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Development of Biosensors for Real Time Analysis of Perchlorate in Water

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Abstract

Perchlorate (ClO_4^-) contamination of ground water is a widespread problem in the U.S., which can adversely affect human health and wildlife. Current methods for detecting and quantifying ClO_4^- in water are time consuming, expensive, and subject to error due to complex procedures and various interferences. Thus, there is an urgent need to develop a method that can accurately detect and measure low concentrations of ClO_4^- in the field. This study reports the construction of a ClO_4^--reductase based biosensor for rapid determination of ClO_4^- in water. Using a 3 mm GCE (glass carbon electrode), we successfully constructed a ClO_4^- sensing bio-electrode by coating an aliquot of the enzyme on nafion (ion-exchange matrix) layer pre-coated on the polished surface of the GCE. Amperometric [i/t] measurements revealed linear increases in current in relation to time and ClO_4^- concentration. The biosensor responded strongly to ClO_4^- at concentrations as low as 1 µg/L and the sensor displayed a linear response to ClO_4^- concentrations in the range 25 to 100 µg/L. Linear response time to ClO_4^- at 100 µg/L was approximately 111 ± 28 seconds. Kinetic evaluation of the sensor response to ClO_4^- revealed linear increases ($r^2 > 99\%$) in 10 min with k values of 10.3, 24.2, 33.9 and 48.2 at 25, 50, 75 and 100 µg/L, respectively. A strong linear correlation was established between biosensor response (nA) and ion-chromatography conductivity readings (µS) in the 25 to 100 µg/L linear domain of the biosensor. Biosensor response to ClO_4^- was maximal at applied potential range of –0.6 to –1.0V. ClO_4^- reduction current increased with an increase in pH and was maximal in the range of 7.6 to 8.0. The ClO_4^- biosensor was significantly stable after repeated use (24 analyses conducted on day 1 over a 10-h period at room temperature). This study indicates great potential for the development of a portable biosensor for real time analysis of ClO_4^- in water in the field.

Introduction and Problem Statement

Perchlorate (ClO_4^-), a strong oxidizing agent, is a major component of rocket and missile propellants as well as explosives and various pyrotechnics. Past industrial usage and disposal procedures have resulted in the release of ClO_4^- into the environment and led to significant contamination of groundwater (Urbanski and Schock, 1999). Wastewater generated from the manufacturing, maintenance, and testing of solid rocket propellants can contain NH_4ClO_4 concentrations in the grams per liter range (Herman and Frankenberger, 1998). ClO_4^- has been detected at high concentrations in surface and ground waters in the U.S and has been estimated to potentially affect the potable water supplies of millions of people in the United States.

ClO_4^- is potentially toxic to various forms of life (Lamm et al., 1999). Recent studies revealed that low concentrations of ClO_4^- in drinking water can inhibit iodide uptake in humans and wildlife (Lawrence et al. 2000), and ClO_4^- contamination of vegetation can have adverse effects on the growth of amphibians (Goleman et al., 2002). ClO_4^- has also been detected in vegetables subjected to irrigation with ClO_4^--impacted water. Known human health risks associated with ClO_4^- are related to adverse effects on the thyroid gland (Capen, 1994; von Burg, 1995), which has spurred the regulation of ClO_4^- in drinking water. A public health goal (PHG) of 6 µg L^{-1} was recently established by the California Department of Health Services (DHS 2004). This PHG is currently being used in development of an enforceable maximum contaminant level, expected later this year. In addition, the U.S. Environmental Protection Agency (EPA) is currently developing a federal drinking water standard, and various site-specific cleanup levels have been imposed ranging down to 1.5 µg L^{-1} (Logan, 2001).

The increasing need to measure ClO_4^- in water has prompted the development of analytical methods such as ion chromatography (IC), capillary electrophoresis (CE) and ClO_4^- ion selective electrodes. These methods are time consuming, expensive, sometimes cumbersome, and subject to

error due to complex procedures and various interferences. Thus, there is an urgent need to develop a rapid method that can accurately detect and quantify low concentrations of ClO_4^- in the field in real time. Biosensors are analytical devices that utilize biomolecules or intact cells in conjunction with a transducer such as an electrode or an optical device. Biosensors are now attracting considerable attention as potential successors to a wide range of analytical techniques due to their unique properties of specificity. Biosensors are based on a system whereby, a biocatalyst converts a substrate to a product and the reaction is detected by a transducer, which converts it to an electrical signal. The output from the transducer is amplified, processed and displayed. Biosensors have been developed for the detection and determination of numerous compounds including: glucose (Delvaux and Demoustier-Champagne, 2003; Pandey et al., 2003), creatine (Stefan et al., 2003), urea (Glab et al., 1994, Walcerz et al., 1996, Kuswandi and Narayanaswamy, 1998), nitrate (Aylott et al., 1997), alcohols (de Prada et al., 2003), chitosan (Wang et al., 2003), asparagine (Li et al., 2002), phenols (Kulys and Vidziunaite, 2003), chromate (Michel et al., 2003) and other compounds.

