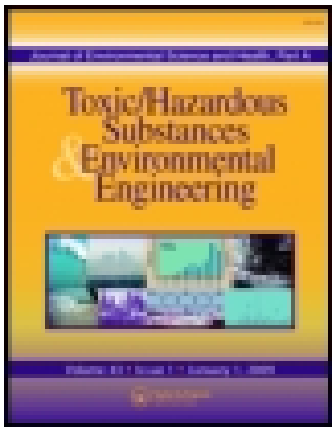

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Oxygen electrode-based single antibody amperometric biosensor for qualitative detection of E. coli and bacteria in water

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Oxygen electrode-based single antibody amperometric biosensor for qualitative detection of *E. coli* and bacteria in water

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Design and performance of an amperometric biosensor for *E. coli* O157:H7 that is based on a common dissolved oxygen probe is discussed. Anti-*E. coli* O157:H7 antibody was conjugated to horseradish peroxidase and immobilized on a nitrocellulose membrane that was placed over the oxygen probe membrane using a custom-fabricated polyvinyl chloride (PVC) insert. Upon bacterial cell binding, a decrease in enzyme activity resulted in a change in oxygen concentration that was detected with a Clark-type oxygen electrode probe. Validation experiments determined the effect of the outer membrane and insert on the Clarke electrode performance and linearity, and the effects of stirring on sensor response. The mechanism of enzymatic disruption is presumably steric hindrance due to binding of the bacterial cell and conformational change in antibody structure. Sampling various dilutions of heat-sterilized *E. coli* O157:H7 cells in water, as little as 50 bacterial cells/mL could be detected in approximately 20 minutes of sampling and processing procedures. Bacterial concentrations from 0 to 5000 cells/mL were tested, with 2.52 mg/L ± 0.37 mg/L equivalents of oxygen produced from as few as 50 cells/mL, versus 6.26 ± 0.64 mg/L when no cells were present in solution. Overall, the developed amperometric biosensor technology offered an efficient means of detection primarily due to its ease of use, cost-effectiveness, portability, and amenability to incorporation at existing water quality gaging stations.

Keywords: Biosensor, *E. coli*, amperometric, enzyme-antibody conjugate, O157:H7.

Introduction

Urban storm water and concentrated animal feeding operations have contributed to increased incidences of bacterial contamination of waterbodies. Numerous recreational advisories are being issued to protect humans and the environment from causative bacterial pollutants. Monitoring the levels of fecal coliform bacteria as an indicator of fecal contamination has been well accepted for several decades. There are currently several established methods to detect and enumerate fecal coliforms. Because these methods vary in speed, accuracy, and complexity, some are more suitable for laboratory use than for detection in the field. Detection schemes involving immunological recognition,[1] polymerase chain reaction (PCR),[2] microscopy or sample culturing are often quite sensitive but require multiple lengthy processing steps. These enrichment processes that serve to amplify the signal for detection can take hours to days and can include separation and extraction techniques, and bacterial growth in selective media. More rapid biosensing methods include optical[3] mass-based[4] or surface plasmon resonance (SPR) based-detectors.[5] Some biosensors employing these schemes offer real-time detection capabilities and integrated sample acquisition, preparation and detection. However, disadvantages of the systems include complex and expensive instrumentation which may require some degree of specialization for use and susceptibility to damage in field deployment. Despite the varied successes in the detection area achieved by several designs, there is still a need for a reliable, rapid, and real-time detection method for pathogens and coliforms in water.[6]

The use of biosensors for the detection of biochemical or microbial targets has grown rapidly in both research and industrial settings.[7] While the common theme amongst all biosensors is the transduction of the biological recognition event into a signal that is easily read, such as an electrical or optical one, there are myriad variations on this theme. The majority of electrical signals are measured after applying a constant or alternating excitation, and fall into three categories: potentiometric, whereby voltage is

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measured, amperometric, whereby current is measured, or impedance spectroscopy whereby resistance, capacitance and/or inductance are measured.[8] There has been much success with amperometric biosensors for the detection of metabolites, much of which has been driven by blood glucose detection.[9] Generally, glucose amperometric biosensors are composed of a base transducer which is normally a hydrogen peroxide or oxygen sensor, an inner membrane selective for hydrogen peroxide or oxygen, and an outer membrane functionalized with specific antibody or enzyme elements. Amperometric biosensors functionalized to detect fecal pathogens such as *E. coli* O157:H7 may offer a fast, reliable, and cost efficient way to quantify the organisms. O157:H7 is a very pathogenic strain of *E. coli* that can grow in the intestines of cattle and is commonly detected in runoff from beef and dairy farms.[10] Technology is needed for point-source tracking of enteric microbes in waterways.[11] Amperometric immunosensor technology, known for its ease of use, sensitivity, and quick response time[12] could fulfill this need.

The majority of immunohistochemical detection methods utilize combinations of multiple antibodies to confirm the presence and/or quantify the analyte or microbial organism. The traditional sandwich technique requires immobilization of an antibody to capture the target, followed by addition of a secondary recognition antibody functionalized with a fluorophore or enzyme to produce a measurable signal. An example of an amperometric biosensor for *E. coli* O157:H7 is a sandwich ELISA-based design by Abdel-Hamid et al.,[13] where antibodies were immobilized on carbon rods followed by bacterial sample incubation, then incubation with an antibody-enzyme conjugate. While this design improved the response time by lifting the electrode from the solution to concentrate analytes in the wetted layer of the protein-coated electrode, the sandwich-type assay was still required, which can complicate and lengthen processing steps. Other enzymatic amperometric biosensor approaches avoid the sandwich scheme by co-immobilizing redox enzymes with antibodies to a surface, such that binding alters enzymatic activity. For example, Xu and Suleiman correlated cocaine[14] or cortisol[15] concentrations in the sub-micromolar levels to reduced enzymatic turnover. More recent biosensor systems detect subtle changes in antibody conformation that result upon antigen binding.[16] While FRET-[17] or electrochemical-based methods[18] have been shown to detectt antigen binding, decreased amperometric signal from a standard ELISA antibody-enzyme conjugate has not been utilized for biosensing.

