of NADH in the detection reaction. During growth of an oxygenase-expressing cell, NADH is regenerated through catabolism; however, biosensors are normally stored in the absence of an energy source and thus NADH levels would be expected to decline with time (through maintenance metabolism) and use (through the oxygenase reaction) of a biosensor. Furthermore, all biosensors are subject to the denaturation of their biocomponent.

To further investigate these issues, 24 biosensors were stored in measurement solution without toluene at 4 °C or 20 °C (twelve at each temperature). At intervals, biosensors were removed from the storage solution and used to measure 92 µM toluene. For both storage temperatures, the biosensor performance decreased over time, and eventually no significant signal was obtained. Biosensors stored at 4 °C retained activity for a longer period than those stored at 20 °C (Figure 3.3). The loss of biosensor activity in this experiment may have been caused by either enzyme denaturation or depletion of NADH via maintenance metabolism when the biosensors are stored. The reuse of biosensors for multiple measurements of 92 µM toluene was also investigated. The signal was found to decrease by approximately 40% each time a biosensor was used, indicating the significance of NADH consumption in the TOM reaction on subsequent measurements.

Since supplying NADH externally in each measurement is expensive, inconvenient, and not well suited for *in-situ* measurements, NADH regeneration within the immobilized cells is desirable. One means of regenerating NADH in *E. coli* without the large oxygen consumption that would accompany glucose feeding relies on NAD^+ reduction via the reaction of formate catalyzed by intracellular formate dehydrogenase (Berrios-Rivera et al. 2002; Slusarczyk et al. 2000). To test this approach, regeneration experiments were conducted by storing biosensors at 4 °C for two weeks in measurement solution, and then supplemented with 1 M formate for 24 h.

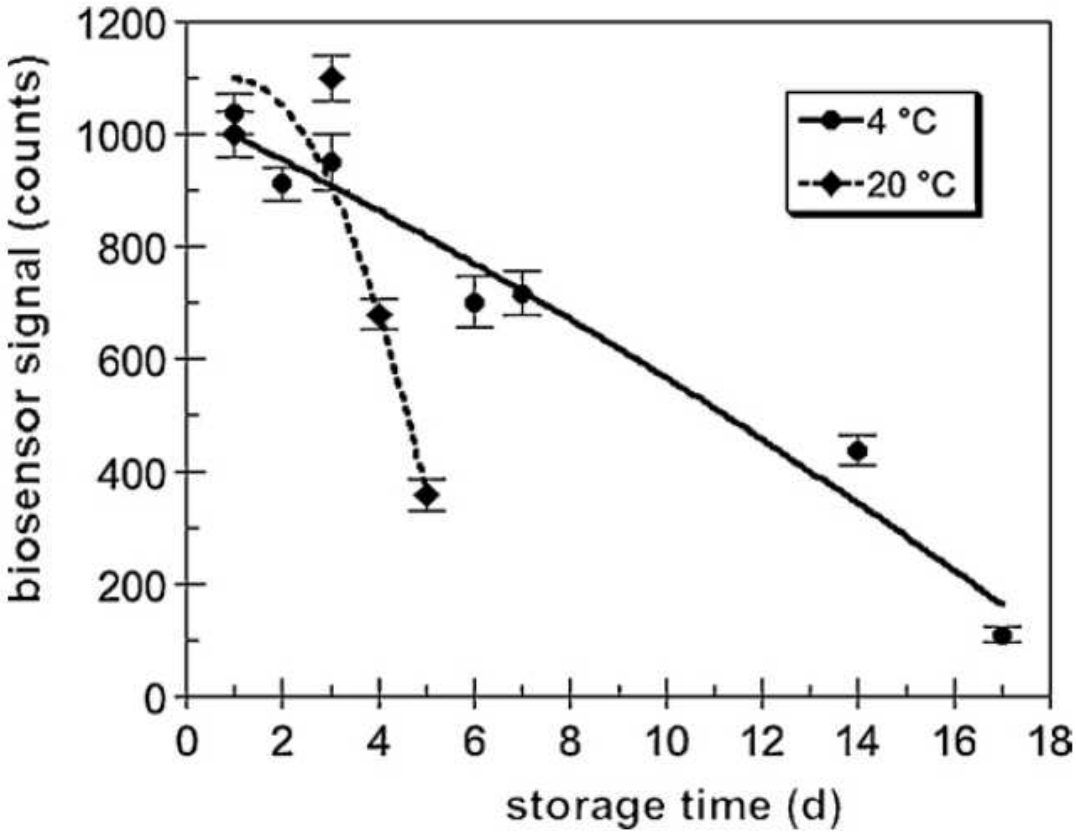

Figure 3.3 - Activity retention of TOM biosensor stored at two temperatures in measurement solution (without formate); each point represents the reading for a 92 µM toluene solution. Error bars represent ±1 standard deviation. The average relative standard deviation over all data points shown was 5.8%.

Solutions of 92 µM toluene were then measured with both regenerated biosensors and controls (stored under the same conditions in formate-free measurement solution). The signal from the regenerated biosensors to the toluene solution was at 350±40 counts, a 25% increase compared with controls. Further optimization using measurement solution supplemented with 1 M formate and 0.1 M ammonium nitrate yielded a signal (620±50 counts) twice that of controls. The increased biosensor activity after regeneration suggests that formate might serve as a potential reagent to regenerate intercellular NADH in this biosensor design. The regeneration efficiency

was greatly improved with the supply of nitrogen, although the basis for this effect is not yet known. The regeneration results also provided additional evidence that the depletion of NADH was the primary factor in the loss of activity during storage.

3.5 Conclusions

The TOM-based optical biosensor developed in this study provides a rapid, reagentless, and simple method to detect toluene in aqueous solutions. This biosensing concept could be extended to other analytes by using different mono- or dioxygenases. Compared with recent binding-based immunoassay or induction-based bacterial biosensors (Table 3.3), this biosensor design has the advantages of each method: The TOM-based biosensor provided a linear response to toluene over a wide concentration range, as is the case with most immunoassays (Eremin et al. 2005; Kim et al. 2001), while the induction-based biosensors normally have a nonlinear calibration curve with a small linear range (Li et al. 2008; Stiner and Halverson 2002; Willardson et al. 1998). Furthermore, the TOM-based biosensor could continuously monitor the change of analyte concentration as can induction-based biosensors (Kim et al. 2005; Paitan et al. 2004; Tizzard et al. 2006; Willardson et al. 1998), while the immunoassay methods are usually discrete (Eremin et al. 2005; Gerlach et al. 1997; Kim et al. 2001). Although activity retention for the TOM-based biosensor was limited by NADH consumption, a method to partially regenerate the signal was demonstrated. In contrast, the measurements of induction-based biosensors must be conducted in growth medium so that the energy for transcription and translation can be provided.

This is the first report of an enzymatic toluene sensor and of an oxygenase-based biosensor. Along with the successful demonstration of this biosensor design concept, this study also highlights the need to address the limited biosensor lifetime, either by improving NADH regeneration or by implementing a different detection scheme that avoids the requirement for NADH. The development of biosensors capable of continuous, *in situ* measurement of toluene

and other hydrocarbons would have many environmental applications, including the monitoring of ground water and measurement of effluent from waste water treatment plants.

Measurement principle	LOD (μM)	Range of detection (μM)	Pretreatment required	Reference
GC/MS	**0.001**	**0.001 - 0.1**	Yes	EPA method 8260a
Immunoassay	**0.02**	**0.02 - 20**	No	Eremin et al. (2005)
Induction-based biosensor	**11**	**11 - 22**	No	Willardson et al. (1998)
	7.5	**7.5 - 100**	No	Li et al. (2008)
Reaction-based biosensor	**3**	**3 -100**	No	This study

Table 3.3 - Comparison of different analytical methods for toluene measurements.

3.6 Acknowledgments

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Chapter 4 Toluene *ortho*-Monooxygenase as the Biocomponent of Fiber Optic Enzymatic Biosensors for Measurement of Trichloroethene in Aqueous Solution

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4.1 Abstract

A fiber optic enzymatic biosensor for the fast, reagentless, and simple measurement of trichloroethene (TCE) concentration was developed. The novel design concept of this biosensor included a variant of toluene ortho-monooxygenase (TOM-Green) as the biological recognition component, and an oxygen-sensitive ruthenium-based phosphorescent chemical immobilized on an optical fiber as the transducer. TCE concentrations were measured on the basis of the enzymatic reaction catalyzed by TOM-Green, which resulted in the consumption of oxygen and corresponding changes in the phosphorescence intensity. The biosensor had a limit of detection of 0.009 µM, a linear detection range up to 0.076 µM, and an average response time of 2 h. The performance was reproducible with different biosensors (RSD=12.8%, n=9). The biosensor signal decreased with each successive measurement and with storage time, and the decrease was also pronounced more rapidly at raising temperature. The activity loss over repeated measurements could be attenuated by co-expressing either of two epoxide-consuming pathways, indicating that TCE epoxide toxicity could be a limiting factor. This is the first report of an optical enzymatic TCE biosensor with a limit of detection of environmental relevance. The design concept of this enzyme-based fiber optic biosensor has the potential to expand biosensor use in water monitoring applications that require continuous, in situ, and cost-effective measurements.

4.2 Introduction

TCE is one of the most commonly used industrial solvent and degreasers in the world. The annual U.S. demand of TCE was 245 million pounds in 2005, with 4.5 percent per year increase through after (Chemical-market-reporter 1996). As a consequence of its extensive use, spillage, and improper disposal, TCE is one of the most commonly found chemicals in contaminated sites, about 34% of the drinking water supply sources and most groundwater contamination sites may contain TCE and 75% of EPA National Priority List (NPL) hazardous waste sites and Suprefund sites have TCE pollution (Jollow et al. 2009). TCE is a suspected carcinogen, as well as a known kidney and liver toxin (Burg and Gist 1999; Scott and Chiu 2006; Tabrez and Ahmad 2009). In addition, TCE can be transformed to vinyl chloride via microbial anaerobic dehalogenation in groundwater (Wackett and Gibson 1988), increasing the concerns regarding of TCE contamination in groundwater.

TCE concentration measurement with Chromatographic analysis such as Gas Chromatography (GC) (Poli et al. 2005; Rosell et al. 2006; Williams et al. 2002) are the most popular TCE detection methods with good selectivity and low limits of detection (LOD, as low as 0.02 µg/L with EPA method 8260b for volatile organic compounds), while absorption spectroscopic-based techniques (e.g. Fourier transform infrared spectroscopy (FTIR)) (Bangalore et al. 1997; Vohra et al. 1996) could also detect trace amount of TCE with fast acquisition time and high signal-to-noise ratio. However, these methods are usually time-consuming and expensive with additional pretreatment steps often required prior to sample analysis.

Biosensor, as one of the recent technology advance in analytical chemistry and environmental monitoring, is considered as an excellent alternative. By integrating biological process and transduction together, a biosensor is capable of real-time analysis with simplicity of operation (D'Souza 2001; Reardon et al. 2009). In biosensor system, enzymes are primary choice for biocomponents due to their high sensitivity and good specificity (D'Souza 2001; Reardon et al. 2009; Rubianes and Rivas 2005), while optical transduction has potential advantages over

electrical transduction in environmental monitoring because of low signal losses over long distance as well as no reference signal needed (Campbell et al. 2006; Monk and Walt 2004; Wolfbeis 2002). Biosensors are often reagentless , and can thus provide continuous, *in situ* measurements as a cost-effective alternative compared with traditional analytical methods.

Toluene *ortho*-monooxygenase (TOM) was first discovered as a successful enzyme in *ortho*-hydroxylation of toluene in 1995(Shields et al. 1995), further study showed that TOM could also catalyze the first few steps in the first few steps in TCE dehalogenation with the presence of oxygen and NADH(Canada et al. 2002), which begins with the formation of very active intermediate TCE epoxide.

$$C_2HCl_3 + O_2 + NADH + H^+ \xrightarrow{TOM} C_2HCl_3O + NAD^+ + H_2O$$

In this study, fiber optic biosensors based on a DNA shuffled (V106A substitution in the hydroxylase alpha-subunit) toluene *ortho*-monooxygenase from Burkholderia cepacia G4 (TOM-Green) were developed. TOM-Green has doubled the initial TCE degradation compared to TOM and been proven as a beneficial choice in TCE biodegradation in previous studies (Canada et al. 2002; Lee et al. 2006; Rui et al. 2004; Rui et al. 2005). TCE measurement with this biosensor was then conducted by the detection of oxygen consumption during the epoxidation reaction. Calcium alginate gel layer was applied to immobilize whole cells containing TOM-Green on a fiber optic oxygen sensor (oxygen optode). The oxygen optode was based on a phosphorescent indicator chemical that exhibits reduced light emission intensity by molecular oxygen via dynamic quenching (Pieper et al. 2008). As the result of enzymatic reaction, the oxygen concentration within the alginate layer decreases with the presence of TCE, shown as an increase in phosphorescence detector. The TCE epoxide toxicity was also studied by using two types of *E. coli* cells with same TOM-Green expressing plasmid but different secondary plasmid with unique epoxide toxicity mitigation mechanism.

4.3 Materials and methods

4.3.1 Chemicals

Toluene (99%. v/v), TCE (99% v/v), alginic acid (low viscosity, sodium salt) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich. Tris (4, 7-diphenyl-1, 10-phenanthroline)-ruthenium (II) complex (RuDPP) was synthesized at the University of Hannover (Kohls 1995). TCE standard solution (200 mg/L in methanol) was purchased from Sigma-Aldrich and diluted sequentially to prepare calibration standards.

4.3.2 Bacterial strains and growth conditions

The biocomponent of the biosensors, TOM-Green, TOM-Green/EchA, and TOM-Green/GSHI were expressed in *Escherichia coli* strain TG1 harboring with different plasmids from *B. cepacia* G4 (Canada et al. 2002; Lee et al. 2006; Rui et al. 2004). *E. coli* cultures were grown aerobically on agar plates made from Luria-Bertani (LB) medium with 20 g/L Bacto-agar (Difco) and 100 mg/L kanamycin (plus 50 mg/L chloramphenicol in the case of TOM-Green/EchA and TOM-Green/GSHI) at 30 °C for 24 h. A culture tube containing 2 mL LB medium supplemented with same concentrations of antibiotics was inoculated from a separate colony on an agar plate and shaken overnight at 30 °C and 200 rpm, and then transferred to a flask containing 200 mL of the same LB-Kan medium and shaken at 30 °C and 200 rpm. Cell concentration was measured as culture absorbance at 600 nm (optical density at 600 nm, OD600) with a spectrophotometer (Spectronic® 20 Genesys™, Thermo Electron Corporation). IPTG solution was prepared with deionized water and added to the culture with a final concentration of 1 mM to induce TOM-Green, TOM-Green/EchA and TOM-Green/GSHI biosynthesis in the early exponential growth phase (OD600 of 0.6). The culture was harvested 4 h after IPTG was added, centrifuged, and resuspended in 20 mL of a solution containing 10 mM phosphate buffered saline at pH 7.4 and stored at 4 °C until further use.

4.3.3 Biosensor tip construction

Each biosensor tip consisted of a layer of immobilized whole cells over an oxygen optode, which was constructed from a 25-cm section of polymethylmethacrylate (PMMA) optical fiber terminated with a straight tip (ST) connector. The fiber jacket was detached from 1 mm of the distal end (non-connector terminated) and then polished with 2000-grit and 3 μm polishing film (part of a fiber optic tool kit, IF-TK4-RP2, Industrial Fiber Optics) to minimize potential signal loss due to scattering. One mg of the oxygen-sensitive phosphorophore RuDPP, which is classified as phosphorophores since its longer decay lifetime than typical fluorophores (Lakowicz 2006), was dissolved into 1 mL chloroform and mixed with 200 mg silicone gel (clear RTV silicone, Permatex, Inc.). A 1-μL aliquot of this mixture was then added to the polished fiber tip. The RuDPP gel layer was affixed to the optical fiber end as soon as the chloroform evaporated. Previously stored *E. coli* whole cells (with different plasimds) were centrifuged and mixed with sodium alginate solution (2.5%) in a cell-to-alginate ratio (wet cell mass: alginate solution) of 1:1 w/w unless otherwise specified. 2 μL of the cell-alginate mixture was placed on the tip of each oxygen optode and immobilized after immersing the optode in 0.47 M calcium chloride solution for 30 min at 0 °C. All biosensors were stored at 0 °C in a solution of 0.15 M NaCl and 0.025 M $CaCl_2$ at pH 7.0 (hereafter referred to as “measurement solution”)

4.3.4 Biosensor instrumentation system

The biosensor instrumentation consisted of two separate optoelectronic modules: a 470-nm LED and a 450/60 nm optical bandpass filter (Chroma Technologies) as the excitation light source, and a computer-controlled Ocean Optics USB4000-FL spectrometer with 10 nm resolution for detection. The 470-nm excitation light was delivered through one leg of a bifurcated optical fiber assembly that has two 1-mm fibers side-by-side in the common end (Ocean Optics, Inc.), which was connected with the biosensor via an ST connector. The phosphorescent emission light (peak at 620 nm) from the biosensor was directed back into the

detector through the other leg of the bifurcated optical fiber and measured by the spectrometer (sensitivity of approximately 60 photons/count at 600 nm). The spectrometer output from 615 nm to 625 nm was integrated over 200 ms and five such values were averaged to yield one measurement value per second. The change in the intensity of the emission light over time correlates to the oxygen concentration change in the RuDPP layer of the biosensor.

4.3.5 Biosensor measurement protocols

All biosensor experiments were performed in glass vials (5 mL) containing 4 mL of measurement solution saturated with air at room temperature with a small magnetic stir bar for agitatiton thoroughly. The biosensor tip was immersed in this solution, sealed in the glass vial with a rubber septum, and shielded from external light sources. Aliquots (0.1 mL) of a TCE solution (0.1 – 4 mg/L) were injected to the measurement solution after the sensor had produced a steady output, defined as the time when the variation in the output was no larger than the peak-to-peak noise for a period of at least 5 min. All measurements were performed at room temperature unless otherwise specified. Each measurement was performed with a fresh biosensor to distinguish the effect in question (e.g., temperature, pH, cell/alginate mass ratio). Biosensors were not reused unless otherwise specified.

4.3.6 TCE concentration measurement by gas chromatography

To assess the accuracy of the TCE concentration data obtained from the biosensors, GC analysis was performed via a modification of EPA Method 8260b. After a biosensor measurement, 0.75 mL of aqueous solution was collected from the measurement vial and transferred into a 2-mL glass screw-top GC vial containing 0.75 mL of chloroform. The GC vial was then capped with a Telfon-coated septum and mixed on a rotating wheel for 15 min. One µL of the chloroform phase was injected into a Hewlett Packard 5890 gas chromatograph equipped with a HP model 5971A mass spectrometric (MS) detector. A calibration curve of the GC-MS total ion count peak

area vs. the TCE concentration in solution was obtained using dilutions of the 200 mg/L TCE standard solution. The GC calibration curve was linear over the range of TCE concentrations from 1 to 1000 µg/L (R^2 = 0.973).

