Fiber optic monooxygenase biosensor for toluene concentration measurement in aqueous samples

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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 *ortho*-monooxygenase (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.

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1. 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 con-

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taining 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

$$C_7H_8 + O_2 + NADH + H^+ \rightarrow C_7H_7OH + NAD^+ + H_2O$$
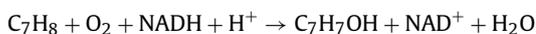
while oxygen is the electron acceptor in oxidase-catalyzed reactions (Ellis et al., 2006). Additionally, there are only a few oxidases available for biosensor applications (Azevedo et al., 2005; Komathi et al., 2009), while the oxygenase family is diverse and may be used to construct biosensors for a wide range of analytes (Park, 2007; van Beilen and Funhoff, 2005).

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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 CaCl₂ at pH 7.0 (hereafter referred to as "measurement solution").

2.4. 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 closely spaced 0.6-mm diameter cores in the common end (Ocean Optics, Inc.) and 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.

2.5. 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.

2.6. Toluene concentration measurement by gas chromatography (GC)

To assess the accuracy of the toluene concentration data obtained from the biosensors, GC analysis was performed via a
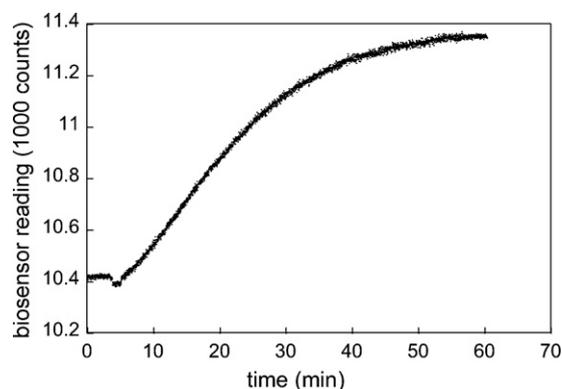
Fig. 1. Time course of a TOM biosensor response to the addition of 92 μM toluene.

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. Results and discussion

3.1. 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 (Fig. 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 in this biosensor design 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.

Two sets of control experiments were performed to further test the proposed biosensing principle (Table 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 1 mM acetate 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 1 mM acetate.

3.2. Analytical characteristics

3.2.1. 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%.

3.2.2. 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 2–4 h

Table 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.

Sample	Toluene concentration (μM)	Acetate concentration (mM)	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
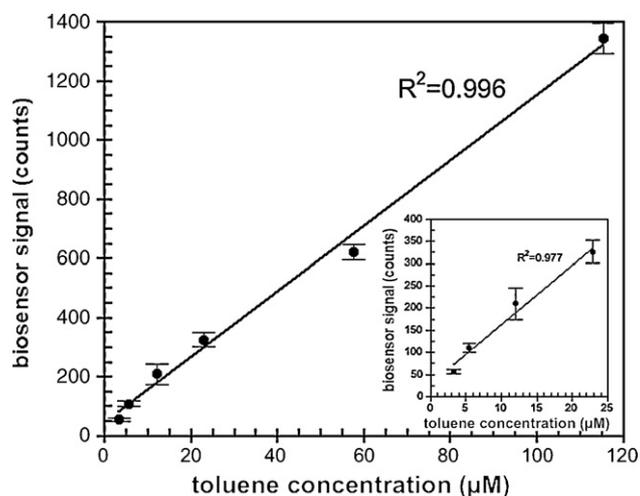
Fig. 2. TOM biosensor signal as a function of toluene concentration. Inset: biosensor signals in the low range of toluene concentrations (0–25 μM). Error bars represent ±1 standard deviation. Relative standard deviations varied (e.g., 10% at 3.3 μM toluene and 4% at 115 μM toluene).

Table 2
Comparison of toluene measurements in spiked water samples. Three biosensors were used for each measurement.

Sample	Toluene concentration (μM)	
	TOM biosensor	GC–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

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.

3.2.3. Calibration curve and limit of detection

A series of toluene solutions were analyzed with TOM-based biosensors. Each biosensor was used only once, each concentration point was measured in triplicate, and all biosensors were constructed in one batch. The biosensor signal was linear over the range from 3 to 100 μM toluene with $R^2 = 0.996$ (Fig. 2). The limit of detection (LOD), calculated as three times the standard deviation of the noise obtained from control experiments, was 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., 11–22 μM (Willardson et al., 1998). Furthermore, the current LOD of the TOM-based biosensor could be improved by increasing the sensitivity of the optoelectronic instrumentation, by replacing the TOM enzyme with another oxygenase that has higher activity at low toluene concentrations, or by increasing the reproducibility of the measurements.

3.2.4. 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 2. The differences between GC/MS measurement values and biosensor measurement values were 0.2 ± 0.5 μM (95% CI, $n = 18$), indicat-

ing that the TOM biosensor is accurate and reliable for toluene measurement in these aqueous matrices.

3.2.5. 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 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).

3.3. 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.

Table 3
Comparison of different analytical methods for toluene measurements.

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
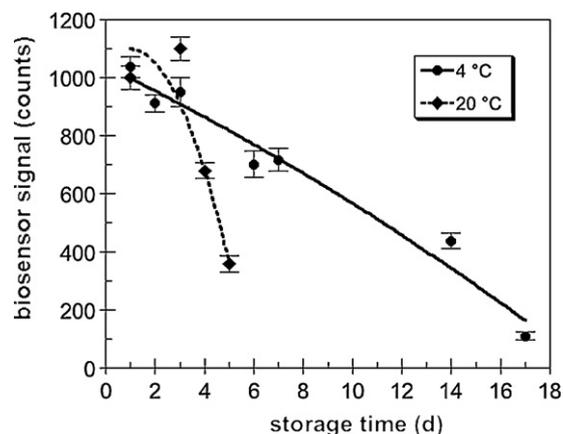
Fig. 3. Activity retention of TOM biosensor stored at two temperatures in measurement solution (without formate); each point represents the reading for a 92 μM toluene solution. Error bars represent ±1 standard deviation. The average relative standard deviation over all data points shown was 5.8%.

3.4. 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 (Fig. 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, then supplemented with 1 M formate for 24 h. 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.

4. 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), 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.

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