In this project, we show an amperometric biosensor design for the detection of *E. coli* O157:H7 that combines a single antibody recognition and redox signaling scheme with a proven amperometric method. This biosensor could be used at stream gaging stations that have existing infrastructure for amperometric measurement to detect fecal-borne pathogens such as O157:H7.

Methodology

Reagents and reagent preparation

Bacterial stock preparation. Heat-sterilized *E. coli* O157:H7 bacteria (Fitzgerald Industries International, Concord, MA) were rehydrated with 1 mL of a 50% glycerol solution to a final concentration of 3.5×10^9 cells/mL. Working standards were made by serial dilutions of this stock *E. coli* 0157:H7 with 0.1M Tris solution (pH 7.0). Heat sterilized bacteria were used in this laboratory study for safety reasons and to ensure a non-proliferating sample population during testing procedures.

Antibody preparation. Lyophilized conjugated goat anti *E. coli*O157:H7- horseradish peroxidase (HRP) antibody (#60-E07, Fitzgerald) was reconstituted with a 1mL of a 50% glycerol solution according to manufacturer's suggestions to a final concentration of 100 μg/mL goat anti *E. coli* 0157:H7-HRP solution. Following gentle agitation for 20 seconds, the conjugated antibody was stored at 4°C for later use (up to 3 months). Unconjugated HRP (#HRP4B, Biozyme Laboratories, San Diego, CA) was also used for concept testing experiments and for custom conjugation with *E.coli* antibody. The listed activity of the enzyme was 254 U/mg material. Unconjugated *E. coli* O157:H7 antibody (#60-E07, Kirkegaard & Perry Laboratories, Gaithersburg, MD) was reconstituted to 1.0 mg/mL using the HRP conjugation buffer supplied.

Nitrocellulose membrane and general reagents. The outer membrane of the sensor was selected based on its ability to provide good hydrophobic-based adsorption of the captured antibody and an optimum pore size for the passage of a small molecule. Nitrocellulose has been used for such biosensing applications, where a 0.45 μm pore size is most commonly used for proteins greater than 10 kDa, but for smaller proteins 0.2 μm is recommended to more efficiently trap the proteins.[19] The GE nitrocellulose membranes used for this study had 0.45 μm pore size (GE Osmonics, Minnetonka, MN, Part# 1212597, Model# WP4HY320F5). Other chemicals such as 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), hydrogen peroxide (3% v/v aqueous solution), and buffers such as Tris (pH 7), sodium citrate (pH 3,4, or 5) and sodium phosphate pH (6.5 or 8.5) were ordered from Sigma-Aldrich and used without further preparation.

Instrumentation

Dissolved oxygen meter

With the exception of "Twin membrane concept testing" and "Colorimetric verification of enzyme activity after

conjugation and immobilization" experiments, all other experiments were conducted using a modified oxygen electrode and an oxygen meter (Model 5739, YSI, Yellow Springs, OH). This meter has a Clark-type electrode consisting of a gold cathode and silver anode composing its electrolytic cell. The electrolytic cell is separated from the probing solution by an oxygen permeable Teflon membrane, which helps to both contain the electrolytic fluid and to allow the oxygen to diffuse through it. The oxygen passing the membrane is then reduced once a potential of 0.7V is applied in reference to the silver electrode. The reduction of the oxygen is proportional to the concentration of dissolved oxygen (in mg/L). The following equation describes this reaction.[20]

$$\text{Cathode reaction} : O_2 + 2H_2O + 4e^- \rightarrow 4OH^-$$
$$\text{Anode reaction} : Ag + Cl^- \rightarrow AgCl \quad (1)$$

Outer insert

Because the inner Teflon membrane has a low affinity for protein binding, which makes it unsuitable for functionalization with antibodies, an additional outer nitrocellulose membrane was used to immobilize antibodies. Several outer inserts (Fig. 1) were made with polyvinyl chloride (PVC), to secure the second nitrocellulose membrane securely over the Teflon membrane on the DO probe. All of the inserts were fabricated with tight tolerances so as to provide a consistent spacing between the two membrane layers, as this could potentially change sensor response characteristics. The outer insert was designed to fit securely over the rubber O-ring securing the Teflon membrane on the YSI 5739 DO probe. The inserts were designed such that they can be attached or detached without replacing the Teflon membrane or refilling the electrolytic cell. The outer insert was incorporated with a groove for holding the outer O-ring, which secured the nitrocellulose membrane to the insert.