Biosensors offer many advantages in comparison to the conventional analytical approaches due to their simplicity, real-time output, lower detection limits and sensitivity (CBC, 2004). The simplicity of biosensors often allows for their use by untrained personnel eliminating many human sources of error. Biosensors normally allow information to be recorded electronically, which enables signal processing and potentially remote monitoring with the transmission of data to a central facility via telemetry.

The mechanism of microbial breakdown of ClO_4^- is believed to proceed through the following pathway: $ClO_4^- \rightarrow ClO_3^- \rightarrow ClO_2^- \rightarrow Cl^- + O_2$ (Logan et al. 2000). The first two reactions are catalyzed by (per)chlorate reductase. Chlorite dismutase catalyzes the disproportionation of chlorite into chloride and oxygen. Thus, ClO_4^- reductase is a key enzyme in the pathway of ClO_4^- breakdown.

Objectives

The overall objective was conctruction of a highly sensitive amperometric biosensor for ClO_4^- using the ClO_4^- reductase of a novel perchlorate-respiring bacterium, *Dechlorosoma* sp. perc1ace, and application of the sensor to monitor ClO_4^- in water.

Procedures

Cultivation of Dechlorosoma sp. perc1ace

Dechlorosoma sp. perc1ace (ATCC 202172) isolated by Herman and Frankenberger (1999) was used in this study. The bacterial culture was pre-grown in 100 ml FTW mineral elements medium using 125 ml Erlenmeyer flasks. The FTW mineral salts medium was comprised of the following (in g/L): K_2HPO_4, 0.225; KH_2PO_4, 0.225; $(NH_4)_2SO_4$, 0.225; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCO_3$, 0.005; $FeCl_2 \cdot 4H_2O$, 0.005, $NaC_2H_3O_4$, 1.39 and 1 ml of trace elements solution (Focht, 1994). The medium was supplemented with 0.100 g/L ClO_4^- and incubated at room temperature (about 22^oC) for five days. The 200 ml culture was used to inoculate sterile 1800 ml FTW mineral medium in a 2L Erlenmeyer flask (Herman and Frankenberger, 1999) and further incubated at room temperature for one week. Cells were washed by centrifugation (4,500 x g, 15 min, 4^oC) and washed using nitrogen saturated buffer (25 mM phosphate buffer pH 7.6, 2 mM dithiothreitol).

Enzyme extraction

Dechlorosoma sp. perc1ace cells were re-suspended in 25 mM phosphate buffer pH 7.6, 2 mM DTT, and lysed in a pre-chilled French pressure cell at 20,000 psi. Cellular debris was separated by centrifugation (4,800 x g, 15 min, 4^oC) and a reddish supernatant fraction was passed through a 0.22 µm filter. The filtrate served as the cell-free enzyme (CFE). ClO_4^- reductase for biosensor

construction was then prepared as follows. The cell-free enzyme extract was concentrated by passing it through a Centricon Plus-20 (polyethersulfone 300, 000 MWCO) centrifugal filter device concentrator (Millipore Corporation, Bedford, MA, USA) to approximately 0.5 ml. The concentrate was applied to a Sephadex ion-exchange column (1.5 x 12.5 cm), previously equilibrated with HEPES (N-Cyclohexyl-2-aminoethanesulfonic acid) buffer at pH 8.5, to which 2 mM DTT was added. Proteins were eluted with 25 ml HEPES buffer at pH 8.5 followed with a linear gradient of 0-1 M NaCl in HEPES buffer at pH 8.5, at a flow rate of 1.25ml/min. One-milliliter fractions were collected at 4^oC and assessed for ClO_4^- reductase activity (Okeke and Frankenberger, 2003). Active fractions (6 to 11) were pooled and dialyzed by passing it through Centricon Plus-20. The purity of the enzyme fractions were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 8 % polyacrylamide. Broad range molecular mass markers (BIO-RAD, Hercules, CA) ranging from 210,199 to 6,461 Da were used. Gels were stained with Coomasie brilliant blue G-250**.** ClO_4^- reductase was assayed in a capped cuvette by monitoring the oxidation of reduced methyl viologen at 578 nm (Kengen et al., 1999) as described by Okeke and Frankenberger (2003). Protein concentration was quantified using Coomassie[R] protein assay reagent (Pierce, Rockford, IL).

Fabrication of electrodes

A 3-mm glassy carbon electrode, GCE (Bioanalytical system Inc., IN, cat. No. MF-2012) was used to construct the ClO_4^- sensing bioelectrode. The electrode surface was first polished by sequentially rubbing over a BUEHLER[R] LTD (Lake Bluff, IL) GAMMA micropolish Π (0.05 micron de-agglomerated alumina) on nanocloth PSA polish paper from BUEHLER[R] LTD. The polished GCE was then sonicated for 5 min in a sonic water bath (AQUASONIC model 75D, VWR Scientific Products). Thereafter, the sensing element was sequentially cast on the surface of the glass electrode. Firstly, 4 µl of nafion (5% nafion, Aldrich, Milwaukee, WI) stock solution (20% v/v in absolute ethanol) was used to coat the surface of the electrode and allowed to air-dry at room temperature for 10 min. Secondly, a 2 µl aliquot of the ClO_4^- reductase preparation (6.7 methyl viologen units/mg protein/ml) was deposited on the nafion layer and air dried for 15 min. Finally, the enzyme adsorbed to the nafion coating was firmly held by using a Spectra/Por[R] dialysis membrane (6-8,000 MWCO).