4.4 Results and Discussion

4.4.1 Demonstration of TOM-Green biosensor for TCE measurement

A 0.1 ml aliquot of 25 mg/L aqueous TCE solution was injected into 4.0 mL of measurement solution in which the biosensor was immersed. The proposed detection mechanism initiates the catalysis of the reaction with TCE and oxygen by the intracellular TOM-Green enzyme immobilized on the biosensor tip, which consumes oxygen in the solution as well as NADH inside the cells (Shields et al. 1995). As a result, the decrease of oxygen in the alginate layer then causes an increase in the phosphorescence intensity of the immobilized RuDPP because of reduced quenching by oxygen decrease (Pieper et al. 2008). The biosensor reading is defined as the measured phosphorescence intensity at a single condition (e.g., measurement solution without no analyte at 1 mg/L dissolved oxygen), while the difference between the readings before and after TCE added is termed the biosensor signal.

The signal of a biosensor with whole cells of *E. coli* TG1 pBS(Kan)TOM-Green was 2000 counts with a response time of 4 h (Figure 4.1), as the result of TCE concentration increase from zero to 0.61 mg/L. When the biosensor reading reached a steady value (variation less than or equal to the system noise), the remaining TCE concentration in the vial was found to be 0.60 ± 0.03 mg/L using GC-MS. This indicates that TCE detection inside the biosensor system is based on a steady-state balance between diffusion and reaction of TCE and oxygen in the biosensor tip region rather than the depletion of TCE in the sample.

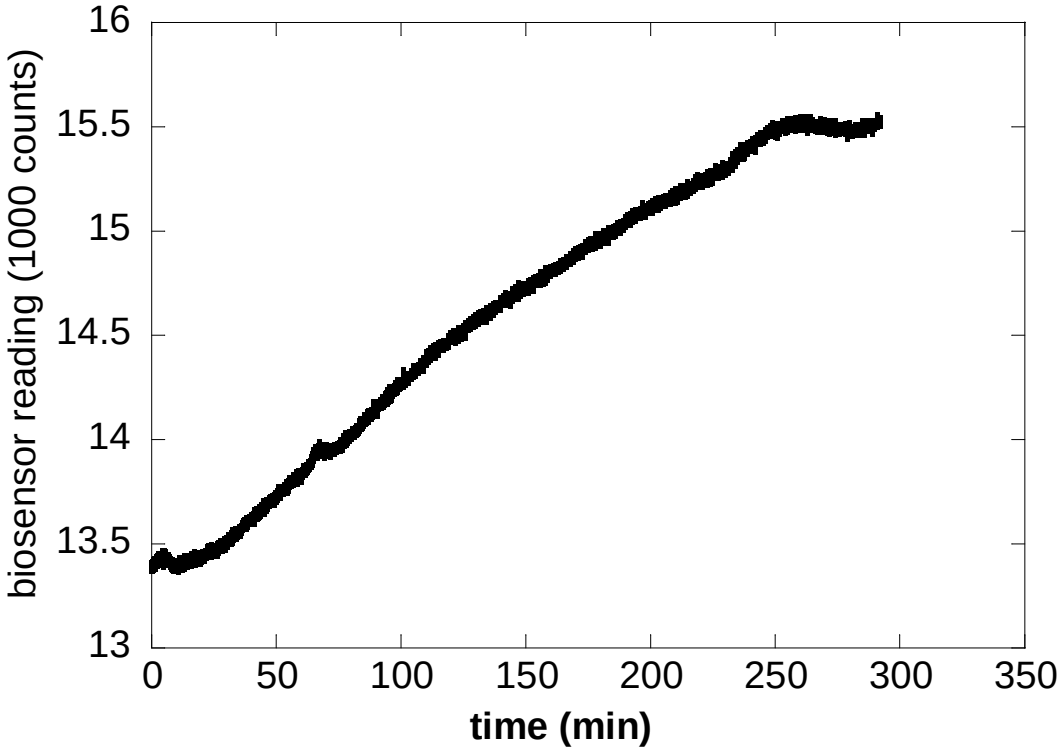

Figure 4.1 - Time course of a TOM-Green biosensor response to the addition of 0.61 mg/L TCE

4.4.2 Characterization of TOM-Green biosensor

4.4.2.1 Reproducibility

Biosensors within a group that were made at the same time under identical conditions were tested with 5 µg/L TCE solutions in order to evaluate reproducibility. The biosensor signal reproducibility was comparable in consistence (RSD=12.8% for n=9, within a batch) with typical enzymatic biosensors, RSD=10.7% for n=3, (Willardson et al. 1998).

In addition, biosensors made at different batch were also tested with same condition to address the reproducibility over the batch. The results showed the biosensor signal reproducibility was also in consistence with a 11% RSD for biosensors made from 5 different batches.

4.4.2.2 Effect of cell concentration

E. coli TG1 pBS(Kan) TOM-Green cells were immobilized at different concentrations in calcium alginate to validate the effect of enzyme concentration on biosensor performance. Triplicate measurements were made for each three different cell-to-alginate w/w ratios (3:1, 2:1, and 1:1). All these biosensors were tested with 5 µg/L TCE and no significant differences in the signal were observed ($p < 0.01$). This result indicates that the oxygen concentration gradient from the alginate layer to the bulk solution is unaffected on cell concentration in the range studied, which could be explained by strong mass transfer limitation dominance.

Similarly, the biosensor response time was not dependent by the cell concentration on the tip. The above measurements with the TCE-based biosensor requires 2 h, as much as a typical enzymatic biosensors needed (Willardson et al. 1998). Therefore, the biosensor response time would probably be contributed by one or more mass transfer processes, rather than a function of the immobilized cell concentrations.

4.4.2.3 Calibration curve and limit of detection

A series of TCE solutions (50 µg/L to 4 mg/L) were measured with TOM-Green biosensors. Each biosensor was used only once, and each concentration point was measured in triplicate. The biosensor signal was monotonically increased with TCE concentration and the overall calibration curve showed as a nonlinear curve. The linear over the range from 1.2 to 9.8 µg/L TCE with R^2=0.962 (Figure 4.2). The limit of detection (LOD), calculated as three times the standard deviation of the noise obtained from control experiments, was equal to 1.2 µg/L, less than the EPA Maximum Contaminant Level Goal for TCE (5µg/L) in National Primary Drinking Water Regulations.

The LOD of the TOM-Green biosensor for TCE was significantly lower than two reported TCE biosensor, 100 mg/L in a thin-film electrochemical biosensor (Chen et al. 2004) and 30 µg/L in a flow-injection based biosensor (Han et al. 2002), which were based on traditional

electrochemical transducer (electrode). Although the linear detection range in this study is narrower than the previous two studies, 100 mg/L to 700 mg/L (Chen et al. 2004) and 30 µg/L to 2 mg/L (Han et al. 2002), it would be much more significant to have a linear detection range in an analyte concentration level close to environment.

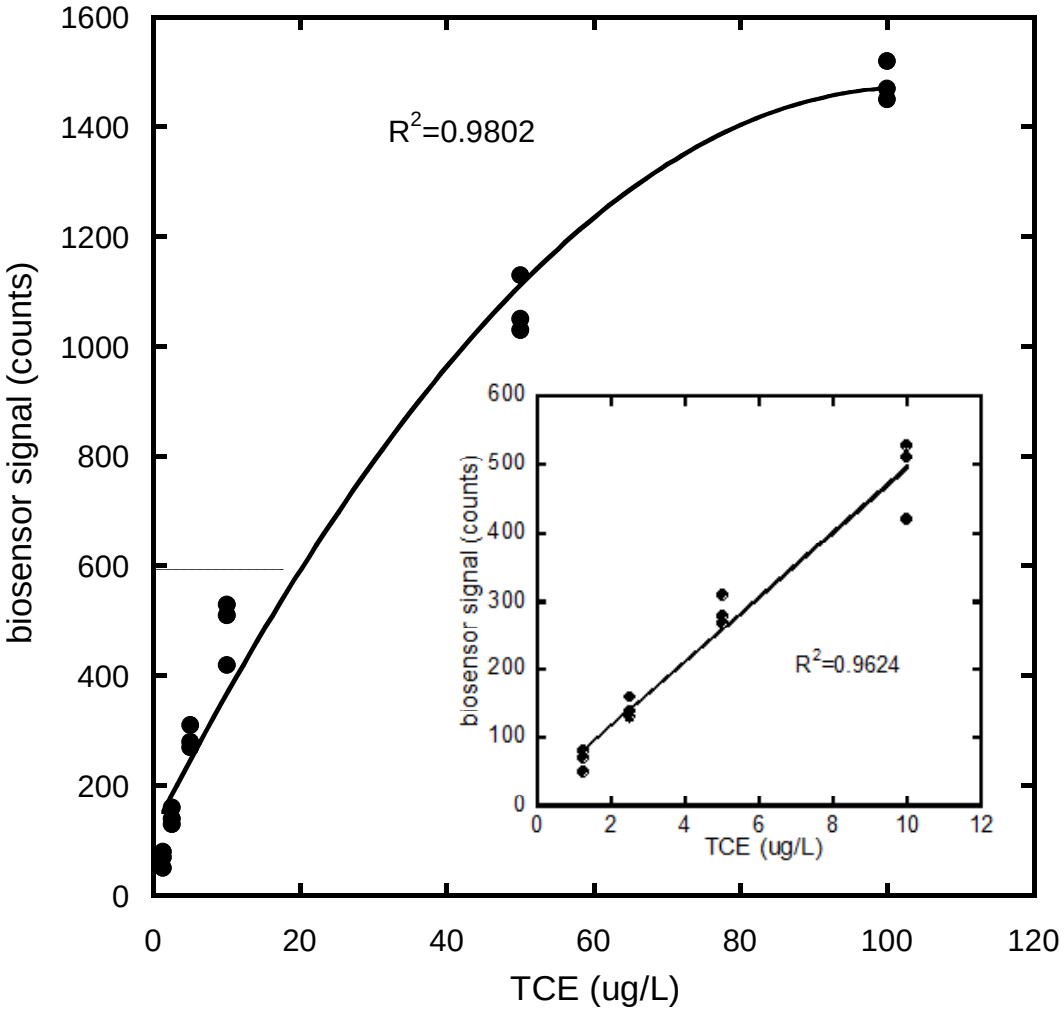

Figure 4.2 - TOM biosensor signal as a function of toluene concentration. Inset: biosensor signals in the low range of toluene concentrations (0–12 µg/L)

4.4.2.4 Accuracy

Water samples from two local lakes (Horsetooth Reservoir and City Park Lake, Fort Collins, CO) were added with TCE to quantify the biosensor performance in real environmental matrices. In each case, three different TCE concentrations were used, locating most of the linear detection range of the biosensor. The concentrations measured by the TOM-Green biosensor and the GC/MS method were compared and reported (Table 4.1). The difference between biosensor measurement and GC/MS measurement were 0.1 ± 0.2 µg/L (95% CI, n=18), indicating that the TOM-Green biosensors provide accurate and reliable measurement for TCE in these aqueous matrices.

Sample	TCE concentration (µg/L)	
	TOM-Green biosensor	GS-MS
Spiked in Horsetooth Reservoir water		
High	**9.8±0.2**	**9.8±0.1**
Medium	**4.9±0.1**	**4.8±0.1**
Low	**1.1±0.1**	**1.2±0.1**
Spiked in City Park Lake water		
High	**9.8±0.1**	**9.7±0.1**
Medium	**4.8±0.1**	**4.8±0.1**
Low	**0.8±0.2**	**1.2±0.1**

Table 4.1 - Comparison of TCE measurements in spiked water samples. Three biosensors were used for each measurement.

4.4.2.5 Selectivity

TOM-Green has been reported to catalyze several chlorinated and aromatic chemicals besides TCE (Canada et al. 2002) in a similar hydroxylation mechanism. Therefore, toluene, benzene and TCE were selected to evaluate the selectivity of the TOM-Green biosensor. All of these analytes were measured at a concentration of 1 mg/L. The biosensor signal was largest for TCE (2280±80 counts), followed by toluene (570±60 counts), and then benzene (40±10 counts).

This trend is consistent with data from a previous study (Canada et al. 2002), in which TOM-Green showed high degradation rate for TCE oxidation among all the analytes. Although the 1mg/L TCE concentration was not located in the linear detection range, it still showed highest biosensor signal in this study, which suggests that the signal increases monotonically when TCE concentration arises.

4.4.3 Effects of temperature and pH on biosensor signal

pH and temperature are two crucial factors in environmental monitoring, since both enzyme activity and mass transfer rates of TCE and oxygen could be affected. In addition, the phosphorescence properties of RuDPP are also temperature dependent. To quantify the effect of pH on the TOM-based biosensor signal, sets of three biosensors were tested in measurement solutions buffered at pH 5.0, 6.0, or 7.0, spanning a common pH range in typical groundwater aquifer. The signals corresponding to 5 µg/L TCE at different pH values were 290±20 counts (pH=5), 280±30 counts (pH=6) and 300±40 (pH=7), indicating that the measurements of the TOM-based biosensor were independent of pH in this range.

Similarly, the signals of a set of three biosensors at three temperatures were investigated. The results of biosensors signals to 5 µg/L TCE were 270±50 counts (at 15 °C), 290±20 counts (at 20°C) and 430±30 counts (at 30°C), which could be contributed by increased enzymatic reaction rate, mass transfer rates or RuDPP itself, although the degrees to this behavior are not known and could be further explored via mathematical models

4.4.4 Activity retention

The retention of activity with use or storage is crucial for any biosensor system. This is a particular concern for TOM-Green biosensor because of the consumption of NADH as well as the formation of TCE epxoide as a toxic intercelluar intermediate during the reaction. In growth of

TOM-Green cells, NADH is regenerated through catabolism; however, biosensors are normally stored without an energy source and thus NADH levels would be expected to decrease overtime as well as consumed after each usage. In addition, all biosensors are destined to the denaturation of their biocomponent

To investigate the retention of activity among fresh made biosensors, two groups of biosensors made within a batch were stored in measurement solution without TCE at 4 °C or 20 °C. At time periods, biosensors were transferred from the storage solution and used to measure 10 µg/L TCE. For both storage temperatures, the biosensor performance declined over time, and eventually not detectable. Biosensors stored at 4 °C kept a longer active period than those stored at 20 °C (Figure 4.3). The NADH starvation or enzyme denaturation would explain the deteriorating biosensor activity overtime, especially at higher temperature.

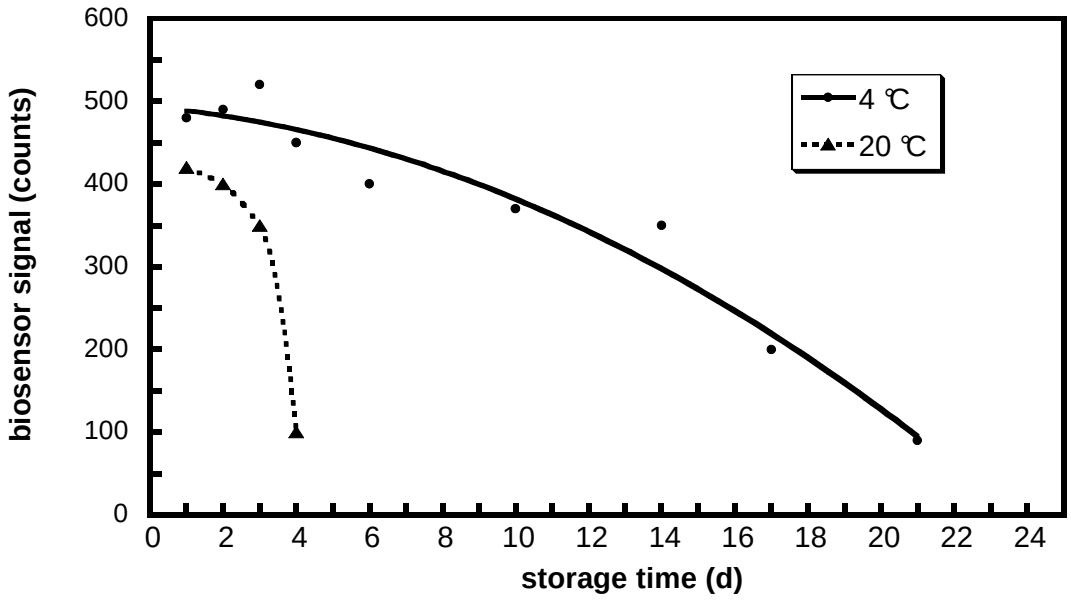

Figure 4.3 - Activity retention of TOM-Green biosensor stored at two temperatures in measurement solution (without formate); each point represents the reading for a 92 µM toluene solution.

4.4.5 TCE Epoxide toxicity mitigation

TCE epoxide, same as other chlorinated epoxyethanes, is electrophilic and could directly or indirectly react with various intracellular biological molecules, such as DNA, RNA, lipids, proteins, and other small molecules (Rui et al. 2004; Vlieg and Janssen 2001). The reactions may result a slowly inactivation of biosensor and permanent damage to enzyme inside biosensor tip.E. coli cells with TOM-Green plasmid and GSHI/EchA plasmids were developed to provide alternative to the problem. In recent report, TOM-Green/GSHI showed higher cell viability than TOM-Green/EchA, while TOM-Green/EchA has more glyoxal formation, in the case of dichloroethene (DCE) (Lee et al. 2006; Rui et al. 2004).

To investigate the effect of GSHI and EchA plasmid for biosensor activity retention and epoxide toxicity mitigation, preliminary NADH regeneration experiments were performed since NADH depletion as discussed in previous section also contributed to the activity loss over time. NADH regeneration with supplying formate externally overtime can partially cover biosensor activity, since intracellular formate dehydrogenase could reduce the NAD^+ to NADH by the oxidation of formate (Berrios-Rivera et al. 2002; Slusarczyk et al. 2000). Regeneration experiments were conducted to test the possibility of regeneration used TOM-Green biosensor after TCE measurement, a modified approach adapted from previous study (Zhong et al. 2010). In comparison with controls (no formate regeneration between repeated measurements), the signal from regenerated TOM-Green biosensors showed 2 ± 3 % increase at high TCE concentration (50 µg/L), -2 ± 4 % increase at medium TCE concentration (10 µg/L) and 5 ± 5% increase at low TCE concentration (2 µg/L), indicating that TCE epoxide could damage the whole cell system and decrease the signal permanently.

Therefore, three different groups of biosensors were made with TOM-Green, TOM-Green/GSHI and TOM-Green/EchA respectively. Biosensors in each group were made within a batch and tested with 50 µg/L TCE, 10 µg/L TCE, and2 µg/L TCE in triplicates, while each biosensor was tested three times at the same TCE concentration.