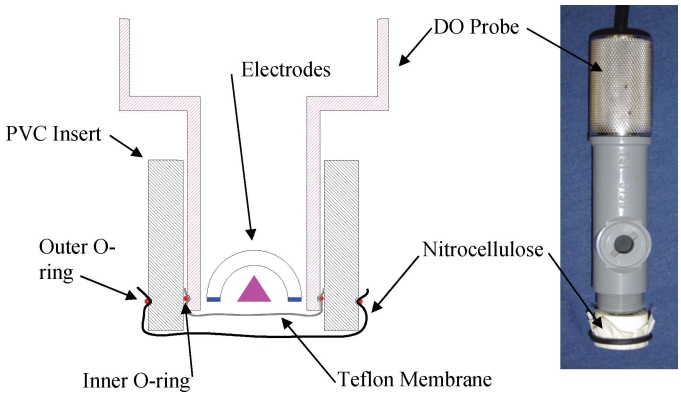


Fig. 1. Schematic (left) and picture (right) of the outer insert over an YSI oxygen electrode. The inner Teflon membrane is held by the DO probe O-ring, while the outer nitrocellulose membrane over the PVC insert is held by the outer O-ring.

Picoammeter and power supply

The Keithley 485 Autoranging Picoammeter was used for the concept testing experiments. These experiments needed an alternative transducer response to confirm the dissolved oxygen readings from the oxygen meter (YSI 5739). The Keithley 485 can accurately detect current in the picoamp range and has a sensitivity of 0.1 pA. The Keithley 485 contains a 4.5" LCD display with front BNC connector input which was necessary for easy readout and probe connection, respectively. A variable voltage, regulated power supply (Mastech Metered Bench Power Supply, Model HY 1803D, with $< 0.01\%$ line/Load regulation) was utilized to apply a potential of 0.7V DC to the DO Probe.

Experimental procedures

Enzyme-antibody custom conjugation

The SureFIRE conjugation kit (#84-01-01, Kirkegaard & Perry Labs) was used to conjugate the HRP to the *E.coli* antibody using a periodate method. Unconjugated antibody solution (100 μl) and 0.3 mg HRP were incubated at room temperature (20–25 C) for 1 hour, followed by addition of 10 μl of sodium cyanoborohydride ($NaBH_3CN$). After 15 minutes, HRP storage buffer (110 μL, #84-04-01, Kirkegaard) was added to the vial and incubated for 15 minutes. The vial was capped tight and stored in a refrigerator until further use. Conjugated antibody was not purified further for use in this application.

Outer membrane antibody immobilization

Using sterile scissors, a $1.75'' \times 1.75''$ piece of nitrocellulose membrane was cut and placed securely over an inverted PVC insert. Conjugated antibody (16 μL) was gently deposited over the membrane surface and incubated for 1 hour at 37 °C. The insert/membrane was later placed in an air-tight plastic enclosure and stored at 4°C until later use.

Twin membrane concept testing

Because envisioned design relies on a second nitrocellulose membrane over the Teflon membrane of the DO probe, concept testing was necessary to: (i) assess the accuracy of oxygen detection with the additional outer membrane, and (ii) asses the variability in sensor response between multiple inserts. These experiments were conducted using the DO meter with an unmodified probe (Model YSI 5739) and a modified DO probe (Probe for YSI 55) connected to a picoammeter. For these initial comparisons, oxygen concentrations were measured by two different meters (DO meter and picoammmeter) without membrane functionalization (Fig. 2). The unmodified probe was connected to the DO meter and yielded direct DO reading. For the modified probe, the outer insert with the nitrocellulose membrane

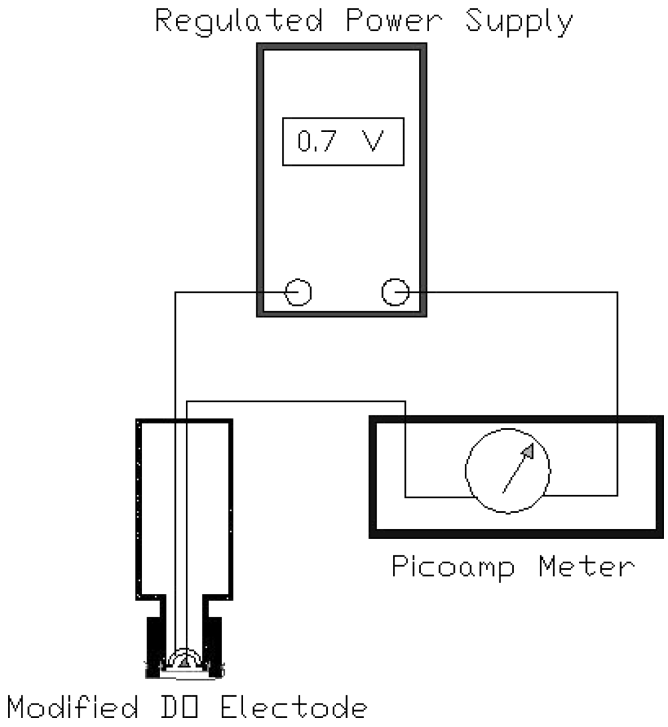


Fig. 2. Electronics schematic of the modified DO probe. A tightly regulated power supply provides the 0.7 V DC voltage potential between the electrodes. The current passing between the probe electrodes, which is proportional to the DO passing the two membranes, is read by a picoamp meter.

was attached. Prior to this attachment, a drop of DI water was added to the inside surface of the nitrocellulose membrane. This drop prevented the inclusion of any air pockets between the two membrane layers during probe assembly. The modified probe was connected to the regulated power supply and the current passing through the electrodes was monitored by a picoammeter.

Water samples with various dissolved oxygen concentrations were prepared either by aerating the samples using an aquarium air pump and an airstone, purging the oxygen by sparging with nitrogen, or depleting with sodium sulfite (1g in 500 mL).[21] For each of these water samples, the DO meter readings from the unmodified DO electrode and the current passing through the modified electrode was read with the picoammeter. A calibration curve was developed to depict the correlation between these two signals. A high correction coefficient would indicate that the response from the two-membrane probe is identical to the single-membrane probe (Teflon). For assessing the effect of using multiple inserts, the same calibration experiment was conducted with multiple inserts.

Colorimetric verification of enzyme activity after conjugation and immobilization

Colorimetric testing of HRP activity was used to determine effectiveness of the conjugated enzyme before and after immobilization to the nitrocellulose membrane. If the active sites of the enzyme were blocked totally during the immobilization process, the proposed single-antibody sensor concept would not be valid. The conjugated *E.coli* antibody was immobilized onto three outer membranes using the aforementioned procedure. The membrane inserts were gently placed into a 20 mL beaker with 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate, a chromogenic substrate for HRP.[22] Production of the chromophore was compared between membrane-immobilized conjugated HRP-*E. coli* antibody complex and the free complex in solution. Two different HRP concentrations (1 mg/L and 2 mg/L) and TMB substrate were evaluated in triplicate.

Effects of solution stirring on sensor response

After successful concept testing and enzyme effectiveness verification experiment, the DO response-based experiments were conducted. The first set of experiments was focused on quantifying the oxygen uptake by the probe and the effect of stirring to prevent localized oxygen depletion at the membrane-liquid boundary. These experiments were conducted with the modified oxygen electrode connected to an oxygen meter and commercially conjugated antibody immobilized onto the membrane. A substrate consisting of distilled water, 0.001 M ABTS, and 0.005 M hydrogen peroxide, and tris buffer (pH 7) was prepared to a final volume of 6 mL. The two-membrane DO probe with the antibody immobilized on the outer membrane was gently introduced into the beaker and the DO was monitored. For experiments that needed stirring, a 6 mm magnetic stirrer was used to gently stir (100 rpm) the solution in the beaker. The change in DO was recorded over 10 minutes.

Oxygen response amplification

The next set of experiments was conducted to compare the oxygen response from: 1) unconjugated, custom-conjugated, and commercially-conjugated HRP, and 2) effect of ABTS addition. The conjugation protocol that yields the closest oxygen response to unconjugated HRP is the preferred option. Metered quantities of HRP stock solution (in 0.1 M tris, pH 7) were added to the substrate solution consisting of distilled water, 0.001 M ABTS, and 0.005 M hydrogen peroxide. The total solution volume with the addition of HRP for all experiments was 6 mL. Using the YSI 55 DO probe and meter, the dissolved oxygen concentration was monitored at steady state for the substrate addition ($t = 0$) for 10 minutes beginning with the point of enzyme addition. All experiments were performed in triplicate. The same procedure was followed for both the commercial and custom conjugated antibody-HRP. Because the HRP is already conjugated with the antibody, no free HRP was added to the beaker initially. The amount of antibody-HRP in the testing solution (as free solution

or conjugated with antibody) was kept constant between tests at 10^{-8} M. All the above experiments were repeated with a substrate system that contained ABTS.

Escherichia coli *O157:H7 testing*

Diluted solutions of bacteria ranging in concentration from 1-5000 cells/mL were prepared in tris-buffered distilled water (pH 7). The outer insert was attached to the YSI 55 probe and submerged into a stirred solution of a given bacterial concentration for 5 minutes. The probe and insert were then removed and placed into a wash solution containing 0.1 M tris buffer at a final volume of 20 mL for 2 minutes. The probe was then submerged into a 6mL solution with 0.001M ABTS. After reaching steady state, the DO concentration was recorded (t = 0). Hydrogen peroxide was added to obtain a 0.005M final concentration and dissolved oxygen (mg/L) readings were recorded every 30 seconds for 10 minutes. This procedure was repeated for every bacterial concentration tested in triplicate.

Results and discussion

Twin membrane concept testing

The transducer response of the modified probe for various DO concentrations was read using the picoammeter and compared to DO measured with the YSI meter (Fig. 3). Several data points were collected within the 0–8 mg/L DO range using three different outer inserts. The responses from both probes were very similar, with a linear fit of data points from all three inserts of:

$$y = 0.8773x + 0.2178 \quad (2)$$

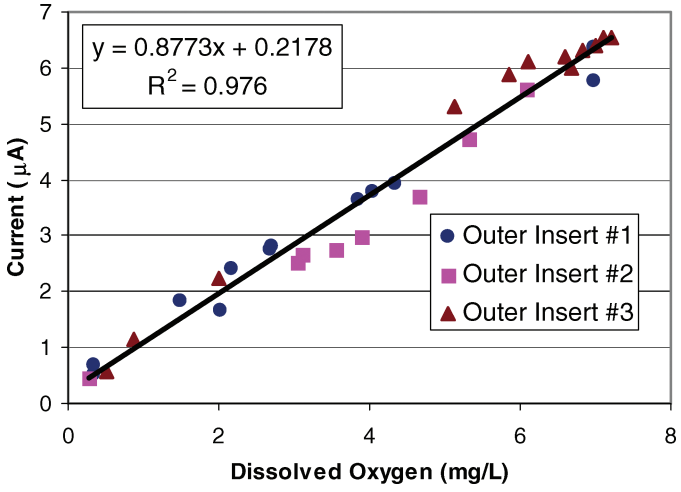


Fig. 3. Dissolved oxygen response between a standard DO meter and the modified electrode using three different custom PVC and nitrocellulose membrane inserts.

and a high degree of correlation ($R^2 = 0.976$). These results reaffirmed the notion that: (i) the addition of a second nitrocellulose membrane does not interfere with the oxygen permeability, and (ii) use of multiple inserts during the actual bacterial testing does not introduce additional variability to the transducer responses.