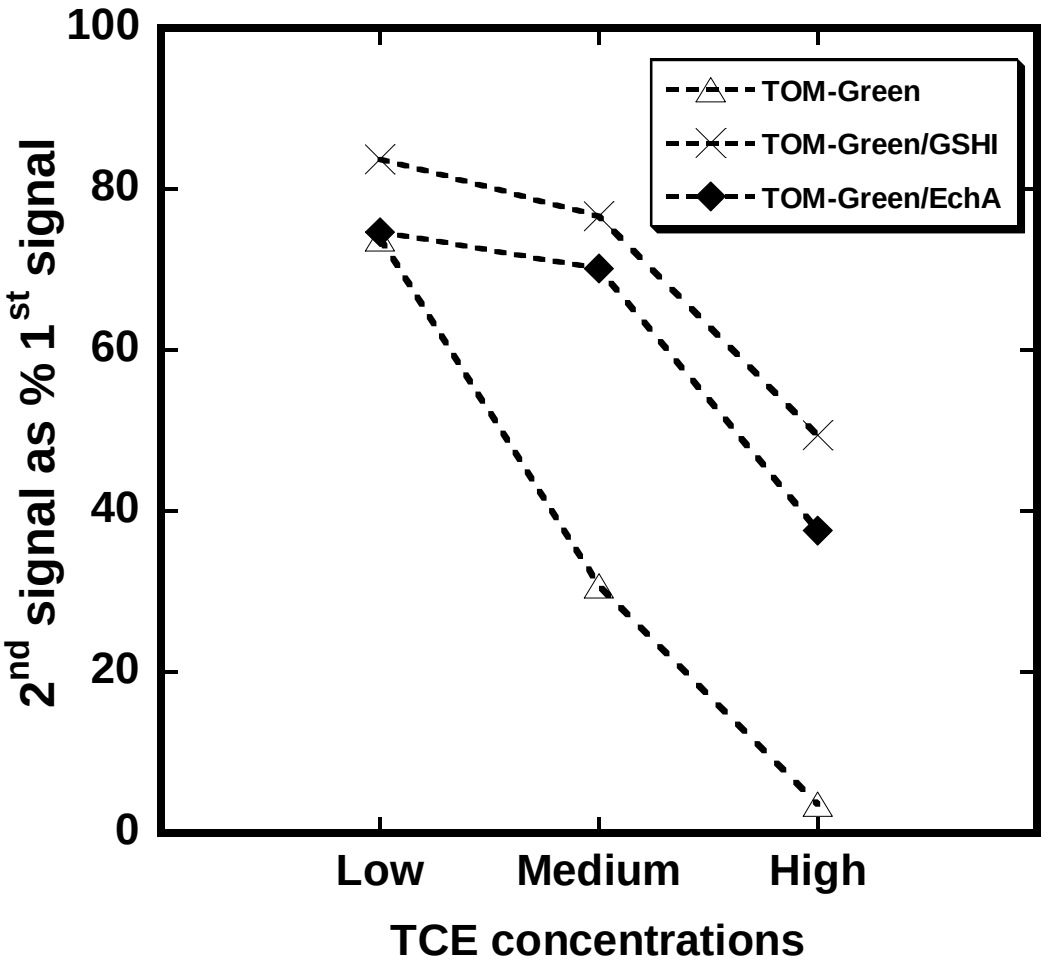

Figure 4.4 – 2nd signals as % 1st signals at different TCE concentrations for all three types of TOM-Green biosensors.

The results (Figure 4.4) showed that at high TCE concentration, TOM-Green biosensor become inactive after the very first usage, while the second measurement of TOM-Green/GSHI and TOM-Green/EchA biosensors had about 50% of initial signals, and the third measurement had about 10% of initial signals. At medium TCE concentration range, 2nd measurement signals were about 75-80% as that of 1st measurement in the case of TOM-Green/GSHI and TOM-Green/EchA biosensors, when TOM-Green biosensor could only retain about 30% activity after first usage. At low concentration range, the TCE toxicity effect was less obvious since all three

kinds of biosensors shared the same range of activity retention after first time usages. In addition, it is also clear than TOM-Green/GSHI had higher biosensor signal than TOM-EchA in all different conditions, which suggested that cell viability is more important in the biosensor system than pure glyoxal formation rate (Rui et al. 2004).

4.5 Conclusions

The development of TOM-Green biosensor in this study demonstrates a fast, reagentless measurement of TCE in aqueous solution with simplicity. The design concept of biosensor could become a promising alternative in detection of other chlorinated ethenes as well as common environmental pollutants with the help of appropriate mono- or dioxygenases. Compared with recent flow-injection biosensor or typical electrochemical sensor, this enzymatic biosensor design showed its unique advantages. TOM-Green biosensor could not only provide a much lower LOD than flow-injection biosensor(Han et al. 2002) or other electrochemical sensor(Chen et al. 2004; Noda et al. 2008), which showed advantages of enzymatic reaction-based detection in chlorinated ethenes, but also monitor the change of analyte concentration continuously while type flow-injection based biosensor and electrochemical-based sensor are usually discrete. In addition, the success of TOM-Green/GSHI and TOM-Green/EchA in solving TCE epoxide toxicity strengthens the promising future of TOM-Green biosensor in application of TCE detection.

4.6 Acknowledgments

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Chapter 5 Construction and Characterization of Fiber Optic Toluene *ortho*-Monooxygenase Biosensors with Formate Dehydrogenase Plasmid Transformation

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(In preparation for submission to Talanta)

5.1 Abstract

NADH consumption overtime in biosensor storage and repeated measurements limited the application fiber optic enzymatic biosensors in continuous, *in situ* measurement. Two different NADH regeneration systems were discussed while the FDH system from *Mycobacterium vaccae* N10 was selected and integrated into TOM cells via plasmid transformation by chemical approach. Successful transformants were validated with agarose gel electrophoresis imaging, while biosensor was subsequently constructed and characterized for activity retention and regeneration capability. Important parameters for regeneration were also discussed, suggesting an optimal regeneration condition for FDH-based system by storing biosensors in measurement solution supplemental with 0.1 M of formate for 24 h. 90% of TOM+FDH biosensor activity retained after stored at optimal regeneration condition for two weeks and 70% remained after one month storage at room temperature. TOM+FDH biosensor activity could be regenerated by preserving at optimal regeneration condition after each measurement, resulting in 80% activity remained during two weeks intervals. TOM-Green+FDH biosensors were performed with similar measurements and obtained comparable results in activity retention but different outcome in activity regeneration due to toxicity intermediate during measurement. PTDH system was also discussed in brief as the other alternative for NADH regeneration. This is the first report of an

enzymatic biosensor with the ability of NADH regeneration supplemental with formate. The potential of this type biosensor could extend the scope of biosensor application in environmental monitoring.

5.2 Introduction

Microbial biosensors have great potential in environmental monitoring and related area and become more accepted as measurement techniques because of the advantages they have in continuous, *in situ* monitoring (Reardon et al. 2009b). Enzymatic reaction-based biosensor is the first kind of biosensor developed in history (Clark and Lyons 1962) and enzyme has been widespread used as biorecognition element in biosensor system because of its high specific activities and analytical specificity (D'Souza 2001; Wanekaya et al. 2008). However, in many enzyme-based biosensors, the inherent biochemical reactions are simple, such as glucose oxidations (Choi 2004; Lim et al. 2005) and halogenations (Campbell et al. 2006; Reardon et al. 2009a), since complicated enzymatic reactions often require one or more cofactors that are expensive to add extracellular while hard to self-regenerated in the system.

NADH and NADPH are two most common cofactors in microbial cell metabolism and function in over 300 reduction-oxidation (redox) reactions (Berrios-Rivera et al. 2002; Foster et al. 1990). It is considered expensive and impractical to supply these cofactors externally in many cases, while a variety of in situ regeneration methods have been developed (van der Donk and Zhao 2003). The most successful and widespread used enzymatic NADH regeneration system is based on *Candida boidinii* formate dehydrogenase (Schutte et al. 1976), which has been used in industrial production of L-*tert*-leucine (McCoy 2001). The formate dehydrogenase gene were found in the system of *Pseudomonas sp. 101* (Tishkov et al. 1993) and *Mycobacterium vaccae* N10 (Galkin et al. 1995) as well.

Previous study on a fiber optic enzymatic biosensor for measurement of toluene suggested that NADH depletion could become a limiting factor after biosensors being stored prolong time or used over times, while supplying formate externally showed partial regeneration of biosensor

activity (Zhong et al. 2010). In addition, there are also native cofactor-independent formate dehydrogenase pathways existing in central metabolic pathway of *E. coli* system (Berrios-Rivera et al. 2002). Hence, introducing a secondary NAD^+ - dependent formate degradation pathway can significantly increase the availability of intracellular NADH *in vivo*, which improves the biosensor activity regeneration and retention consequently.

Plasmid transformation occurs naturally and can be manipulated by increasing the cell competence via two primary approaches: chemical induction and electroporation (Thomas and Nielsen 2005). Chemical approach relies on treatment with polyvalent cations and incubation at low temperature in order to transiently open gated membrane channels. Heat shock during the transient periods result in a rapid influx of extracellular medium into the bacterium (Casali and Preston 2003). Electroporation was initially invented as a physical method to introduce DNA into eukaryotic cells (Neumann et al. 1982), and was developed for bacterial transformation subsequently (Fiedler and Wirth 1988). Electroporation is based on electrical pulse forming reversible transient pores through which DNA molecules travels into the cell (Casali and Preston 2003). Both transformation methods work for *E. coli* system, while chemical induction is simple, low cost and ease of use compared with electroporation.

In this study, chemical induction was selected as transformation techniques to deliver a *Mycobacterium vaccae* N10 formate dehydrogenase (FDH) plasmid into *Escherichia coli* strain TG1 harboring the plasmid pBS(Kan)TOM. Fiber optic enzymatic biosensors were made with successful transformants in order to compare NADH regeneration capability and activity retention with original TOM biosensors. Additional plasmid DNA separation and imaging with agarose gel electrophoresis were performed as verification for plasmid transformation.

5.3 Materials and methods

5.3.1 Chemicals

Toluene (99%. v/v), alginic acid (low viscosity, sodium salt), isopropyl β-D-1-thiogalactopyranoside (IPTG), sodium formate (≥99.0%), $CaCl_2$ (≥99%), Glycerol (99%. v/v), Tris base (≥99.9%), Acetic acid (≥99.7%), EDTA (≥99%) and Agarose (≤1.0% Ash, ≤10% water) were purchased from Sigma-Aldrich. Tris (4, 7-diphenyl-1, 10-phenanthroline)-ruthenium (II) complex (RuDPP) was synthesized at the University of Hannover (Kohls 1995). Minipreps DNA purification kits were purchased from Promega. SYBR safe DNA gel stain (10,000x DMSO) was purchased from Invitrogen. Toluene standard solution (2 mM in methanol) was purchased from Sigma-Aldrich and diluted sequentially to prepare calibration standards.

5.3.2 Microorganisms

Escherichia coli strain TG1/pBS(Kan)TOM and *Escherichia coli* strain TG1/pBS(Kan)TOM-Green were from our own strain collection and One Shot® TOP10 chemically competent *Escherichia coli* was obtained from Invitrogen. *Mycobacterium vaccae* N10 formate dehydrogenase (FDH) plasmid obtained from University of Kyoto (Galkin et al. 1995) was transformed into TOP10 cells in order to amplify the quantities of FDH plasmids for further transformation.

5.3.3 Preparation for transformation

Chemical treatments were performed to convert normal *E. coli* cells into to chemically competent *E. coli* cells with an modified protocol (Sarkar et al. 2002) for TOM, and TOM-Green. TOM and TOM-Green cultures were maintained aerobically on agar plates made from Luria-Bertani (LB) medium with 20 g/L Bacto-agar (Difco) and 100 mg/L kanamycin at 30 °C for 48 h, A culture tube containing 2 mL LB medium supplemented with 100 mg/L kanamycin was inoculated from a single colony on an agar plate and shaken overnight at 30 °C and 200 rpm. The culture was then transferred to a flask containing 200 mL of the same LB-Kan medium and shaken at 30 °C and 200 rpm. Cell concentration was measured as culture absorbance at 600 nm

(optical density at 600 nm, OD600) with a spectrophotometer (Spectronic® 20 Genesys™, Thermo Electron Corporation). 25 ml of selected culture was placed in a cold (4 °C) 50 ml conical tube at early exponential growth phase (OD600 of 0.6).The culture was centrifuged for 5 min at 4 °C and 3000 rpm after cooling the tube for 30 min. After supernatant decanted, the cell pellets were gently resuspended in 10 ml of 100 mM $CaCl_2$ at 0 °C. The cells were centrifuged at the same condition after 30 min. The pellets were resuspend gently in 1 ml of 100 mM $CaCl_2$ + 15% glycerol (v/v) at 0 °C for overnight.

5.3.4 Plasmid purification

TOP10 *E. coli* cells harboring *Mycobacterium vaccae* N10 FDH plasmid were grown under the same protocol except using 50 mg/L of ampicilin as antibiotics instead of 100 mg/L of kanamycin. 2 ml of cell culture was collected early exponential growth phase (OD600 of 0.6) and centrifuged at room temperature and 3000 rpm. FDH plasmid DNA were extracted and purified from the cell pellets using the Miniprep DNA purification systems (Promega). Subsequent DNA extraction and purifications were also followed using the Miniprep DNA purification systems.

5.3.5 Transformation protocols

10 ng of FDH plasmid DNA was added to 100 µL of chemically competent cells (TOM or TOM-Green). The combined solution was mixed by swirling or tapping the tube gently and then incubated at 0 °C for 1 h. The mixture solution was transferred to a heat-block at 42 °C for 60 sec and returned to ice-cold condition (0 °C) for another 2 min. 900 µL of super optimal broth (SOC) medium was added into the cell culture to maximize the transformation efficiency of competent cells. The cells were regenerated at 30 °C with shaking at 200 rpm for 1 h and spreaded on agar plates made with LB medium with 20 g/L Bacto-agar (Difco), 100 mg/L kanamycin, and 50 mg/L of ampicilin at 30 °C for overnight.

5.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis of plasmid DNA was prepared using 0.7% (w/v) agarose gel with 1X TAE buffer (Tris base, acetic acid and EDTA, pH = 8) (Brody and Kern 2004). The gel was running in plastic tank under 100 volts for 1h. The gel was transferred to gel container with 100 ml of TAE buffer and stained with 10 µL of 10,000x SYBR safe DNA gel stain (Invitrogen) while under slowly shaking and shielded from light.

5.3.7 Biosensor construction, instrumentation system and measurement protocols

Fiber optic enzymatic biosensors were made with successful transformants whole cells cultured and immobilized on biosensor tip as biocomponent, while the oxygen optodes served as transducer. The biosensor detection system consists of a computer-controlled Ocean Optics USB4000-FL spectrometer with 10 nm resolution and an excitation light source containing a 470-nm LED with a 450/60 nm optical bandpass filter (Chroma Technologies). The detailed biosensor construction, instrumentation system and measurement protocols were described in previous study of TOM biosensor for measurement of toluene in aqueous solution (Zhong et al. 2010).

5.4 Results and Discussion

5.4.1 Demonstration of biosensor measurements with successful TOM+FDH transformants

Biosensors made with successful transformants of TOM+FDH cells and normal TOM cells were performed for measurements of toluene concentration in aqueous solutions. Biosensors measurement protocols were adapted from previous study (Zhong et al. 2010). TOM+FDH biosensors and TOM biosensors were conducted by measurements of 92 µM toluene in triplicates respectively. TOM+FDH biosensors were regenerated by storing biosensors at 4 °C and supplemented with 1 M formate solution for 24 h after first measurement and analyzed with same toluene concentration for additional two measurements with formate regeneration for 24 h

between 2nd and 3rd measurements for each biosensor. TOM biosensors behaved as controls and no regeneration treatments were applied in between each measurement.

The relative signals of 2nd and 3rd measurements were calculated as:

Relative signal = Absolute signal / Initial signal × 100%

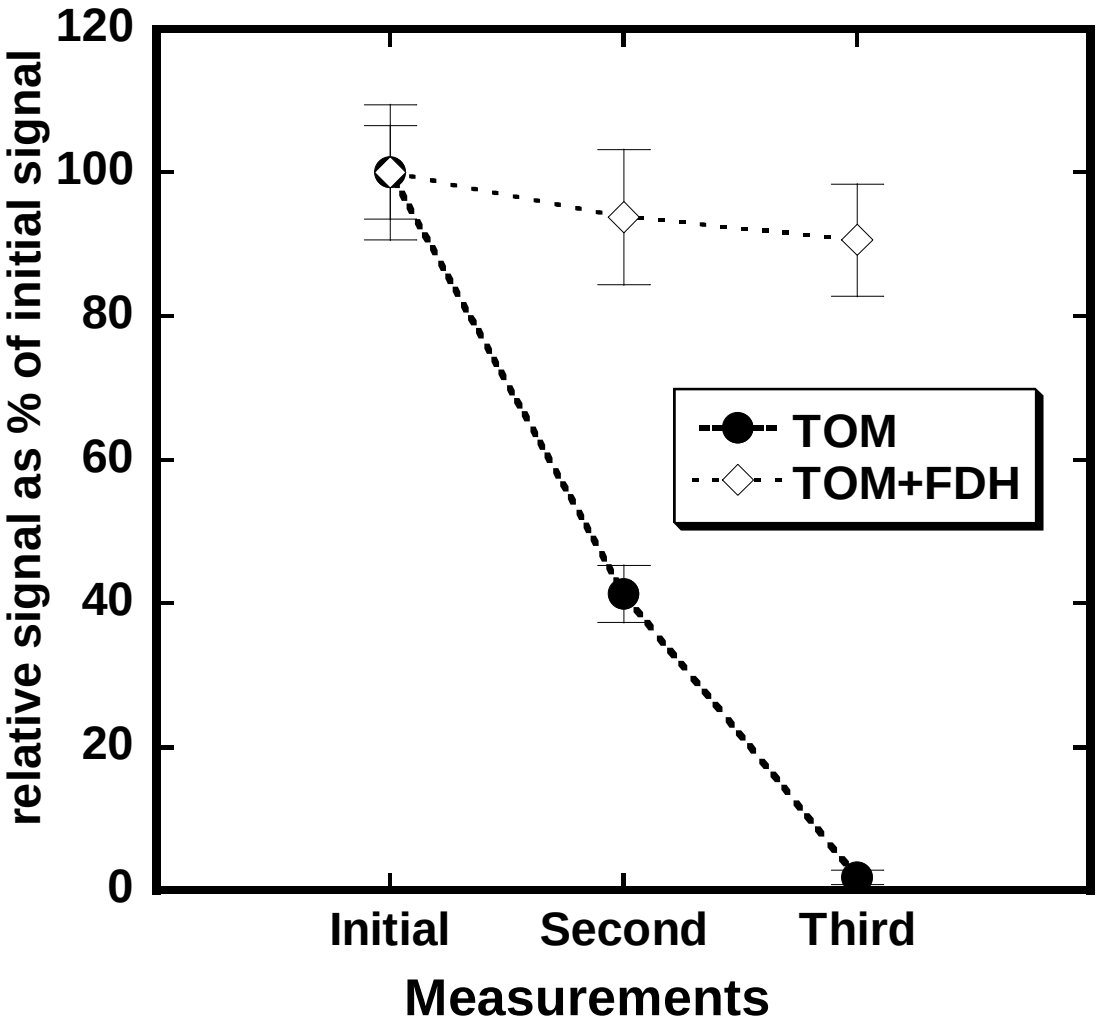


Figure 5.1 – Demonstration of TOM+FDH and TOM biosensors for measurements of 92 μM toluene solutions: TOM+FDH biosensor was performed for three times together with 1 M formate regeneration for 24 h twice in between each measurement. TOM biosensors were controls with no regeneration treatment applied

The subsequent results (Figure 5.1) suggested successful biosensor activity regeneration of TOM+FDH biosensors since the signal from third measurement of same biosensor was about 90% of initial signal. The control TOM biosensor signals decreased after each measurement since the intracellular NADH was consumed without any regeneration. However, the absolute signal of TOM+FDH biosensor was about 1/3 of that of TOM biosensor at the first measurement. The less absolute signal could indicate that the total TOM enzyme expressed in TOM+FDH whole cells was less than control TOM cells, since the introduction of FDH plasmid in TOM cells deviated partial energy and resource to FDH plasmid expression when both plasmids were induced by IPTG.

5.4.2 Plasmid DNA extraction and agarose gel electrophoresis

FDH plasmids were first transformed into TOP10 *E. coli* cells in order to retain and amplify the plasmids. FDH plasmids DNA were extracted and separated after successful transformants were grown and cultured in LB+Amp medium. Agarose gel electrophoresis was performed to verify the FDH plasmids DNA presence in the TOP10 *E. coli* cells.

The results (Figure 5.2) showed three obvious bright bands (nick, linear and supercoiled DNA) for both duplicates samples. The plasmids DNA samples ran from the top part (negative electrode) to the bottom part (positive electrode) of the gel image, driven by electric current since the phosphate backbones of plasmids DNA were negatively charged. Small DNA strands moved faster through gel than large DNA strands, while plasmid DNA in supercoiled form moved faster than the other two formations.

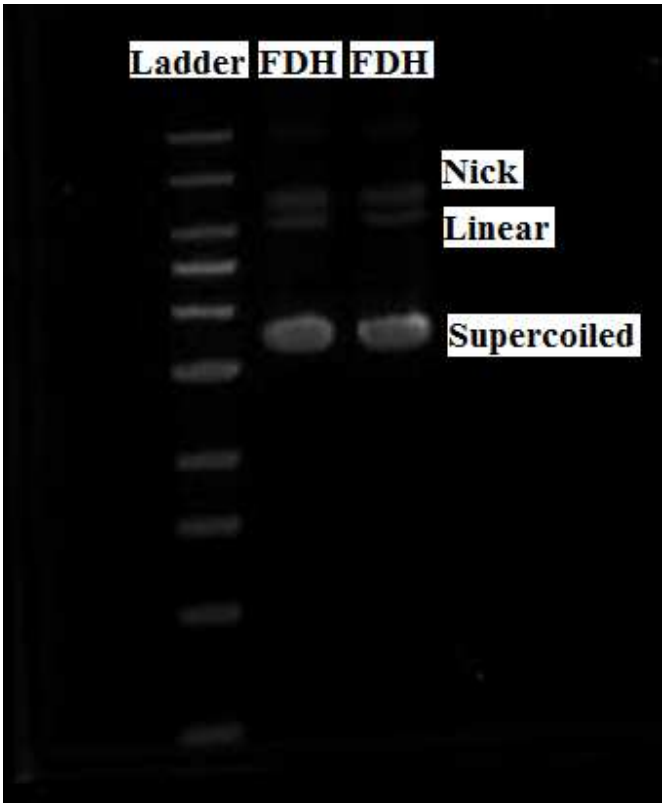


Figure 5.2 – Agarose gel electrophoresis image of plasmids DNA extracted from successful TOP10 *E. coli* transformants: The ladder used was 1 kb DNA ladder from Biolabs kit

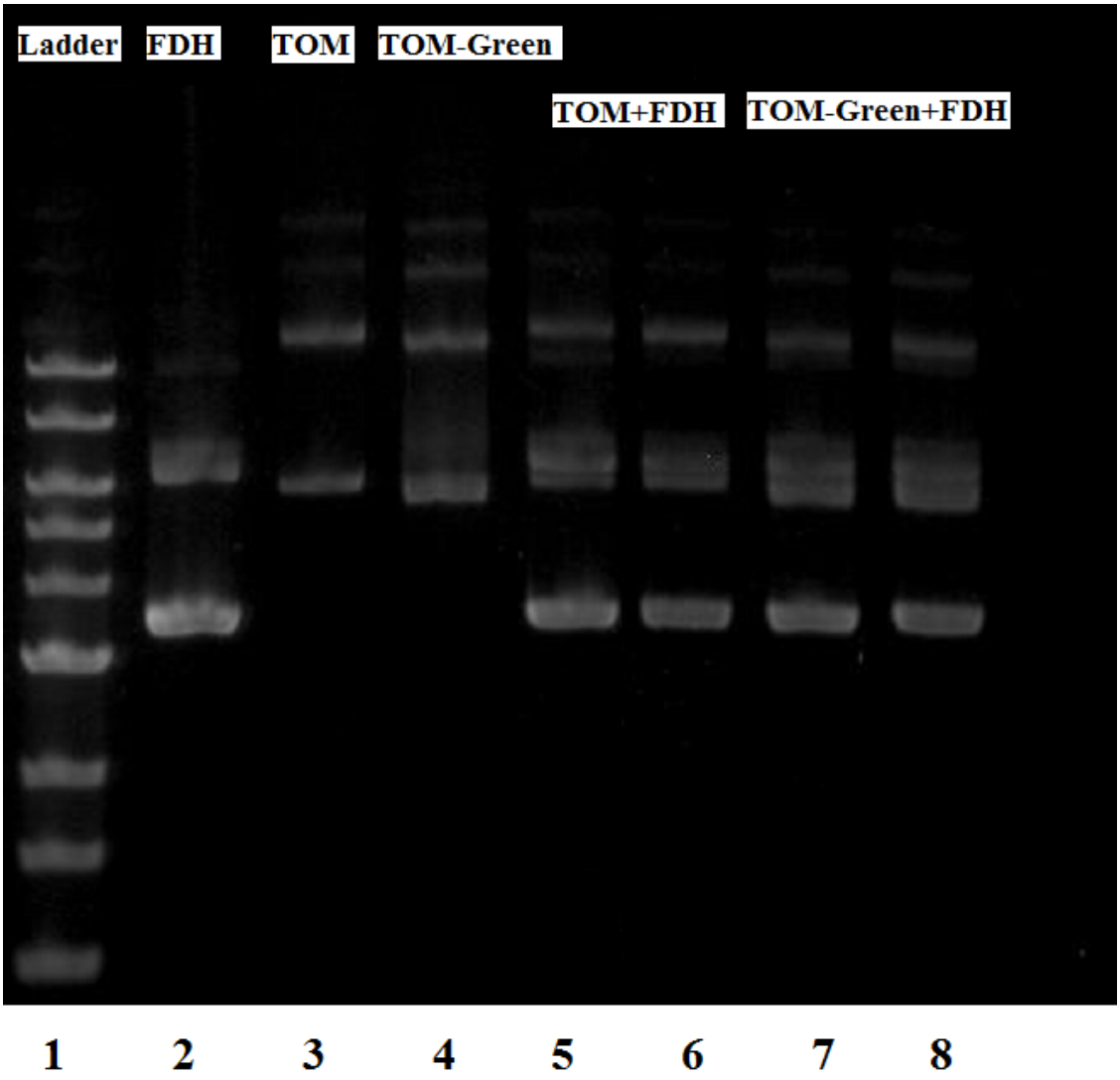


Figure 5.3 - Agarose gel electrophoresis image of plasmids DNA listed as following: 1 - 1kb ladder, 2 - FDH, 3 - TOM, 4 - TOM-Green, 5 and 6 - TOM+FDH, 7 and 8 - TOM-Green+FDH.

Plasmids DNA from TOM+FDH and TOM-Green+FDH transformants were extracted and separated under the same procedure and subjected to agarose gel electrophoresis together with control DNA of TOM plasmid only, TOM-Green plasmid only and FDH plasmids only. The results (Figure 5.3) suggested that TOM+FDH and TOM-Green+FDH transformants contained FDH plasmid and their original plasmids. Hence, the biosensors constructed with TOM+FDH and TOM-Green+FDH should have potentials of activity regeneration with the presence of formate.

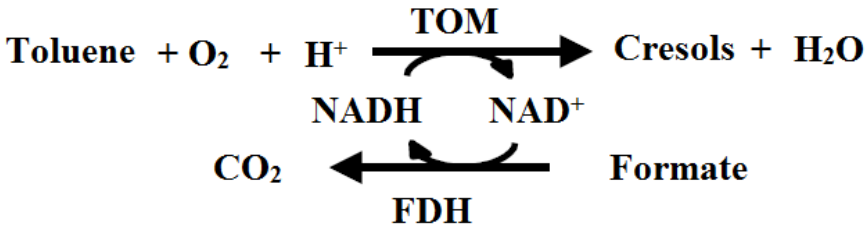
5.4.3 Biosensor activity retention and regeneration

The biosensor activity retention and further regeneration is of great interest in this oxygen-based biosensor since the central enzymatic reaction for detection is cofactor related. However, intracellular NADH levels in whole cells immobilized on biosensors decreases overtime due a variety of different reasons, such as absence of energy source, denaturation, maintenance metabolism of viable cells. In addition, NADH levels drops significantly after each biosensor usage through the monooxygenase reaction.

In previous study, the 1 M formate regeneration for 24 h resulted in 25% increase of biosensor signal compared with controls (Zhong et al. 2010), which was encouraging as the first regeneration approach for this type of biosensors. Prompt NADH regeneration via FDH plasmid transformation is a great alternative since the introduction of secondary FDH plasmid could immensely increase the FDH regeneration rate with expression of FDH enzyme.

5.4.3.1 Formate concentration

Formate served an electron donor in the following redox reaction for NADH regeneration:



Three different formate concentrations (1 M, 0.5 M, and 0.1 M) with measurement solution were supplied during TOM+FDH biosensors regeneration in 24 h at room temperature (20 °C). All the biosensors were subjected to measurement for 92 µM toluene solutions for three times with two regeneration period between each measurement, while control TOM+FDH biosensors were supplied with measurement solution for regeneration only. The results showed biosensors regeneration levels were the same for three formate concentrations (Table 5.1), indicating 0.1 M formate concentration would be sufficient for biosensor regeneration.

Formate concentrations (M)	TOM+FDH signals (counts)		
	Initial signal	2nd signal	3rd signal
1.0	**320**	**300**	**290**
0.5	**310**	**290**	**280**
0.1	**320**	**290**	**290**
control (no formate)	**310**	**120**	**20**

Table 5.1 - The effect of formate concentrations in biosensor regeneration at 20 °C for 24 h

5.4.3.2 Regeneration time

The initial setup for biosensor regeneration time with formate was 24 h. The time was primarily influenced by how fast formate could diffuse into the intracellular area and the reaction rate of formate dehydrogenase catalyzing the redox reactions. The formate diffusion was contributed to the diffusion constant as well as the concentration gradient if formate concentration was uniformly distributed in bulk solution. The experiments setup was same compared with previous experiments in section of formate concentration, except the formate concentration was fixed at 0.1 M while three different regeneration periods were investigated.

Regeneration time (h)	TOM+FDH signals (counts)		
	Initial signal	2nd signal	3rd signal
4	**310**	**200**	**90**
24	**300**	**280**	**270**
48	**300**	**310**	**300**

Table 5.2 - The effect of regeneration time in biosensor regeneration with 0.1 M formate at 20 °C

The results (Table 5.2) showed that biosensor regeneration for 24 h or more had about the same regeneration effect while 4 h regeneration time was insufficient for complete regeneration.

5.4.3.3 Biosensor activity retention

Fresh made biosensors with TOM+FDH cells were stored at 20 °C in measurement solution supplemental with 0.1 M formate solutions prior to analysis. All the biosensors were subjected to measurement for 92 µM toluene solutions for only once. The results showed that TOM+FDH

biosensors retained about 90% of their activities in two weeks after stored at 20 °C in measurement solution supplemental with 0.1 M formate, while original TOM cells completely lost activities in a week. Further experimental data suggested that TOM+FDH biosensors could retain about 70% of their activities after stored for a month, while typical microbial biosensors could last no longer than a week after construction (D'Souza 2001; Li et al. 2008; Willardson et al. 1998).

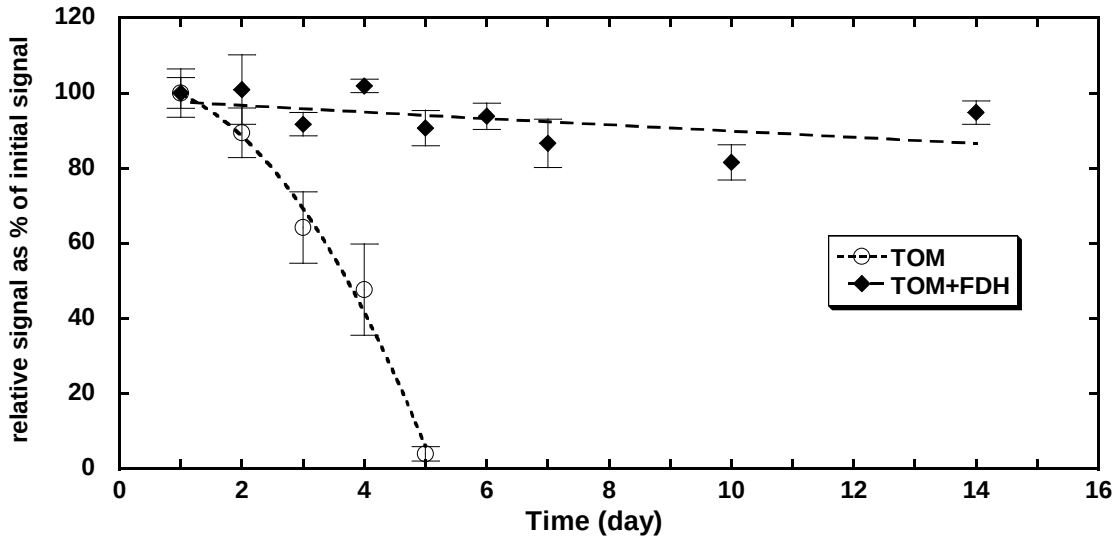


Figure 5.4 - TOM+FDH and TOM biosensors activity retention at 20 °C in measurement solution supplemental with 0.1 M formate. Error bars represent ±1 standard deviation. The average relative standard deviation over all data points shown was 6.5%.

5.4.3.4 Biosensor regeneration after usage.

Fresh made biosensors with TOM+FDH cells were performed for regeneration after usage overtime. At intervals, each biosensor was measured with 92 µM toluene solutions once and regenerated with 0.1 M formate subsequently at 20 °C for 24 h before the next analysis. The results suggested that TOM+FDH biosensor maintained about 80% of their activities after being used and stored at high temperature. The success of biosensor regeneration was of great

importance since most microbial biosensors especially induction-based biosensors were only good for one-time assaying (Kim et al. 2005; Li et al. 2008; Willardson et al. 1998).

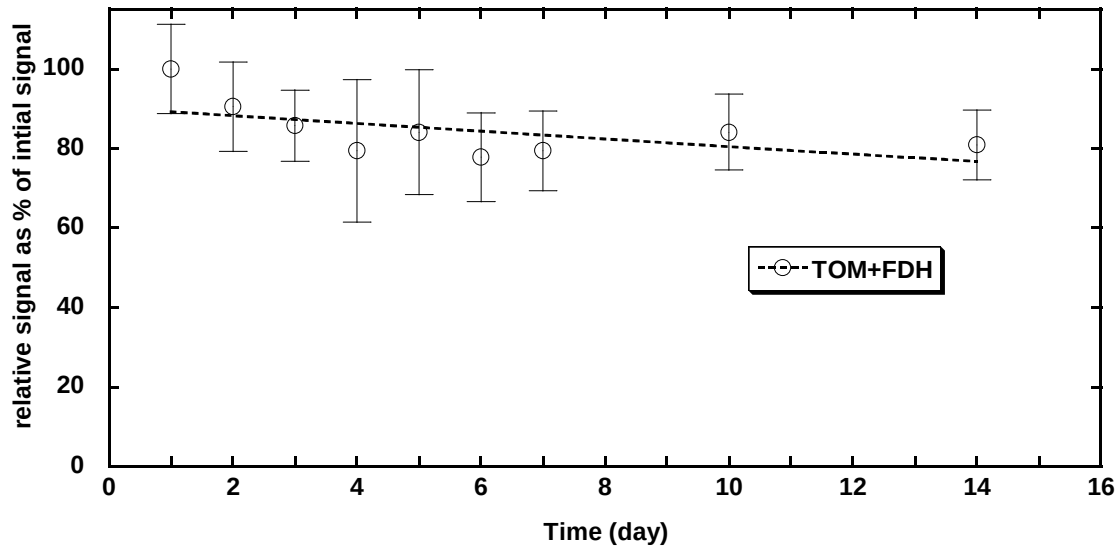


Figure 5.5 - TOM+FDH biosensors regeneration after repeated usage at 20 °C in measurement solution supplemental with 0.1 M formate. Error bars represent ±1 standard deviation. The average relative standard deviation over all data points shown was 8.7%.

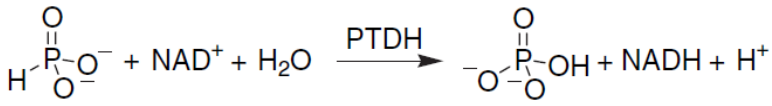
5.4.3.5 TOM-Green+FDH biosensor

Similar experiments (10 µg/L of TCE solution instead of 92 µM of toluene solution) on biosensor activity retention and regeneration were performed for biosensors made with TOM-Green+FDH cells. The activity retention experiments showed a similar trend as TOM-Green+FDH biosensor retained 90% of their activities after being stored in measurement solution supplemental with 0.1 M formate at 20 °C for 2 weeks. However, the biosensor regeneration showed that TOM-Green+FDH biosensors activities were regenerated at limited levels, and became inactive shortly after multiple usage, indicating that the formation of toxic intermediate TCE epoxide damaged the intracellular enzyme system (Lee et al. 2006; Rui et al. 2004; Vlieg

and Janssen 2001) at some degree after each measurement and resulted in complete loss of biosensor activities due to the accumulation toxic effect.

5.4.4 Discussions alternative NADH regeneration system

Phosphite dehydrogenase (PTDH) is a unique, aerobic enzyme that oxidizes inorganic phoshpite to phosphate and reduces NAD^+ to NADH, shown as following equation (Relyea and van der Donk 2005).