Colorimetric verification of enzyme activity after conjugation and immobilization

Colorimetric tests of antibody-enzyme conjugate activity in the immobilized and free form were compared across two concentrations of conjugate (Fig. 4). When 1 μg/mL of conjugated HRP-*E. coli* antibody was used, an absorbance of 0.491 ± 0.012 AU (mean $\pm$ standard error) was observed from conjugate in solution, while 0.543 ± 0.002 AU was observed from the membrane-bound form. When 2 μg/mL conjugated antibody was used in solution versus attached to the membrane, average absorbance readings were 0.785 ± 0.027 and 0.721 ± 0.008 AU, respectively. These results indicate that the conjugation process does not significantly affect the activity of the HRP enzyme at the 2 μg/mL loading concentration ($P > 0.05$, Student's t-test).

Effect of solution stirring on sensor response

The effect of stirring the sampling solution was measured on the modified probe output. In Figure 5, the average DO concentrations are shown for the ten minute testing period. There is a reduction in oxygen in both cases, although the effect is greater in the unstirred case. This may be contributed to oxygen consumption by the electrode. The larger decrease in DO from samples without stirring is possibly due to localized DO depletion at the membrane. In samples that were stirred, the DO drop was distributed throughout the entire sample volume. This study clearly demonstrated the need for moderate stirring, without which the localized DO drop (due to consumption at the electrode) could distort the true oxygen production from HRP-peroxide reaction.

Oxygen response amplification

Based on the combination of DO probe size, beaker size, immersion depth, and other practical issues, the minimum volume of solution needed for accurate testing of the new amperometric biosensor system was determined to be approximately 6 mL. Experiments conducted with free HRP enzyme in solution indicated that a 0.6×10^{-6} M HRP was needed for a discernable change (>5 mg/L) in the DO reading (Fig. 6a). Thus at the scale of this sensor with a 6 mL minimum liquid volume, using the commercially available HRP would be cost-prohibitive. From a reagent conservation point of view, there is a need to reduce the amount of antibody used in a single sensor application. Ideally, 1 mL of antibody applied at 6×10^{-8}M HRP would last for at

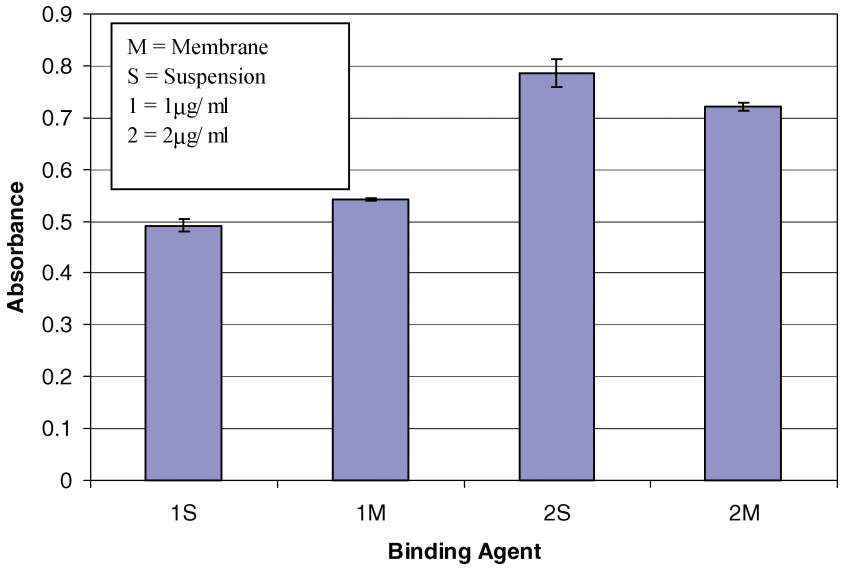


Fig. 4. Comparison of enzyme activity before and after conjugation with *E. coli* antibody. The "S" and "M" in the figure indicate the location of the enzyme, namely "solution" and "membrane, respectively.

least 10 applications (to functionalize outer sensor membranes). In order to do so, a lower concentration than 0.1 μM HRP (as conjugated HRP) would be necessary. However, lowering the final HRP concentration under 0.1 μM did not allow a distinguishable change in dissolved oxygen concentration. Hence, a reagent that would increase the rate of oxygen production was necessary. ABTS was chosen to enhance oxygen production based on Barr and Rust's findings[23] that ABTS dramatically increased the rate of oxygen evolution with the use of horseradish peroxidase and hydrogen peroxide.

The dissolved oxygen production experiment was then repeated with ABTS and lower concentrations of conjugated HRP-*E. coli* antibody and the substrate (Fig. 6b). To further lower the quantities of antibody needed for each insert, a custom conjugation was done in order to increase the HRP:antibody molar ratio. The combined effect from the presence of an oxygen enhancer (ABTS) and custom conjugation lowered the amount of HRP needed for a detectable signal (of 5 mg/L DO change) to 4×10^{-8} M. One explanation for the larger response seen from the custom conjugate is that the commercial conjugated antibody (Fitzgerald Industrial International) had a HRP:antibody molar ratio of 4:1, whereas the custom conjugated antibody presumably had a larger ratio of enzyme:antibody (up to 25:1 with suggested ratio of 10:1; based on personal communication with KPL). These larger signals favored the use of custom conjugated antibody for the final *E. coli* O157:H7 detection tests.

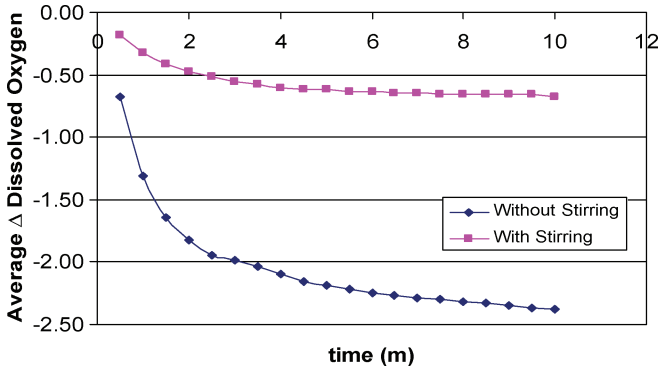


Fig. 5. Observed DO decrease with and without stirring. The stirring distributed the localized DO drop due to oxygen consumption at the electrode.

E. coli *testing*

Figure 7a depicts the results obtained when using the amperometric biosensor to detect 0-5000 cells/mL concentrations of *E. coli* O157:H7. The curves are hyperbolic in nature, reaching steady state in this configuration after approximately 6 minutes after HRP begins reacting with hydrogen peroxide. The results from this test indicated that the oxygen generation was not linearly correlated to the bacterial concentration. However, the results clearly and repeatedly reflected the ability to qualitatively indicate the presence of 50 cells/mL or higher concentrations.

The initial reaction rate of DO production calculated at 1 minute (Table 1) also failed to clearly demonstrate a linear response, although also suggested a significantly lower oxygen production rate at 50 cells/mL or higher concentrations. In order to more quantitatively assess the results, the change in dissolved oxygen concentration at the 10 minute time period was compared over bacterial concentration (Fig. 7b). For 50 cells/mL and 100 cells/mL, the average change in dissolved oxygen (±standard error) at

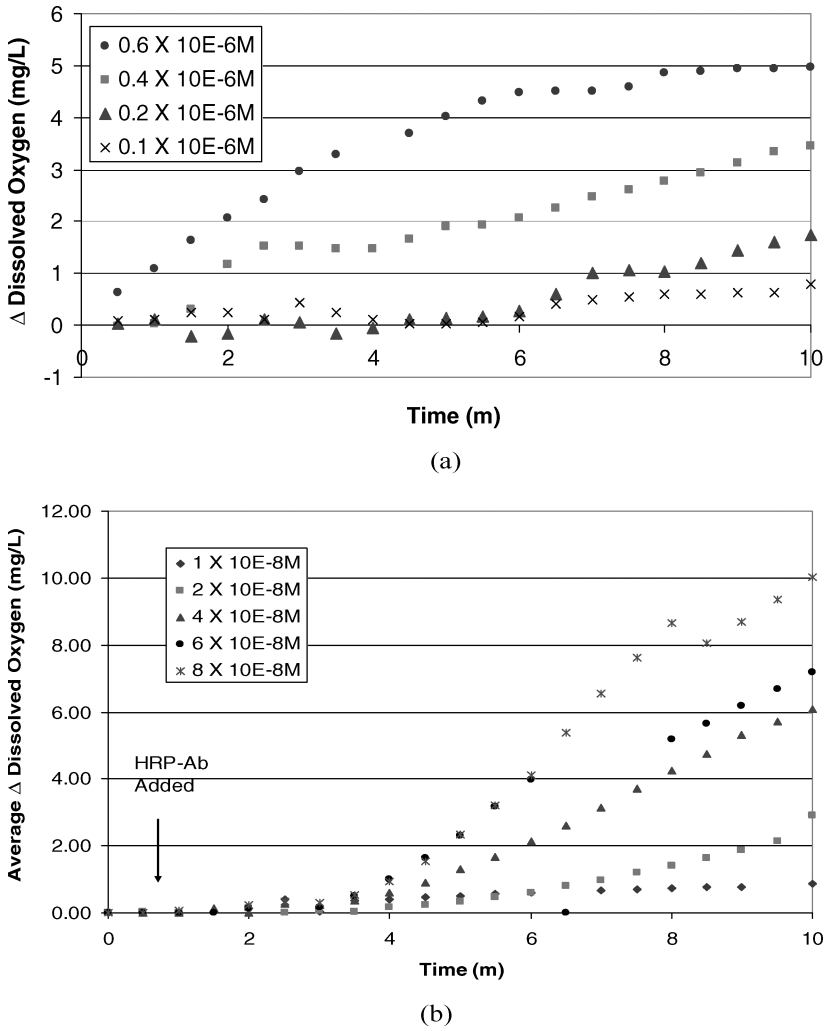
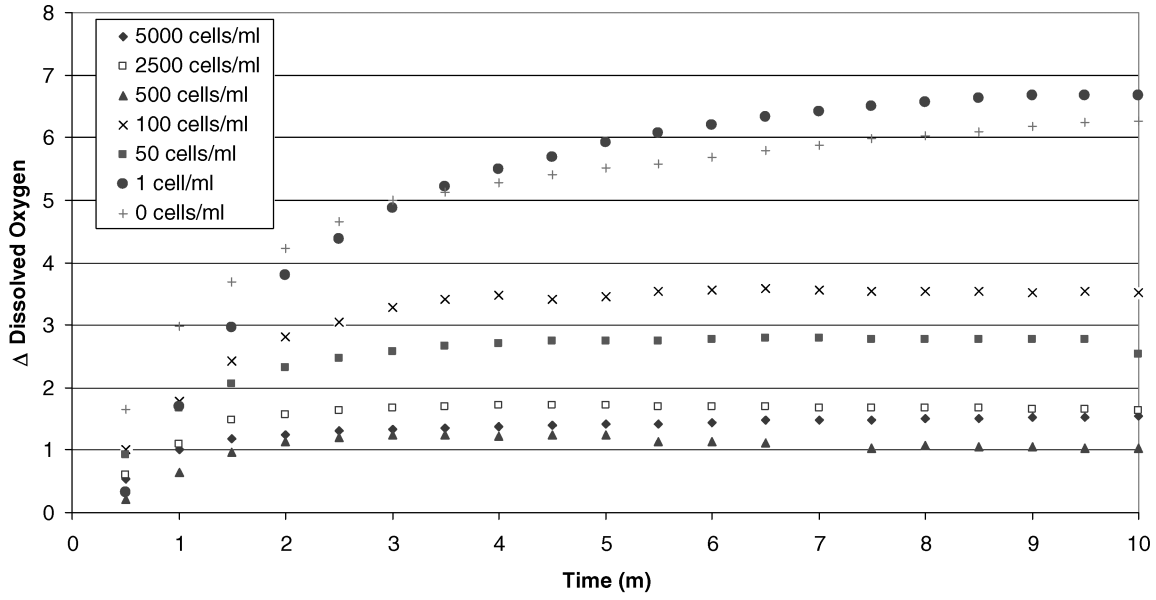