The catalytic efficiency of PTDH with NAD^+ in wild-type *Pseudomonas stutzeri* WM88 was about the same as that of FDH with NAD^+ (Johannes et al. 2005; Woodyer et al. 2006). The subsequent mutants of PTDH family were primarily focused on improving the catalytic efficiency of PTDH with $NADP^+$ since wild-type PTDH strains had a preference for NAD^+ over $NADP^+$ by about 100-fold (Woodyer et al. 2006).

Direct addition of PTDH gene into TOM or TOM-Green via plasmid transformation could be adapted from previous experience on FDH plasmid transformation. However, it could be different in transformation procedure design since PTDH plasmid consists of a T7 promoter-based expression system. The transformation was conducted by add PTDH plasmid in a competent cells with T7 promoter-based expression system (e.g. BL21 Star™ (DE3) from Invtrogen), transforming extracted TOM or TOM-Green plasmid DNA into the successful PTDH transformants subsequently.

Compared with FDH system, PTDH based regeneration system has similar features such as inexpensive substrate, easily removable byproduct, and both substrate and byproduct are harmless to enzymes (Woodyer et al. 2006). Hence, development of biosensor made with TOM+PTDH

transformants or TOM-Green+PTDH would be another promising alternative in solving the NADH depletion by prolong storage time or repeated measurements overtime.

5.5 Conclusions

The TOM+FDH biosensor developed in this study has better activity retention as well as regeneration capability compared with TOM biosensor. The success of biosensor construction with TOM+FDH transformants is considered as another solid proof-of-concept for fiber optic enzymatic oxygen-based biosensor design, which could be further broadened to other alternative enzyme systems for intracellular NADH regeneration. The TOM+FDH biosensor overcomes the limitation of NADH consumption overtime and thus extends the potential application of this type of biosensors to a new level. Traditional microbial biosensors are often limited to repeated measurements, especially for induction-based luminescent biosensors (Endo et al. 2008; Lee 2008; Li et al. 2008; Reardon et al. 2009b), which could often be used only once. The PTDH system discussed in this study is a promising NADH regeneration alternative and has been of great interest in recent years (Relyea and van der Donk 2005; Woodyer et al. 2006). New PTDH-based biosensor regeneration system would be achievable once suitable PTDH mutants are developed.

This is the first report of an enzymatic biosensor with the ability of NADH regeneration supplemental with formate. This work shows great potential of fiber optic enzymatic biosensor after successful integration with intracellular NADH regeneration system, resolves the problems of NADH consumption overtime during storage as well as multiple utilization with same biosensors. This report also highlights the advantages of chemical approach of plasmid transformation as a simple, low-cost, and reliable method. The consequent development of biosensors for toluene and other environmental contaminants would broaden the scope of biosensor application as well as providing a continuous, *in situ* with quantitatively measurements techniques for groundwater and wastewater monitoring.

5.6 Acknowledgments

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5.7 Reference

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Chapter 6 Development and Application of Fiber Optic Enzymatic Biosensors Array for Measurement of Halogenated Alkanes Mixture

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6.1 Abstract

Fiber optic enzymatic pH-based biosensor array was developed in this study to provide continuous, *in situ*, regeantlenss measurements. An array-based system was constructed and characterized for the measurement of haloalkanes mixture concentrations in aqueous solutions. The biological recognition elements were consisted of three different dehalogenases expressed by *Escherichia coli*, while an optical fiber coated with a pH-sensitive fluorescent dye served as the transducer. Haloalkanes were detected based on the enzymatic reaction catalyzed by dehalogenase, which resulted in the generation of pH and changes in the fluorescence intensity. The biosensor array measurement was conducted by an 8-channel optoelectronic detector. Different measurement principle was discussed and linear approach appeared to be simple but less accurate due to the inherent competitive inhibition during biosensor array measurement. Nonlinear approach described the array system with precision but in sacrifice of efficiency. A multivariate chemometric approach was performed for measurement of a 3X3 array system with 24 different concentration combinations. The results showed good consistency between calculated values and true values in computing two analytes concentration, while the third analyte have more variation because of outliers during measurements. This is the first report of an

enzymatic pH-based biosensor array. The design concept of this pH-based biosensor has the potential to broaden biosensor array applications in environmental monitoring and related areas

6.2 Introduction

Biosensors have been developed and used for a wide variety of applications, such as environmental, clinical, and medical monitoring, industrial process control, and many other fields. A biosensor is a device integrating a biorecognition element with a suitable transducing system to produce a quantifiable and processable signal in proportion to the concentration of the analyte in a sample (D'Souza 2001; Nakamura and Karube 2003). Many biosensors are normally designed for measurement of single analyte in a sample but incapable of detecting multianalyte in mixture sample due to the limitation of single-channel design (Tsai and Doong 2005).

Array-based biosensors are generally consist of multiple detection elements or channels and able to provide high-throughput analysis and a means of quantitatively assessing matrix effects with minimal preparation of complex samples (Ligler et al. 2007).Biosensor array systems have been developed over decades with a variety of application in clinical and environmental monitoring (Bally et al. 2006; Lucarelli et al. 2008; Rowe et al. 1999a; Rowe et al. 1999b; Taitt et al. 2005; Tsai and Doong 2005). Enzyme-based biosensor array system are often considered less popular than immunoassay and other binding-based array system since chemical cross-talk between biosensors, especially at low anaylte concentrations, may interfere the channel-to-channel detection (Palmisano et al. 2000; Suzuki and Akaguma 2000). However, binding-based techniques are lack of potential in continuous monitoring since these detections often occur with the sacrifice of irreversible binding (D'Souza 2001; Monk and Walt 2004). Hence, enzymatic biosensor array is yet promising in the scope of continuous, *in situ* monitoring of mixture measurement.

The goal of this research to develop a ternary fiber optic enzymatic biosensor array and study optimal approaches based on different kinetic models. The ternary array system consisted

of three dehalogenases (DhlA (Campbell et al. 2006), DhaA (Prokop et al. 2003), and LinB (Nakamura et al. 2006)) and their corresponding halogenated alkanes (1, 2-dichloroethane, 1-chlorohexane, and 1, 2-bibromoethane), with pH-based optical transducers (pH optodes) based on a fluorescent indicator chemical that exhibits reduced fluorescence intensity as pH decresed since the protonated form of fluorescent indicator emited less fluorescence (Leiner and Hartmann 1993). In the presence of halogenated alkanes, dehalogenase catalyzed the following reaction where R represents the alkane function group and X refers to the halogen element:

$$R\text{-}X + H_2O \rightarrow R\text{-}OH + H^+ + X^-$$

The dehalogenation reaction caused a decrease in pH within sensor tip region and corresponded to the decrease in fluorescence.

The dehalogenases used in this study have been well-studied and represented suitable biorecognition components for this ternary biosensor array system, since each dehalogenase had its unique catalytic activity towards different analytes (Damborsky and Koca 1999; Marek et al. 2000; Nagata et al. 1997; Prokop et al. 2003). However, the competition among different analytes over the limited catalytic active sites for each enzyme could also result competitive inhibition that increased the complexity of data analysis and quantification. A number of different approaches have been discussed in this research to simplify the data calculation with minimal measurement required.

6.3 Materials and methods

6.3.1 Chemicals

1, 2-dichloroethane (DCA) (99%. v/v), 1, 2-dibromoethane (EDB) (99%. v/v), 1-chlorohexane (CH) (99%. v/v), poly (vinyl alcohol) (MW: 30,000-70,000. 4% in H_2O), fluoresceinamine isomer I, cyanuric chloride (≧98.0%. (T)), glutaraldehyde solution (50%. w/v), alginic acid (low viscosity, sodium salt) and isopropyl β-D-1-thiogalactopyranoside (IPTG) were

purchased from Sigma-Aldrich. All solutions were prepared with deionized water and when necessary their pH was adjusted with a NaOH or HCl solution. DCA, EDB and CH standards were prepared by manual sequential dilution from pure products (99%. v/v).

6.3.2 Bacterial strains and growth conditions

The biocomponent of the biosensors, haloalkane dehalogenase DhlA, was expressed in *Escherichia coli* strain BL21 (DE3) harboring the plasmid pGELAF^{+} with the *dhlA* gene from *Xanthobacter autotrophicus* GJ10 (Janssen et al. 1989), while the DhaA and LinB were expressed in similar *Escherichia coli* strains with *dhaA* gene from *Rhodococcus rhodochrous* NCIMB 13064 (Kulakova et al. 1997) and *linB* gene from *Sphingomonas paucimobilis* UT26 (Nagata et al. 1993) respectively. E. coli cultures were grown aerobically on agar plates made from Luria-Bertani (LB) medium with 20 g/L bacto-agar (Difco) and ampicillin (100 mg/L) at 37 °C for 48 h. A test tube holding 2 ml LB medium supplemented with 100 mg/L ampicilin was inoculated from a single colony on an agar plate and shaken overnight at 37 °C and 200 rpm. The culture was then transferred to a flask containing 200 ml of the same LB-Amp medium and shaken at 37 °C and 200 rpm. Cell concentration was measured as culture absorbance at 600 nm (optical density at 600 nm, OD600) with a spectrophotometer (Spectronic® 20 Genesys™, Thermo Electron Corporation). IPTG solution was prepared with deionized water and added to the culture with a final concentration of 1 mM to induce dehalogenase biosynthesis in the early exponential growth phase (OD600 of 0.6). The culture was collected 4 h after IPTG induction, centrifuged and resuspended in 20 mL of a solution containing 10 mM phosphate buffer saline at pH 7.4 and stored at 4°C until further use.

6.3.3 Biosensor tip construction

Each biosensor tip consisted of a layer of immobilized one type of halogenase over an optical pH sensor (pH optode). Each pH optode was constructed from a 25-cm section of

polymethylmethacrylate (PMMA) optical fiber terminated with a straight tip (ST) connector. The fiber jacket was removed from 1 mm of the distal end (non-connector terminated), and then the fiber was polished with 2000-grit and 3 μm polishing film (IF-TK4-RP2, Industrial Fiber Optics) to reduce potential light loss due to scattering. The pH-sensitive fluorophore used in this study was synthesized by attaching the fluorescenamine dye molecules onto poly (vinyl alcohol) backbones with the help of cyanuric chloride linking, which was modified from the methods of Wangbai and coworkers (Murray 1989). 2 μL of the resulting product was further crosslinked with 2.5% glutaraldehyde in the presence of 6 M HCl to affix the pH-sensitive fluorophore to each optical fiber end.

Previously stored *E. coli* dehalogenase whole cells were centrifuged and mixed with sodium alginate solution (2.5%) in a cell-to-alginate ratio (wet cell mass : alginate solution) of 1:1 (w/w) unless otherwise specified. Biosensors were constructed by placing 2 μL of the cell-alginate mixture on the tip of a pH optode and then immersing the optode in 0.47 M calcium chloride solution for 30 min at 0 °C. All biosensors were stored at 0 °C in a solution of 0.15 M NaCl and 0.025 M $CaCl_2$ at pH 7.0 (hereafter referred to as “measurement solution”).

6.3.4 Biosensor array and instrumentation

Biosensor array was made with different halogenase biosensors bundled together with a reference pH optode in center. Biosensor array instrumentation included an 8-channel Optoelectronic measurement device that integrated excitation light source (470 nm) and photomultiplier tube (PMT) detector. The 470-nm excitation light from each channel was controlled by data acquisition module inside the device as only one channel was able to gain excitation light at a time. Biosensor was directly connected to each channel via an ST connector. The fluorescent emission light (peak at 520 nm) from the biosensor was directed back into the detection channel and measured by the inherent PMT detector. The date collected by the optoelectronic device was transferred to lab computer with appropriate software for data

recording. The data acquisition occurred as excitation light cycled from channel to channel in numerical order with 200 ms intervals in between, when PTM signal for each channel was also integrated. Channel signal was the change in the intensity of the emission light over time correlates to the pH value change in the fluorescent dye layer of the biosensor.

Biosensor array experiments were performed in glass vials (5 mL) containing 4 mL of measurement solution with pH adjust to 7 at room temperature. A small magnetic stir bar was used to agitate the solution thoroughly. The bundled biosensor tips were immersed in this solution, sealed in the glass vial with a rubber septum, and shielded from external light sources. Aliquots (0.1 mL) of a variety of analyte mixture solution with known concentrations were added to the measurement solution after the sensor had produced a steady output, defined as the time when the variation in the output was no larger than the peak-to-peak noise for a period of at least 5 min. All measurements were performed at room temperature unless otherwise specified. Each measurement was performed with a fresh biosensor to distinguish the effect in question (e.g., temperature, pH, cell/alginate mass ratio).

6.4 Results and discussion

6.4.1 Fiber optic enzymatic pH-based biosensors measurements of halogenated alkanes mixture

The initial experiments with the dehalognase biosensor were performed to characterize each enzyme-analyte combos (a total of nine a ternary biosensor array system).DhlA, DhaA and LinB biosensors were calibrated with DCA, EDB, and CH standard solutions respectively using channel 1as single-channel biosensor detection. Four different concentrations for each analyte standard solutions were measured with each type of dehalogenase biosensor in triplicates.

The dehalogenation reaction required no external energy or cofactor to cleave the halogen and happened spontaneously with the presence of analyte and enzyme in aqueous solution

(Campbell et al. 2006; Reardon et al. 2009). Therefore, the reaction could be described as following based on Michaelis-Menten kinetics:

$$V = \frac{V_{max}[C]}{K_M + [C]}$$

Where V was the reaction rate, V_{max} was the maximum reaction rate, K_M was the Michaelis constant and [C] was the analyte concentration. The above equation was simplified when the concentration of analyte was low, [C] << K_M, and a linear relationship between [C] and V was derived, while the reaction rate V (equal to rate of proton generation) had a linear correlation to biosensor signal. Hence, a total of nine different enzyme-analyte combos, the linear correlation parameters were observed as all the biorecognition reaction were assumed with low concentration range that much small than the corresponding Michaelis constant K_M.

$$Biosensor\ signal\ (S) = k \times [C]$$

Analyte	Enzyme		
	DhlA	DhaA	LinB
DCA	**0.60**	**0.09**	**0.18**
EDB	**0.40**	**1.13**	**0.64**
CH	**0.14**	**0.95**	**0.62**

Table 6.1 - k-value (mV / µM) for nice enzyme-analyte combos

6.4.2 Demonstration of the measurement for biosensors array

The measurements of the biosensor array system were performed to measure three-analyte mixture. All eight channels of optoelectronic detector were calibrated with same pH optodes in triplicates by measuring a known pH value change from 6.8 to 6.7. Channel to channel variations were adjusted subsequently prior to measurements. The ternary mixture with known concentrations were then measured with biosensor array using 4 channels in detector with DhlA,DhaA, LinB, and reference pH optode respectively. Three different mixture concentrations

(DCA: EDB: CH = 50 µM: 5 µM: 5 µM, 5 µM: 50 µM: 5 µM, and 5 µM: 5 µM: 50 µM) were prepared with standard solutions and 0.1 mL of these mixture aliquots were subjected to biosensor array measurement.

6.4.2.1 Simplified linear approach

Biosensor signal from mixture measurement could be considered as a combination signal that was attributed to every analyte in the mixture during measurement. Therefore, the equations for a system of three biosensors and three analytes could be written as following:

$$S_1 = k_{1A}C_A + k_{1B}C_B + k_{1C}C_C$$

$$S_2 = k_{2A}C_A + k_{2B}C_B + k_{2C}C_C$$

$$S_3 = k_{3A}C_A + k_{3B}C_B + k_{3C}C_C$$

Where S_i is the total signal of biosensor *i*, k_{ij} is the response factor (slope) of biosensor *i* to analyte *j*, and C_j is the concentration of analyte *j*. which was the ideal case of biosensor array measurement in a 3X3 system. The theoretic biosensor signals could then be calculated based on above equations since k_{ij} was known from Table 6.1, C_j was also known from the mixture preparation step.

Biosensor array	DCA : EDB : CH					
	50 µM: 5 µM: 5 µM		5 µM: 50 µM: 5 µM		5 µM: 5 µM: 50 µM	
	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
Channel 1 (DhlA)	**32.9**	**31.0**	**23.5**	**19.0**	**12.1**	**10.0**
Channel 2 (DhaA)	**15.0**	**9.0**	**61.8**	**56.0**	**53.6**	**46.0**
Channel 3 (LinB)	**15.2**	**13.0**	**36.0**	**33.0**	**35.1**	**36.0**
Channel 4 (pH optode)	**0**	**0.1**	**0**	**0.3**	**0**	**0.1**

Table 6.2 - Predicted and experimental values for each channel of biosensor array in 3X3 system based linear approach. Channel 4 was reference channel as pH optode was used to monitoring the bulk solution pH changes.

The results showed in table 6.2 showed that the experimental signals were smaller than the predicted signals, while control channel suggested no significant pH value change in bulk solution during measurements. However, the mismatch between experimental and theoretic data indicated that the linear correlation assumption for biosensor signal in mixture measurement was biased.

6.4.2.2 Competitive inhibition approach

Introducing competitive inhibition kinetics would increase the accuracy of data predication while sacrificing the efficiency since complicated nonlinear equations were often time-consuming to resolve. The reaction kinetics model for enzyme 1 could be written as following by incorporating purely competitive substrate kinetics:

$$V_1 = \frac{V_{max1A}C_A}{K_{M1A} + C_A + \left(\frac{K_{M1A}}{K_{M1B}}\right)C_B + \left(\frac{K_{M1A}}{K_{M1C}}\right)C_C} + \frac{V_{max1B}C_B}{K_{M1B} + C_B + \left(\frac{K_{M1B}}{K_{M1A}}\right)C_A + \left(\frac{K_{M1B}}{K_{M1C}}\right)C_C} + \frac{V_{max1C}C_C}{K_{M1C} + C_C + \left(\frac{K_{M1C}}{K_{M1A}}\right)C_A + \left(\frac{K_{M1C}}{K_{M1B}}\right)C_B}$$

Where V_i is the total reaction rate of enzyme 1 (which will generate the signal for biosensor 1), V_{maxij} and K_{Mij} were the maximum reaction rates and Michaelis constants for Enzyme 1 acting on analyte *j*.

The competitive inhibition equations could be further converted into one that would represent the signal from a biosensor, while mass transfer effects was taken in consideration and parameters would no longer be true Michaelis-Menten parameters but rather apparent ones (Bailey 1986; Gomez et al. 2003). The signal of biosensor 1 could be shown as below if the general equation format can be assumed to remain the same:

$$S_1 = \frac{k_{1A}C_A}{m_{1A} + C_A + a_{1AB}C_B + a_{1AC}C_C} + \frac{k_{1B}C_B}{m_{1B} + C_B + a_{1BA}C_A + a_{1BC}C_C} + \frac{k_{1C}C_C}{m_{1C} + C_C + a_{1CA}C_A + a_{1CB}C_B}$$

Where k_{ij} and m_{ij} were the apparent V_{mij} and K_{Mij}, while a_{1ij} exhibited the impact of analyte *j* on the reaction rate of enzyme 1 on analyte *i*.