Fig. 6. Average change in DO from free (a) commercially conjugated HRP in solution, and (b) custom-conjugated HRP in solution with ATBS.

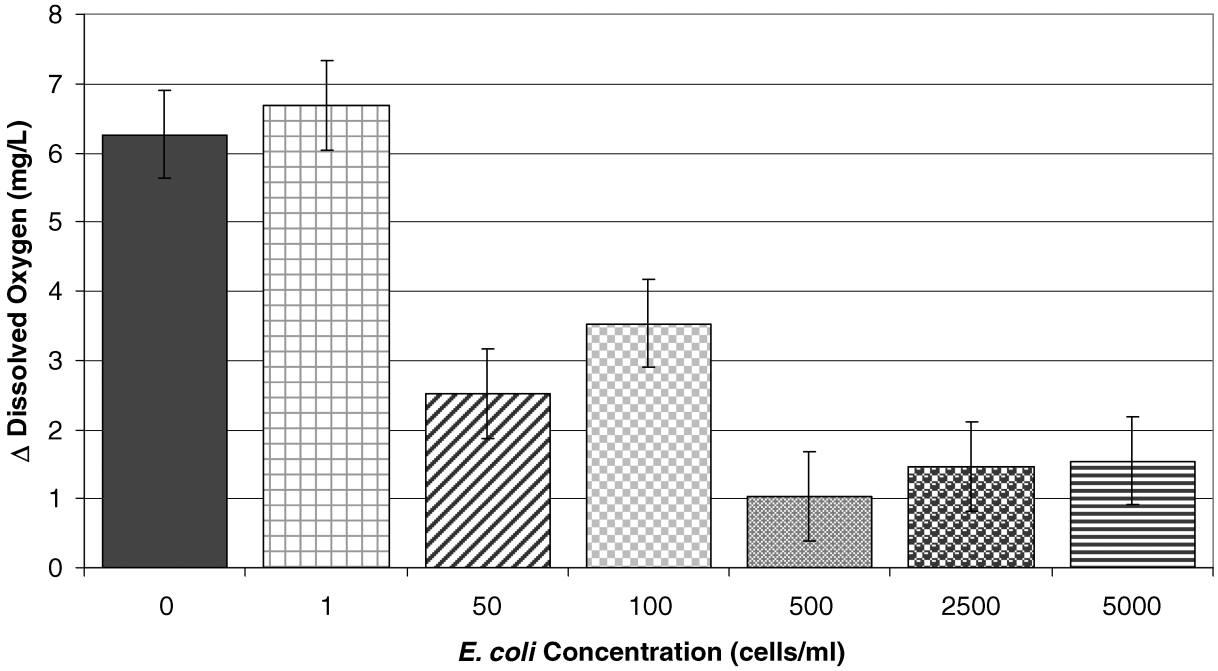
steady state was 2.52 ± 0.37 mg/L and 3.53 ± 0.26 mg/L, respectively. In general, the 0 cells/mL and 1 cell/mL had the highest DO increases, followed by 50 cells/mL and 100 cells/mL. As anticipated, the highest cell concentrations (500 and 2500 cells/mL) had the smallest increase in DO.

Figure 7b has a certain degree of linearity when the cell concentrations are categorized as ranges (0–1 cells/mL, 10-100 cells/mL, and 500–2500 cells/mL). A clear explanation as to why 100 cells/mL concentrations had a higher DO change than 50 cells/mL (or 2,500 over 500 cells/mL) could not be inferred from this study. An additional study was conducted to explore this inconsistency. The *E. coli* detection test was repeated with addition of TMB (rather than H_2O_2 and ABTS) to verify DO output with the more widely-accepted colorimetric response. If the blocking of the enzyme activity due to bacterial attachment was truly occurring, the colorimetric response should agree with the previous DO responses. A plot between both the colorimetric response and DO response at the 10-minute point versus the cell concentration is depicted in Figure 8. This figure reflected a slightly improved degree of correlation (R^2 of 0.93) between the absorbance and cell concentration than was observed with DO measurement (R^2 of 0.88). This result indicated that the steric hindrance concept is plausible, although improvements are needed in the DO-based detection in order to achieve a more linear sensor response. Thus a possible alternative to the amperometric DO detection is to use an optical sensor capable of colorimetric quantification. However, our hopes were to rely solely on amperometric electronics for simplicity and practicality of reading a DO probe at existing waterway gaging stations.

Additional experiments are necessary to improve the robustness of the DO response. Controlling the initial DO concentration in the sample would allow for a larger dynamic signal to be achieved. For example, if the ambient DO in the sensor solution is 5 mg/L, a production of 5 mg/L DO is likely to push the DO concentrations to well above the DO saturation point at room temperature (22°C).



(a)



(b)

Fig. 7. Average DO production from biosensor at varying concentrations of *E. coli* O157:H7 (a) during the first 10 minutes, (b) at the 10th minute (steady-state). The error bars reflect standard error.

As supersaturated oxygen will be lost to the atmosphere, the DO reading will not reflect the true response from the enzyme-substrate (HRP vs. H_2O_2) reaction. Future experiments will also assess the effect of stirring speed, room temperature, and activity of ABTS in solution. Although the ABTS solution (a known photosensitive reagent) was protected from light, its effectiveness appeared to drop significantly within a few days.

Conclusions

Here we report the use of an amperometric sensor for detecting the presence of *E. coli* O157:H7 based on an immobilized antibody-enzyme conjugate. Initial evaluation of the design indicated that the response from the twin-membrane probe had a high degree of correlation with the unmodified DO probe response, thereby endorsing the use

Table 1. Initial velocity for hydrogen peroxide and ABTS with HRP after 1 minute.

E. coli *O157:H7 (cells/mL)*	*Vo (μM/minute)*
0	93.13
50	52.19
100	55.94
500	19.69
2500	34.38
5000	31.56

of a second membrane for protein immobilization. Results also indicated that the conjugation process does not significantly ($P > 0.05$) affect the effectiveness of the HRP enzyme. There is a clear need for stirring the sampling solution with this sensor configuration, without which the localized DO drop (due to consumption at the electrode) could distort the true oxygen response from HRP-peroxide reaction. The use of a custom conjugated antibody offered many benefits to the amperometric biosensor, such as ability to control the molar ratio of HRP:antibody and minimize the volume of antibody used. A concentration of $4 \times 10^{-}8$ M HRP generated a distinguishable change (~6 mg/L) in dissolved oxygen when attached to a nitrocellulose membrane and conjugated to *E. coli* antibody.

The bacterial testing process consisted of a three step process: exposing of sensor into water sample, wash, and exposing to a substrate. This process lasted a total of 17 minutes, with 10 minutes for signal (dissolved oxygen change) generation. The detection limit for the biosensor system was 50 cells/mL. Although a high degree of linearity between DO production and *E. coli* concentration was not noticed, results supported the generalized notion that the bacterial attachment lowered the enzyme activity. The blocking notion was further validated by results from the colorimetric (TMB) experiments. The exact mechanism of blocking is not evident from this research and remains to be ascertained.

Although this research focused on detection of heat-sterilized *E.coli*, there is no reason to believe that this concept is limited to *E.coli* O157:H7, since a plethora of antibodies to various analytes are becoming available. An improved and automated version of this biosensor can be incorporated at various USGS gaging stations to generate real-time data on bacterial concentrations. Such data will allow the regulatory agencies to take remedial actions or pinpoint the stream(s) with high bacterial concentrations.

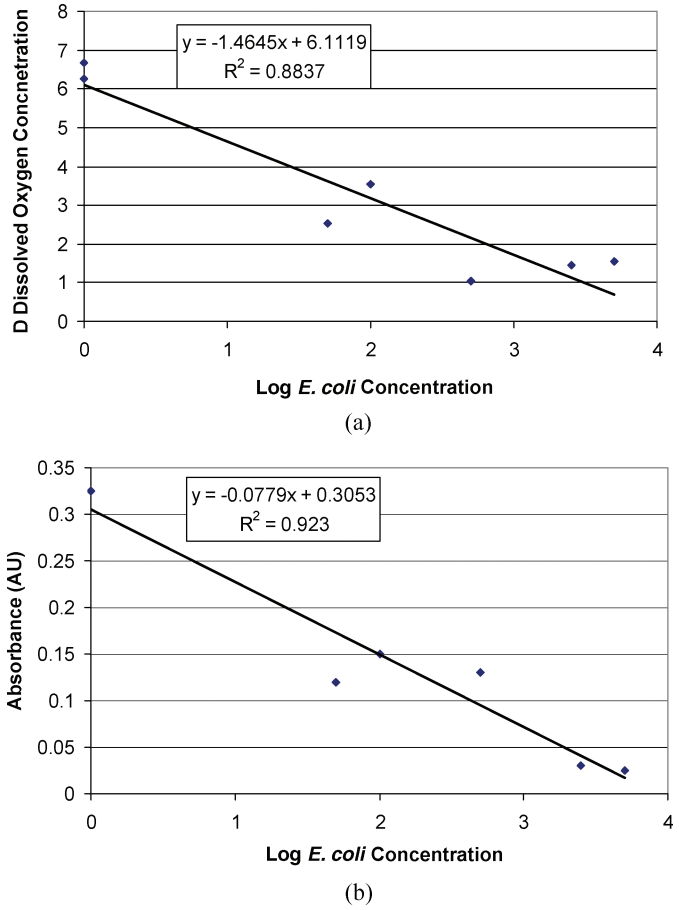


Fig. 8. Average increase in (a) DO as a function of log *E.coli* concentration, and (b) absorbance (from TMB reaction) as a function of log *E.coli* concentration. The oxygen concentration is read by the DO meter.

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