Although the complexity of above equation was could be reduced by making additional assumptions (e.g., $a_{1ij} = a_{1ji}$) , the competitive inhibition model for a 3X3 system would consist at least 18 or more unique parameters, which required additional calibration measurement in order to provide accurate analysis via biosensor array. In addition, high-throughput-measurement would become necessary as the dimension of complexity for biosensor array increased rapidly by extra biosensors or analytes being introduced into the array system.

6.4.2.3 Chemometric approach for biosensor array

A multivariate chemometric approach was performed based on linear correlation assumption for a 3X3 biosensor array system. All 8 channels of optoelectronic detector were used as each type of biosensor was duplicated as well as the control pH optode. The mixture concentration pattern was designed with a total of 24 concentration combinations, including inherent replicates. A ternary biosensor array (DhlA, DhaA and LinB) system measured all 24 concentrations and calculated the predicted concentrations in comparison to the known values based on multi-linear regression.

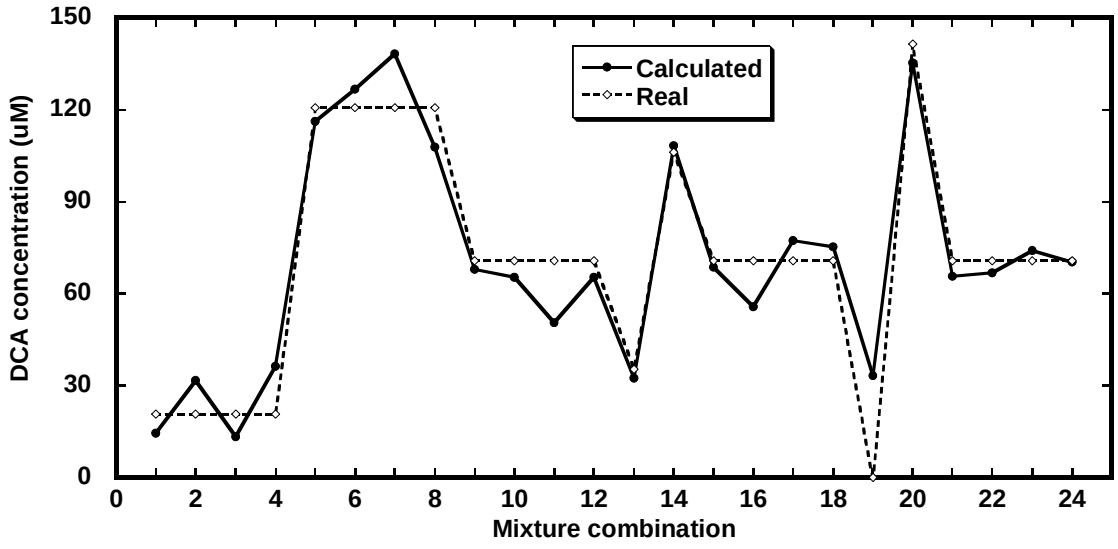

Figure 6.1 - Comparison between calculated and real DCA concentrations by cheometric approach

The results showed that calculated DCA concentrations were far from the true DCA values in the mixture at quite a few data points (Figure 6.1). In addition, the root mean square error (RMSE) was 11.1 µM, indicating the measurement of DCA concentration was inaccurate in this case. The variation between predicted and real DCA values could be affected by abnormal biosensor output, which could also be easily found in Figure 6.1: In mixture 19, the true value of DCA was zero while the calculated value of DCA was around 30 µM.

The other two analyte concentration calculation (Figure 6.2 and 6.3) matched better than that of DCA, with much smaller RMSE values for both EDB (3.1 µM) and CH (1.8 µM).

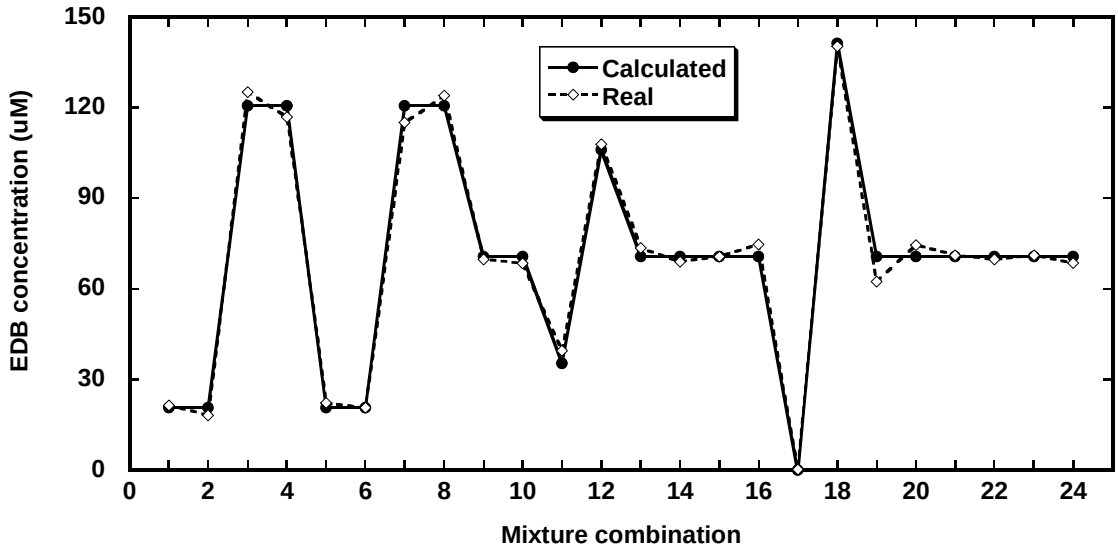

Figure 6.2 - Comparison between calculated and real EDB concentrations

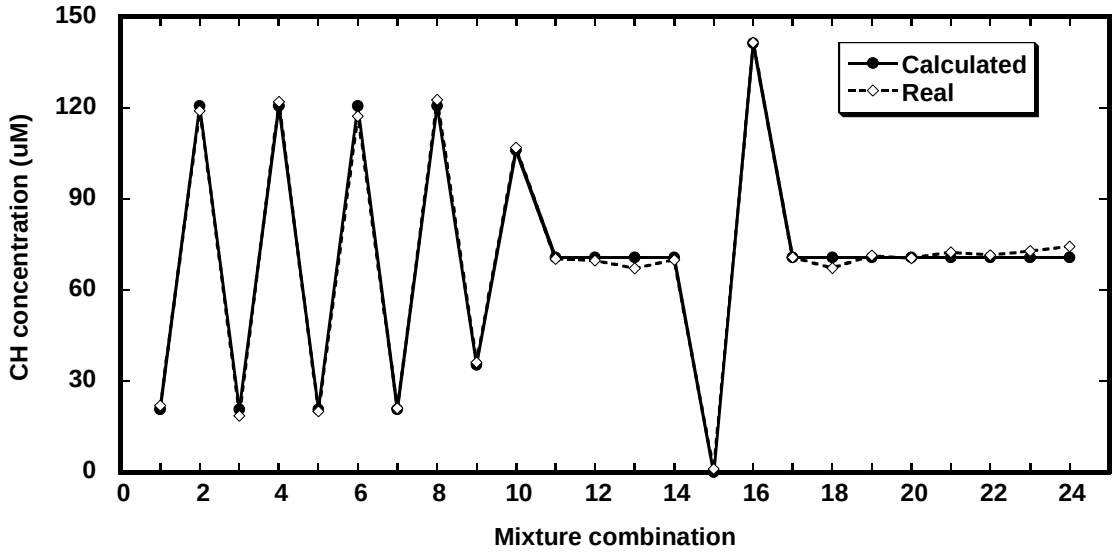

Figure 6.3 - Comparison between calculated and real CH concentrations

6.5 Conclusions

The fiber optic enzymatic pH-based biosensor array developed in this study provides a rapid, reagentless, and quantitative method for measurement of haloalkanes mixture. This biosensor array approach showed great potential compared with other binding-based immunoassay (Ligler et al. 2007; Rowe et al. 1999a; Rowe et al. 1999b), DNA biosensor array (Elsholz et al. 2009) or microchips (Sakaguchi et al. 2007)with the highlight of continuous and repeatable usage of array since the enzymatic reaction required no cofactor and could be functional even under cell death as long as the enzyme were intact(Campbell 1998). Different data acquisition and analysis approach were discussed and complex approach (e.g. nonlinear approach) was close to the reality in sacrifice of efficiency, while simplified approach, such as linear approach, decreased the difficulty of data analysis by giving up accuracy. Mathematical and statistical tools such as chemometrics (Yonzon et al. 2004), artificial neural network (Gutes et al. 2005), principal component regression (Sandstrom et al. 2001; Thompson et al. 2003), or combination among

these methods (Bachmann and Schmid 1999; del Valle 2010) could reduce the magnitudes of complexity in biosensor array system with appropriate assumption.

This is the first report of an enzymatic biosensor array for measurement of haloalkanes. Along with the successful demonstration of this biosensor array design concept, this study also highlights the need to select approximate measurement principle with a balance of accuracy and efficiency in multivariable measurement. The development of biosensors array provides an alternative in quantitative mixture assessment as well as capable of continuous and *in situ* measurement, offering promising feature in groundwater monitoring, industrial process control and other related areas.

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Chapter 7 Conclusions

The results of this research project presented in this dissertation have emphasized the advantages of fiber optic enzymatic biosensors and biosensor array for measurement of chlorinated ethenes as well as other analyte of interests in environmental monitoring and other related areas. This research started with oxygen biosensor measurement of toluene with the TOM biosensor, as a proof-of-concept for this new type of fiber optic enzymatic biosensor. The success of the TOM biosensor for toluene measurement was essential for the following TCE biosensor and biosensor optimization, since it showed the advantages of fiber optic enzymatic biosensor system as advanced measurement principle. In addition, potential improvement and optimization was also addressed. Nevertheless, this research has not yet illustrated the measurement of chlorinated ethene mixtures with a biosensor array, since the oxygen biosensor for TCE and other chlorinated ethenes was less robust and required further optimization in performance. Instead, the measurements of halogenated alkanes with pH-based biosensors were performed to mimic the similar measurement scenario in order to provide information of modeling and analysis.

The TOM-based optical biosensor provides a rapid, reagentless, and simple method to detect toluene in aqueous solutions. This biosensing concept could be extended to other analytes by using different mono- or dioxygenases. Compared with other binding-based immunoassay or induction-based bacterial biosensors, this biosensor design has the advantages of each method: The TOM-based biosensor provided a linear response to toluene over a wide concentration range, as is the case with most immunoassays, while the induction-based biosensors normally have a nonlinear calibration curve with a small linear range. Furthermore, the TOM-based biosensor could continuously monitor the change of analyte concentration as can induction-based

biosensors, while the immunoassay methods are usually discrete. Although activity retention for the TOM-based biosensor was limited by NADH consumption, a method to partially regenerate the signal was demonstrated. In contrast, the measurements of induction-based biosensors must be conducted in growth medium so that the energy for transcription and translation can be provided.

The development of the TOM-Green biosensor demonstrates a fast, reagentless measurement of TCE in aqueous solution with simplicity. The design concept of this biosensor could become a promising alternative in detection of other chlorinated ethenes as well as common environmental pollutants with the help of appropriate mono- or dioxygenases. Compared with recent flow-injection biosensor or typical electrochemical sensor, this enzymatic biosensor design has unique advantages. The TOM-Green biosensor could not only provide a much lower LOD than the flow-injection biosensor or other electrochemical sensor, which showed advantages of enzymatic reaction-based detection of chlorinated ethenes, but also monitor the change of analyte concentration continuously while the flow-injection based biosensor and electrochemical-based sensor are usually discrete. In addition, the success of the TOM-Green/GSHI and TOM-Green/EchA biosensors in solving TCE epoxide toxicity strengthens the promising future of biosensor for TCE detection.

The TOM+FDH biosensor design integrated the NADH regeneration capability of better activity retention and regeneration while maintaining the advantages of the previous TOM biosensor. The success of the biosensor construction with TOM+FDH transformants is considered as another solid proof-of-concept for fiber optic enzymatic oxygen-based biosensor design, which could be further broadened to other alternative enzyme systems for intracellular NADH regeneration. The TOM+FDH biosensor overcomes the limitation of NADH consumption over time and thus extends the potential application of this type of biosensors to a new level. Traditional microbial biosensors are often limited to repeated measurements, especially for induction-based luminescent biosensors, which could often be used only once.

The fiber optic enzymatic pH-based biosensor array developed in this study provides a rapid, reagentless, and quantitative method for measurement of a haloalkane mixture. This biosensor array approach showed great potential compared with binding-based immunoassays, DNA biosensor arrays, or microchips with the advantage of continuous and repeatable usage of the array since the enzymatic reaction required no cofactor and could be functional even under cell death as long as the enzymes were intact. Different data acquisition and analysis approaches were discussed. The more complex approaches (e.g., nonlinear approach) were more accurate at the expense of efficiency, while simplified approaches, such as linear approach, decreased the difficulty of data analysis by giving up accuracy. Mathematical and statistical tools such as chemometrics, artificial neural network, principal component regression, or combinations among these methods could reduce the magnitudes of complexity in biosensor array system with appropriate assumptions.

Chapter 8 Opportunities for Future Work

Future research efforts should continue to improve the analytical characteristics of current fiber optic enzymatic biosensor and optimize the biosensor array system with appropriate data analysis approach.

Current fiber optic enzymatic biosensors provide only a few choices of enzyme as biocomponent. Enlarge the enzyme library would greatly extended the potential of biosensor application in many areas, such as food, clinical monitoring, as well as industrial process control. The monooxygenase applied in TOM-biosensor and TOM-Green biosensor served as a perfect template for genetic modeling since its catalytic capability. In fact, quite a few TOM-enzyme mutants have been acquired in the lab with only limited preliminary analysis data. The subsequent trials on these TOM mutants may provide alternative enzymes for different analyte of interests using the same biosensor design concept. In addition to existing TCE enzymes, enzymes for other chlorinated ethenes should also be examined and made into biosensor for test since there are at least 5 different forms of chlorinated ethenes.

The NADH regeneration work require further tailoring in order to use oxygen-based biosensor in practical since one-time usage biosensor is considered as an assay rather than a sensor which could provide continuous monitoring. PTDH system would be the next on the to-do-list since its high efficiency in NADH regeneration. There are several successful PTDH mutants reported, but most were modified for $NADP^+$ rather than NAD^+. Therefore, choosing appropriate plasmid before transformation would be crucial to avoid trouble in advance.

Another promising and urgent topic in fiber optic enzymatic biosensor is modeling. The current work lacks the support of mathematical model and with a limited understanding of

biosensor response time and other parameters. For example, what is the primary parameter to control the biosensor response time: Biochemical reaction rate or analyte/oxygen diffusion rate or the cell-alginate layer thickness? A developed mathematical model could explain many experimental observations as well make precious prediction which would benefit the future biosensor design concept.

The biosensor array study in this dissertation was limited to a simplified 3X3 system with linear approach. The system used a pH-based system to avoid the consumption of NADH levels after repeated usage. There are a lot of work could be extended at different levels. The most important task for biosensor array system is to find out an appropriate approach to simplify the nonlinear model without sacrificing the accuracy too much. Current linear model with multivariable chemometric method could be valuable when analyte concentration was small as well as the mixture was not too complicated. High-throughput-measurement would be necessary if the system contains more than 10 or even 5 different analytes with unique characteristics. Another important aspect could be developed for biosensor array system is instrumentation, current proto-type (McFOFI) system is too big to be carried out in field, the recent advance of OptiEnz device showed great potential as a portable array detector for fiber optic enzymatic biosensors. Future instrumentation development should be focused on improve the dynamic detection as well as device solution, such as spectrometric based detector.

In addition to biosensor and biosensor array improvement, oxygen optode and pH optode improvement could also be implemented in the future. A variety range of oxygen-sensitive dye and pH-sensitive dye were reported. The current oxygen optode and pH optode are reliable but not the best of choice due to many reasons. Discover a new optode with same or similar transduction mechanism could boost the biosensor performance as well as increase alternative for array system choice. It is interesting to apply two or more than two different types of biosensor with unique measuring principle as an array system.

Appendix I

New pH optode preparation (developed by Dr. Michael Frizsche)

Idea:

Aminofluorescein (FLA) is bound to polyvinyl alcohol (PVA) with cyanuric chloride (CC). Before this reaction the carboxyl groups of the dye are protected to prevent them from reacting with the cyanuric chloride.

Optode preparation:

The solvents used in this procedure should be dried before use because acid chlorides will react with water. Acetone can be dried by storing it over $CaCl_2$ (anhydrous) or molecular sieve. Molecular sieve can be used to dry DMF, too.

Work in hood because cyanuric chloride and DMF are toxic by inhalation.

	Procedure	*Desired Reaction*
1)	**Protection of the carboxyl groups**	
	– Dissolve 2 mg FLA in 700 l DMF.	*Thionyl chloride converts carboxylic acids into the corresponding acid chlorides. This reaction is catalyzed by DMF.*
	– To this solution add 300 l DMF containing 3 l thionyl chloride. (For example, dissolve 10 l thionyl chloride in 1 ml DMF and use 300 l of this solution.)	
	– Heat to 50 °C for one hour.	
	– Add 17 l of water free ethanol.	*The ethanol reacts with the FLA-dichloride forming the ethyl ester.*
	– Heat to 50 °C for two hours.	
2)	**Binding of CC to PVA**	
	– One hour before step 1 is completed, add 5 mg CC in 1 ml acetone to 50 mg PVA.	*The cyanuric chloride binds to the PVA.*
	– Stir or shake at 50 °C for one hour.	
	– Allow the PVA to sediment and remove the solvent.	*Unbound cyanuric chloride is removed.*
	– Add 1 ml acetone, mix, and remove the solvent again.	
3)	**Binding of the protected FLA to the PVA-CC conjugate**	
	– Add the solution of the FLA-ester to the PVA-CC.	*The amino group of the protected FLA binds to the CC that is bound to PVA. Unlike the pure PVA the product is hydrophobic enough to dissolve in DMF.*
	– Stir or shake at 50 °C over night. The solid will dissolve after some hours.	
4)	**Immobilization on the optic fiber**	
	– To 25 l of the product solution obtained in step 3 add 5 l of an 2.5 % solution of glutaraldehyde in acetone (prepared from a 50 % aqueous stock solution) and 5 l 6 M HCl.	*Glutaraldehyde will crosslink the modified PVA. Hydrochloric acid is used as a catalyst for this reaction.*

	– Mix by pipetting the solution in and out several times.	
	– Place a small drop of the solution on the tip of an optic fiber.	
	– Check the remaining solution to observe the crosslinking process. It will become a gel after a few minutes.	
5)	**Hydrolysis and storage**	
	– When the polymer is crosslinked, place the optode in 0.1 M Na_2HPO_4 for about 2 hours before use. The hydrolysis may be monitored by measuring the fluorescence intensity (see below).	*The ethyl ester is hydrolyzed to obtain back the free acid of the fluorophore which shows a strongly pH dependent fluorescence.*
	– The pH optode can be stored in 0.1 M Na_2HPO_4.	

Testing the optodes

To check if the appropriate amount of indicator was immobilized on the fiber, the optode is connected to a filtered light source (approx. 470 nm) and to the fiber optic spectrometer using a bifurcated fiber. A thicker layer of fluorescent polymer will give higher signal intensities but a slower response to pH changes. Thus, by varying the amount of the indicator the properties of the optode can be adjusted to the requirements of the application within certain limits.

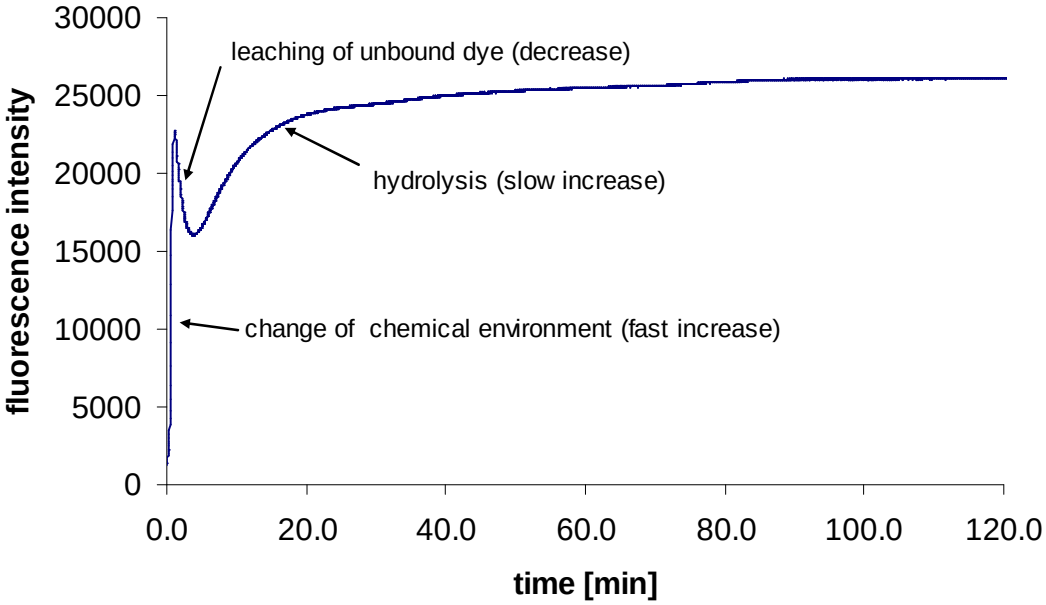
Normally, the fluorescence in a 0.1 M Na_2HPO_4 solution should be strong enough to give signals of several thousand counts at integration times of 1-2 s. If a faster response is required longer integration times can be used to enhance the sensitivity of the spectrometer.

The response time of the optode can be measured by switching between two solutions of different pH.

Additional information:
Fluorescence changes during hydrolysis

The following figure shows the change of the fluorescence intensity during hydrolysis of the immobilized indicator (step 5 of the preparation procedure).

During the first seconds after placing the optode in a 0.1 M Na_2HPO_4 solution a fast signal increase due to the change of the chemical environment of the dye (from DMF/HCl to the aqueous solution) is observed. The following decrease shows the leaching of unbound dye. Finally, the signal increases slowly until hydrolysis is completed.


30000
25000
20000
15000
10000
5000
0
fluorescence intensity
leaching of unbound dye (decrease)
hydrolysis (slow increase)
change of chemical environment (fast increase)
0.0
20.0
40.0
60.0
80.0
100.0
120.0
time [min]

Preparation of oxygen optodes

Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium chloride (RuDPP) is a fluorescent dye whose fluorescence strongly depends on the oxygen concentration. This indicator is immobilized in silicone, a highly oxygen permeable polymer, and attached to an optic fiber to yield an oxygen optode.

Procedure:

- In hood, dissolve 1 mg RuDPP in a few drops of chloroform.
- Mix this solution with 0.2 g silicone (e.g. *Permatex* clear RTV 66B).
- If necessary, add more chloroform until the mixture becomes a highly viscous liquid.
- Place a small drop of this mixture on the tip of an optic fiber.
- Let the silicone harden for about one day. (This process will be faster in a warm and humid atmosphere.)

The concentration of the dye in silicone (here 5 mg/g) and the method of coating the fiber tips (e.g. pipetting, dipping…) may still be subject to optimization. The efficiency of the quenching and consequently the sensitivity of the optodes can be enhanced by adsorbing the RuDPP on silica gel prior to the immobilization in silicone:

- Dry silica gel at 70 to 100 °C for some days.
- Add the silica gel to a solution of RuDPP in chloroform (that has been dried over $CaCl_2$ or molecular sieve) and mix for some minutes.
- Let the silica gel sediment and remove the solvent.
- Mix the product with silicone that has been thinned with chloroform.
- Place a small drop of this mixture on the tip of an optic fiber and let it harden.

The concentration of the dye solution in the adsorption step (e.g. 1 mg/ml) and the amount of silica gel (e.g. 50 mg per ml dye solution) may be optimized as well as the amount of silica gel-dye in the silicone. Too much silica gel may complicate the hardening of the silicone.

If a second dye is used to generate a reference signal, both dyes may be adsorbed on silica gel. This way, hydrophilic dyes as fluorescein can be immobilized in silicone, too.

Testing the oxygen optodes

Signal intensity, response behavior and the sensitivity to oxygen of the optodes can be tested in a single experiment. For this purpose, the optode is connected to a filtered light source (approx. 470 nm) and to the fiber optic spectrometer using a bifurcated fiber. The sensor tip is placed in about 3 ml air saturated water containing a catalytic concentration (traces) of some cobalt (II) salt. After adding 1 ml concentrated Na_2SO_3 solution the oxygen in this solution is rapidly reduced. The intensity of the fluorescence signal is measured before and after the addition of Na_2SO_3. The simultaneous recording of a time scan will show the response behavior of the optode to this switch from air saturation to 0 % oxygen.

General cell growth protocol

	Procedure	*Purpose*
1)	*Prepare LB+agar plate*	
	Dissolve tryptone, yeast extract, sodium chloride and Bacto nutrient agar in 500 ml – 1L DI water. (Ratio: 10 g typtone, 5 g yeast extract, 5 g sodium chloride and 20 g of nutrient agar for 1 L)	*Prepare Medium*
	Mix with magnetic rod in a hot stir plate (40-50 ℃ is enough) to allow full dissolve.	
	Autoclave prepared medium for 30 min liquid cycle	
	Prepare appropriate concentrated antibiotics solution. Dissloved in DI water	*Final Conc of different antibiotics:* *Ampicilin: 100 mg/L* *kanamycin: 100 mg/L*
	After the autoclaved medium cools (if u can handle with your hand), add the concentrated antibiotics soluion	
	Pour 20 ml of LB agar solution into each plate (doing this inside the hood)	
	Seal and stored the plate after the agar solidified	
2)	*Inoculation*	
	Add one drop (1ml) of frozen stock of selected culture to the prepared plate	.
	Spread the cell drop over the agar plate	
	Culture at 37 ℃ / 200 rpm shaker for overnight	
3)	*Growth*	
	Inoculate a separate colony in the plate to a 1 ml of LB+antibioitics medium in a 10 ml test tube or falcon tube	
	Culture at 37 ℃ / 200 rpm shaker for overnight	
	Transfer the culture to 200ml of LB + antibioitics medium in 500 ml of flask to grow cell exponentially	
4)	*Induction*	
	Add IPTG to a final concentration of 1 mM into the medium after cell OD 600 reached 0.6	*It is also a good breakpoint to transfer some culture and store in a 15% glycerol solution for frozen stock*
5)	*Harvest*	
	Harvest the cells after 3-4 hours, and centrifuge the culture to remove extra LB medium	
	Resuspend the cell pellet in 0.1 M PBS solution and store in fridge (4 ℃)	

Chemically [Heat Shock] Competent *E. coli* Cells and Transformation

$CaCl_2$ Method (Longer but More Transformants)

Media to Prepare

At least 60 mL of LB
At least 40 mL of 100 mM $CaCl_2$
At least 4 mL of 100 mM $CaCl_2$ + 15% glycerol
*Autoclave or filter sterilize each solution

**Autoclave 2- 250 mL flasks with foam stoppers and some microcentrifuge tubes

End of Day 1

1. Start a overnight (O.N) culture of *E. coli* cells in 5 mL of LB (usually from a single colony on a plate)

Beginning of Day 2

2. Inoculate 100 μL of the O.N. culture of *E. coli* into 50 mL LB in 250 mL shake flask
3. Grow at 37 C until OD reaches 0.4 to 0.6
4. Place 25 mL in cold 50 mL centrifuge tubes (x4)
5. Place tubes on ice for 30 min, mixing periodically to ensure uniform cooling
6. Centrifuge for 5 min at 4 C and 3000 rpm [important for rotor to be cold before starting]
7. Decant supernatant and resuspend gently in 10 mL of ice cold 100 mM $CaCl_2$
8. Place on ice for at least 30 min [2 hours is optimal]
9. Centrifuge for 5 min at 4 C and 3000 rpm [important for rotor to be cold before starting]
10. Decant supernatant and resuspend gently in 1 mL of ice cold 100 mM $CaCl_2$ + 15% glycerol
11. Place on ice overnight [put ice bucket at 4 C so the ice doesn't completely melt]
12. Ready for transformation or for 200 μL aliquots into cold microcentrifuge tubes which can be flash frozen and stored at -80 C.

Transformations

1. thaw competent cells on ice and add plasmid DNA (100 pg to 10 ng) to 100 μL of cells
2. incubate on ice for 30 min to 1 hr
3. incubate at 42°C for 60 seconds
4. incubate on ice for 2 min
5. Add 900 μL of LB (or SOC) media
6. regenerate cells for 1 h at 37°C with shaking at 225 rpm
7. Spread cells on selective agar plates

Plasmid DNA extraction

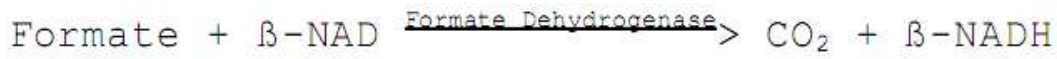
	Detailed procedure	Purpose
Step 1	Pellet 2 ml of cell culture for 5 min using a centrifuge	Remove liquid/solution from the cell culture. (usually the LB medium or the PBS solution used to store cells)
Step 2	Resuspend the pellet with 250 µL of Cell Resuspension Solution (All the solution used in this protocol are from the Minipreps Kits bought from Promega)	
Step 3	Add 250 µL of cell lysis solution to each sample; invert 4 times to mix	Break the cell to allow Plasmid DNA come out
Step 4	Add 10 µL of Alkaline Protease Solution; invert 4 times to mix	Denature the plasmid DNA
Step 5	Add 350µL of Neutralization Solution; invert 4 times to mix	Neutralize the remaining alkaline protease solution
Step 6	Centrifuge at 13,000 rpm for 10 min at room temperature	Pellet the cell debris and anything can't dissolve
Step 7	Insert Spin Colum into Collection Tube, Decant cleared lysate into Spin Colunm	Plasmid DNA is attached to the Spin Column, others are gone with the flow through
Step 8	Centrifuge at 13,000 rpm for 1 min at room temperature Discard flow through and reinsert Column to Collection Tube	
Step 9	Add 750µL of Wash Solution (Ethanol added). Centrifuge at top speed for 1 min and Discard the follow through. Repeat with 250 µL of Wash solution	Wash and purify the plasmid DNA attached to the Spin Column
Step 10	Transfer the Spin Column to a sterile 1.5 ml microcentrifuge tube. Add 100 µL of Nuclease-Free Water to the Spin Column. Centrifuge at top speed for 1 min at room temperature	Elute the plasmid DNA
Step 11	Discard the column and store DNA at – 20 °C	

Plasmid separation via agarose gel

	Detailed procedure	Purpose
Step 1	Pour 0.7% (w/w) agarose gel into 100 ml of 1X TAE buffer (Tris base, acetic acid and EDTA, pH = 8); Heat in microweave to 2 min to allow agarose gel dissolve quickly in TAE buffer	Make the agarose gel
Step 2	Carefully load the gel into the gel box and place the lane cone inside (white side of lane cone face out); then wait the gel to harden (usually takes 1 h)	
Step 3	Place the gel into the electrophoresis box and make sure gels are completely immersed with TAE buffer	Loading gel
Step 4	For each lane, adding a combination of 5 µL of plasmid DNA sample, 4 µL of super nanopure water and 1 µL of dye. For reference lane, use 10 µL of 1 kb DNA ladder (All the supplies are from BioLabs kit).	Prepare the sample
Step 5	Carefully load all the samples to each lane respectively, then run gel at 110 volts for 1 h. (or until you see the blue band approaching the second red line in the electrophoresis box)	Electrophoresis. Higher volts could reduce time, but sacrifice the separation quality
Step 6	After the gel is done, place in gel container with 100 ml of TAE buff and 10 µL of 10,000x SYBR safe DNA gel stain (Invitrogen). Cover the container with foil to protect exposure to light and shake slowly for 30 min	Stain the gel
Step 7	View the gel with UV light and capture the gel image	

FDH activity assay

Detect the light absorbance at 340 nm (NADH absorbance) continuously. (Ref. : Hopner, T. and Knappe, J. Determination with formate dehydrogenase 1974 Methods of Enzymatic Analysis, Volume III, 1551-1555)

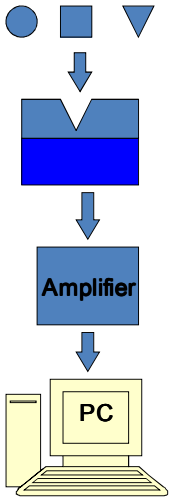


- E coli strains: E coli with FDH, TOM, TOM-Green and no plasmids. Grow the cells in appropriate medium (LB + amp or LB + kan) to an OD 600 = 0.6
- Transfer 1 ml of reaction mixture into cuvettes which contains:
    - 0.36 ml of cell suspension (OD 600 = 0.6) in LB
    - 0.285 ml of 200mM PBS buffer solution, pH = 7
    - 0.25 ml of 200 mM sodium formate solution
    - 0.105 ml of 10.5 mM β-NAD solution
- Mix well and place in the spectrophotometer to measure the A_{340nm} in 5 minutes. (It was suggested in the protocol to use a thermostated spectrometer)
- Record the initial A_{340nm} and the final A_{340nm}.

Appendix II:

Biosensor design concept

Design Concept of Biosensor



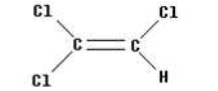
Analyte

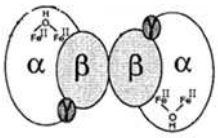
Biocomponent: Enzyme

Transducer: Optical

Signal amplification

Detector and data processor

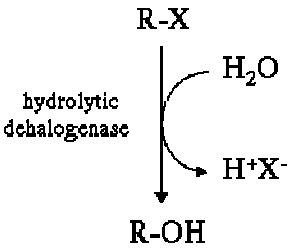


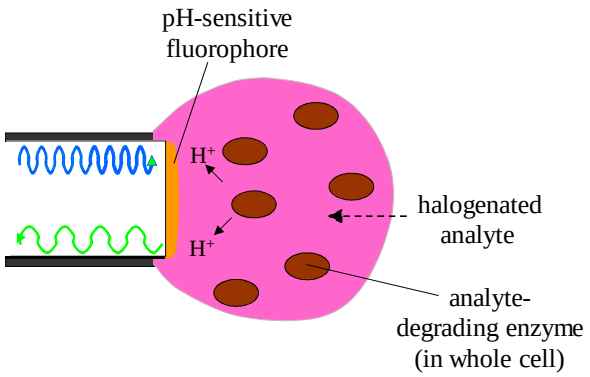


Toluene *ortho*-Monooxygenase

pH-based biosensor scheme

CSU biosensor concept*



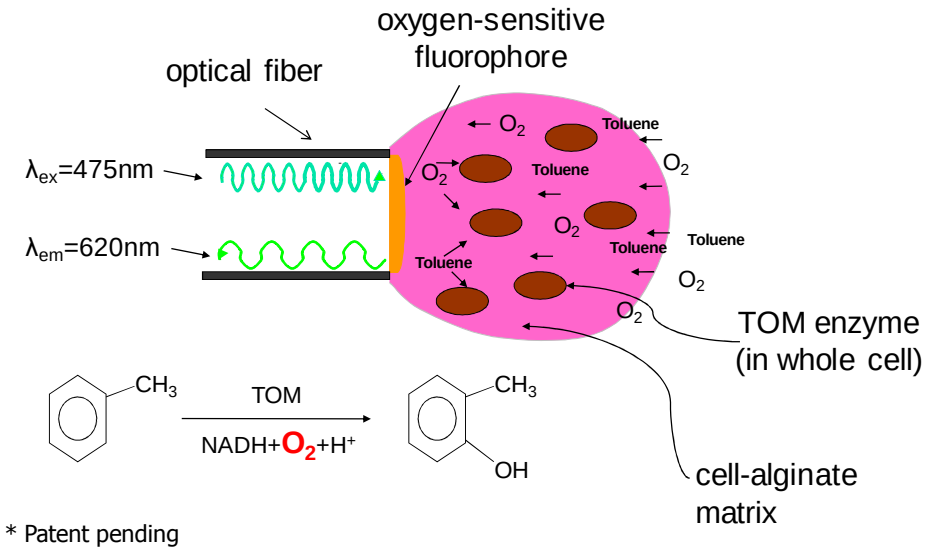


No cofactors
⇒ live cells not required

*patent pending

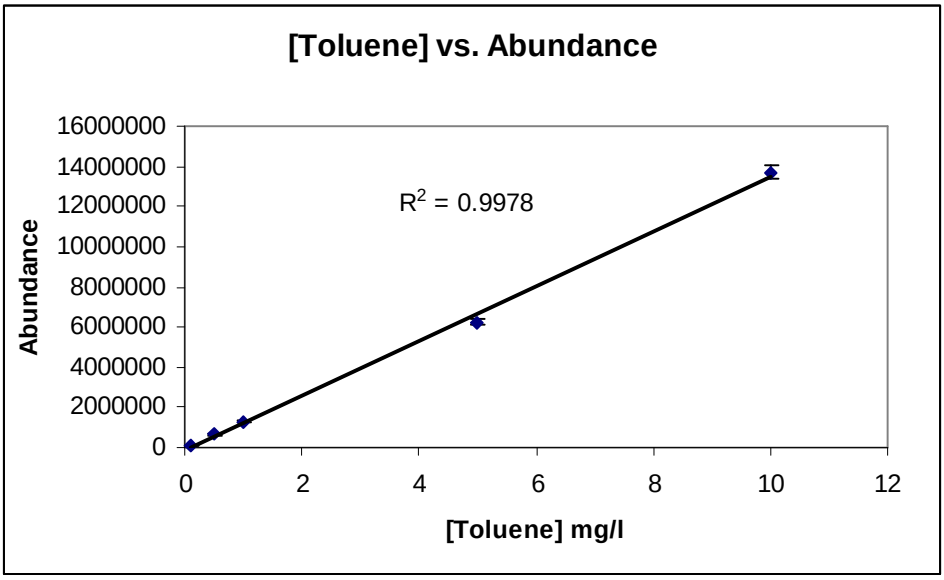
Oxygen-based biosensor scheme

Biosensor concept*

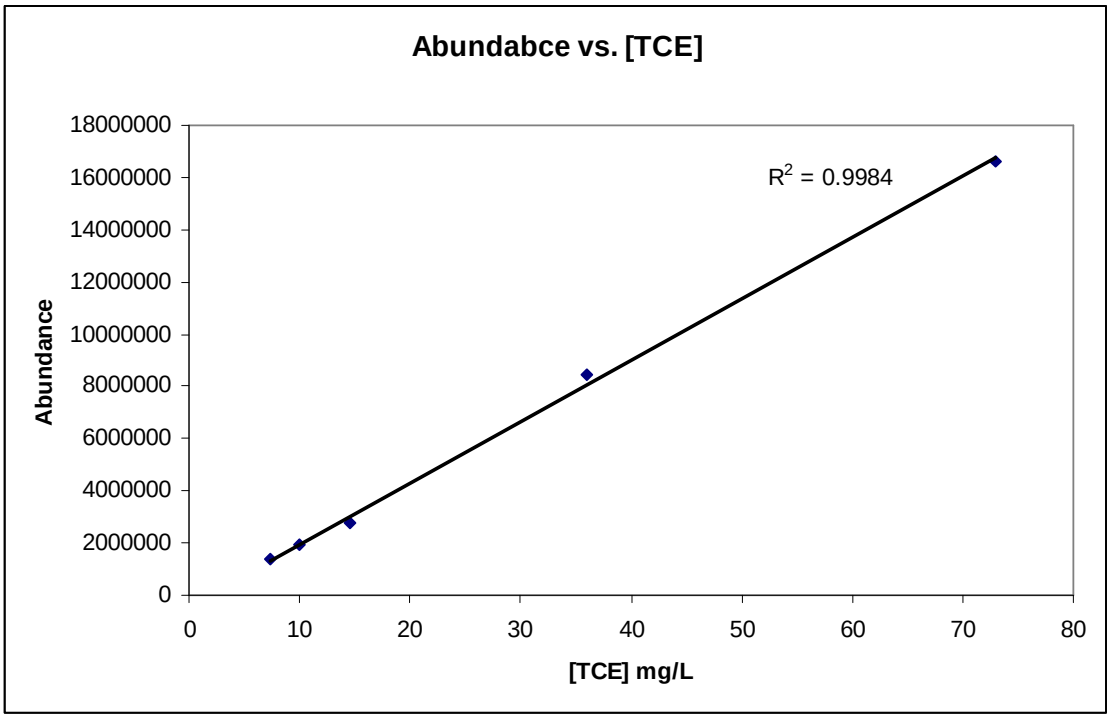


* Patent pending

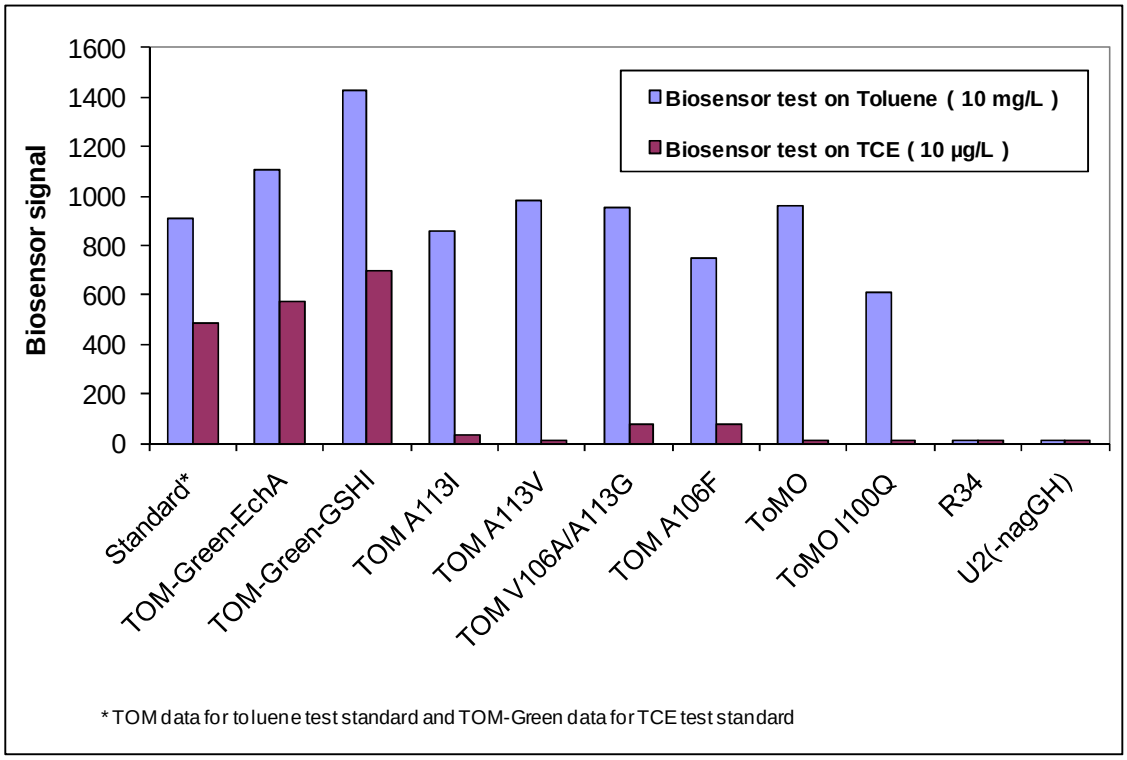
Toluene calibration with GC/MS



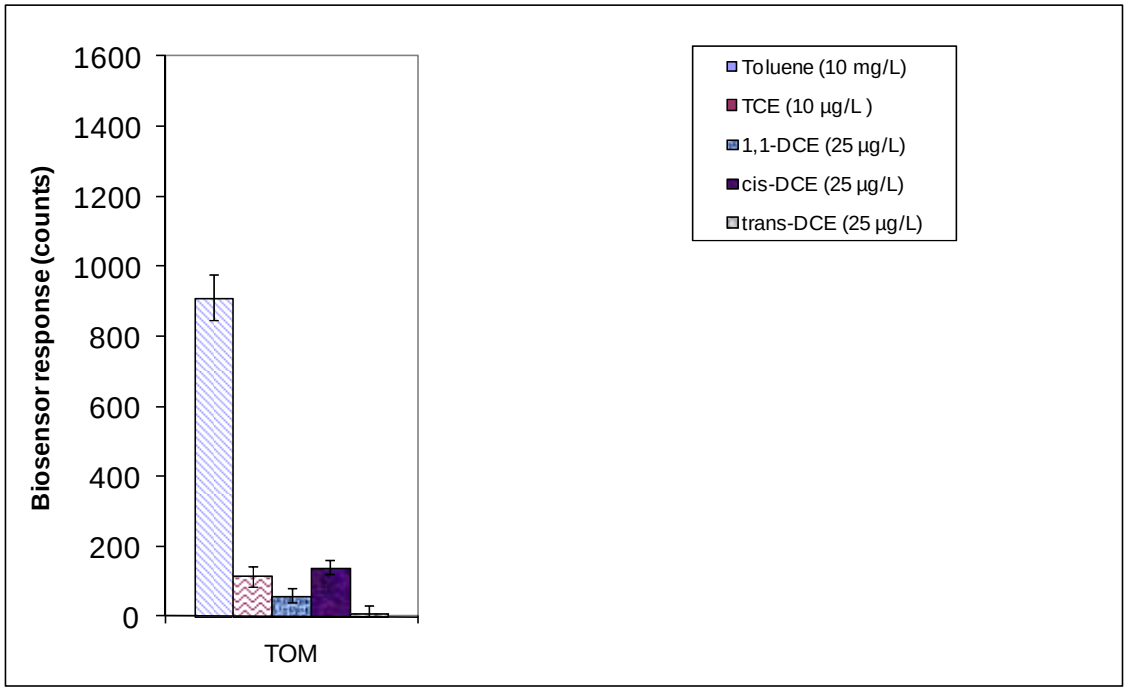
TCE calibration with GC/MS



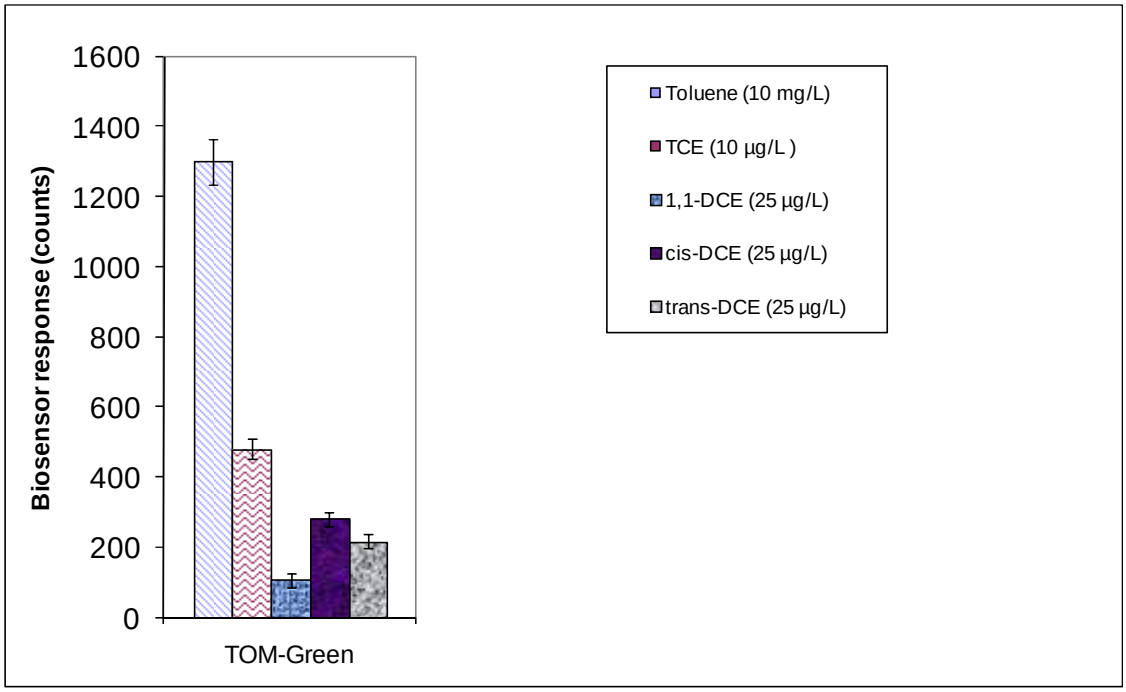
10 different Biosensors measurement of toluene and TCE



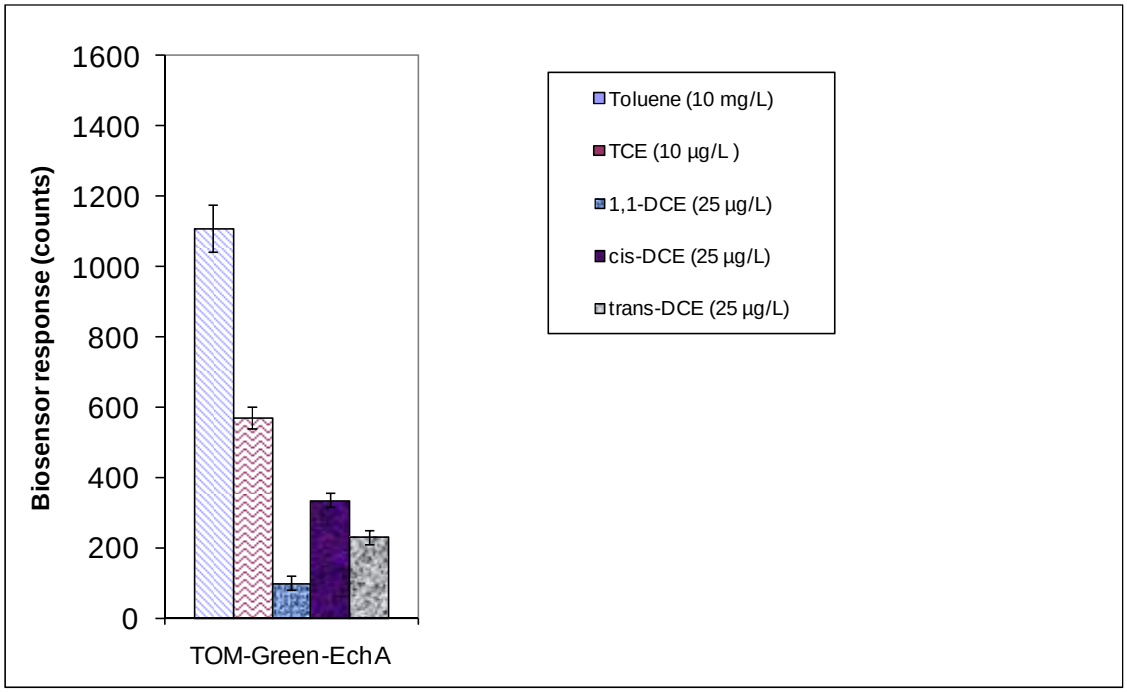
TOM biosensor with detection of five different analyte



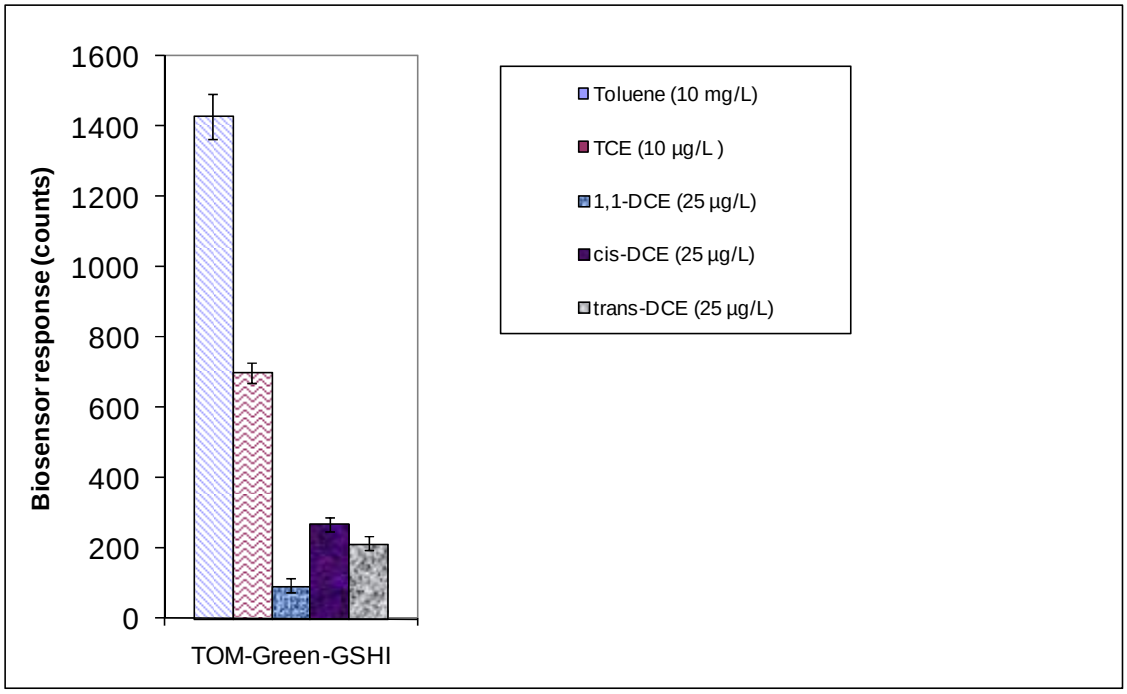
TOM-Green biosensor with detection of five different analyte



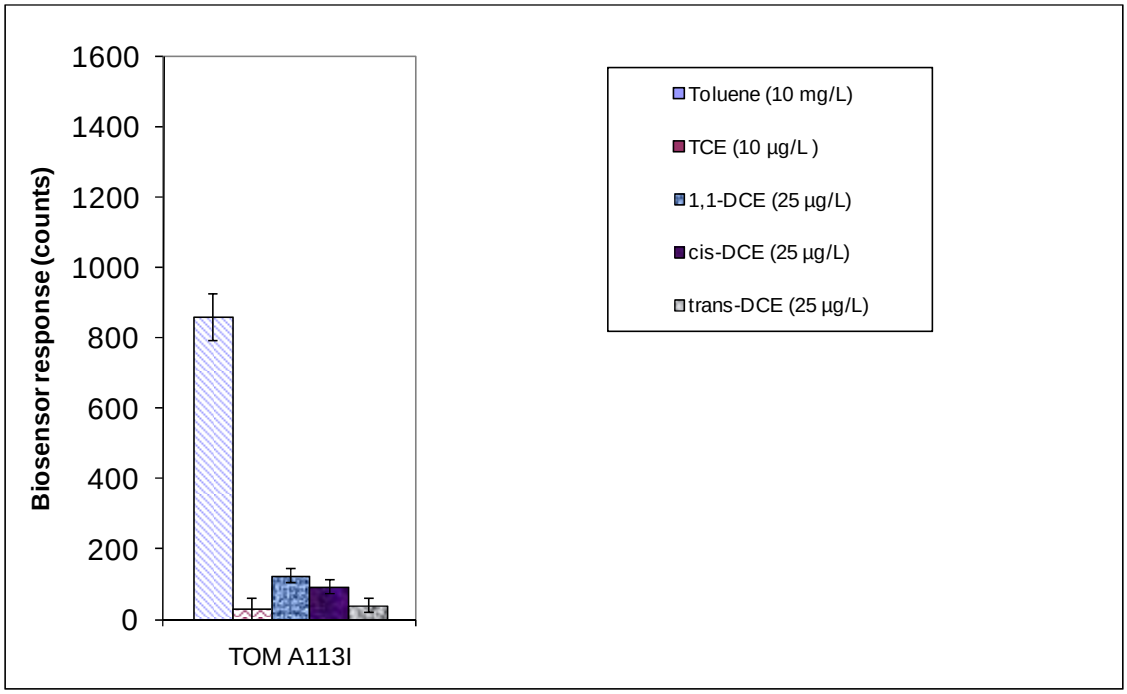
TOM-Green-EchA biosensor with detection of five different analyte



TOM-Green-GSHI biosensor with detection of five different analyte



TOM A113I biosensor with detection of five different analyte



TOM A113V biosensor with detection of five different analyte