Amperometric measurements of ClO_4^- using the biosensor

Amperometric measurements were conducted in 10 ml of 25 mM potassium phosphate buffer pH 7.6. The buffer was initially de-aerated by sonication in a sonic bath for 30 min. Aliquots (10 ml) were transferred to a clean 20 ml electrochemical cell and purged with oxygen-free nitrogen for 5 min. An Ag/AgCl electrode (BAS MF- 2052) was used as the reference and a platinum wire was the counter electrode. Before insertion into the analytical buffer solution, the GCE ClO_4^- biosensor was soaked in a solution of 1mM methyl viologen in 25 mM phosphate buffer for 5 min to saturate the sensing layer. Anoxic condition was maintained by spiking the buffer (pH 7.6) with 10 µl of a 0.25 g/ml cysteine hydrochloride mono hydrate (Atlas, 1993) to give a final concentration of 0.25 mg/ml. Reactions were initiated by spiking the reaction buffer with 10 µl from ClO_4^- stock solutions (1, 5, 25, 50, 75, 100, 125, 150, 175, 200 mg/L) to give the respective final concentrations in µg/L. Electrochemical measurements were then conducted using a CH Model 650A Electrochemical Workstation (CH Instruments, Inc., Austin, TX). The program was an amperometric i-t curve using the following parameters: sample interval: 0.1 sec, run time: 600 sec, sensitivity (A/V): 1 E^{-6} and applied potential (E) was -0.8V except for the study on effect of applied potential tested in the range –0.4 to –1.2 V." Experiments were conducted at ambient temperature (approximately 24^oC). Biosensor response (change in current; Δ i) was calculated as the difference in current from 2 min to

10 min. In the linear range, the reaction was zero order and $k = di/dt$ was calculated from the curve ($i = kt + c$).

Effect of applied potential

Applied potential was applied from -400 to –1200 mV. Amperometric measurements were conducted as described above. The 10 ml analytical phosphate buffer (pH 7.6) solution was spiked with ClO_4^- to a final concentration of 100 µg/L.

Effect of pH

The effect of pH on the performance of the biosensor was tested using 25 mM phosphate bufffer at pH 6.0, 6.4, 6.8, 7.0, 7.2, 7.6 and 8.0. Amperometric measurements were conducted as described above. The 10 ml buffer solutions were spiked with ClO_4^- to a final concentration of 100 µg/L.

Effect of nitrate on ClO_4^- detection

The effect of nitrate on ClO_4^- detection in water was evaluated using 100 µg/L in 25 mM phosphate buffer pH 7.6. The analytical buffer was then spiked with varying nitrate levels to give final concentrations of 100 µg/L to 100 mg/L. Amperometric measurements were conducted as described above.

Ion chromatography analysis of ClO_4^-

Samples were filtered using a 0.2 µm membrane filter (PALL Corporation, Ann Arbor). ClO_4^- was analyzed using a Dionex ion-chromatography 600 system (Dionex, Sunnyvale, CA), equipped with a GP40 gradient pump, an AS40 automated sampler, 1000 µl injection loop, an AS16 analytical column and an ED40 conductivity detector. The eluent was 50 mM NaOH at 0.4 ml min^{-1}. An ASRS-II suppressor, operated by external regeneration with 50 mM H_2SO_4 was used to suppress the eluent.

Statistical analysis of data

The means and standard errors of replicates were calculated. Linear regression analysis was performed on relevant data sets. The significance ($p<0.05$) or non-significance ($p>0.05$) of variable effect was analyzed by Mann-Whitney non-parametric test using the InStat 3 statistical software (GraphPad Software, Inc., San Diego, CA, USA).

Results
ClO_4^- biosensor

We successfully constructed a ClO_4^- sensing bioelectrode by coating aliquots of a perchlorate reductase enzyme on a nafion layer (ion-exchange matrix) on the surface of a 3 mm GCE. The ClO_4^- biosensor is based on the mixed function redox property of the system in which the immobilized sensing element (ClO_4^- reductase) oxidizes the mediator (an electrochemically reduced methyl viologen) and the reduced enzyme consequently reduces ClO_4^- (Fig. 1). Thus, at an applied potential, increases in reduction current can be used to measure ClO_4^-. With the initiation of the reaction by adding aliquots of predetermined concentrations of ClO_4^-, the background current was initially high, but rapidly decayed to stable readings. The current subsequently increased due to ClO_4^- reduction. The average response time (defined as the onset of steady increases in reduction current) to ClO_4^- at 100 µg/L was 111±28 seconds. The biosensor chronoamperogram for ClO_4^- with and without ClO_4^- reductase in the linear response range is presented in Fig. 2.
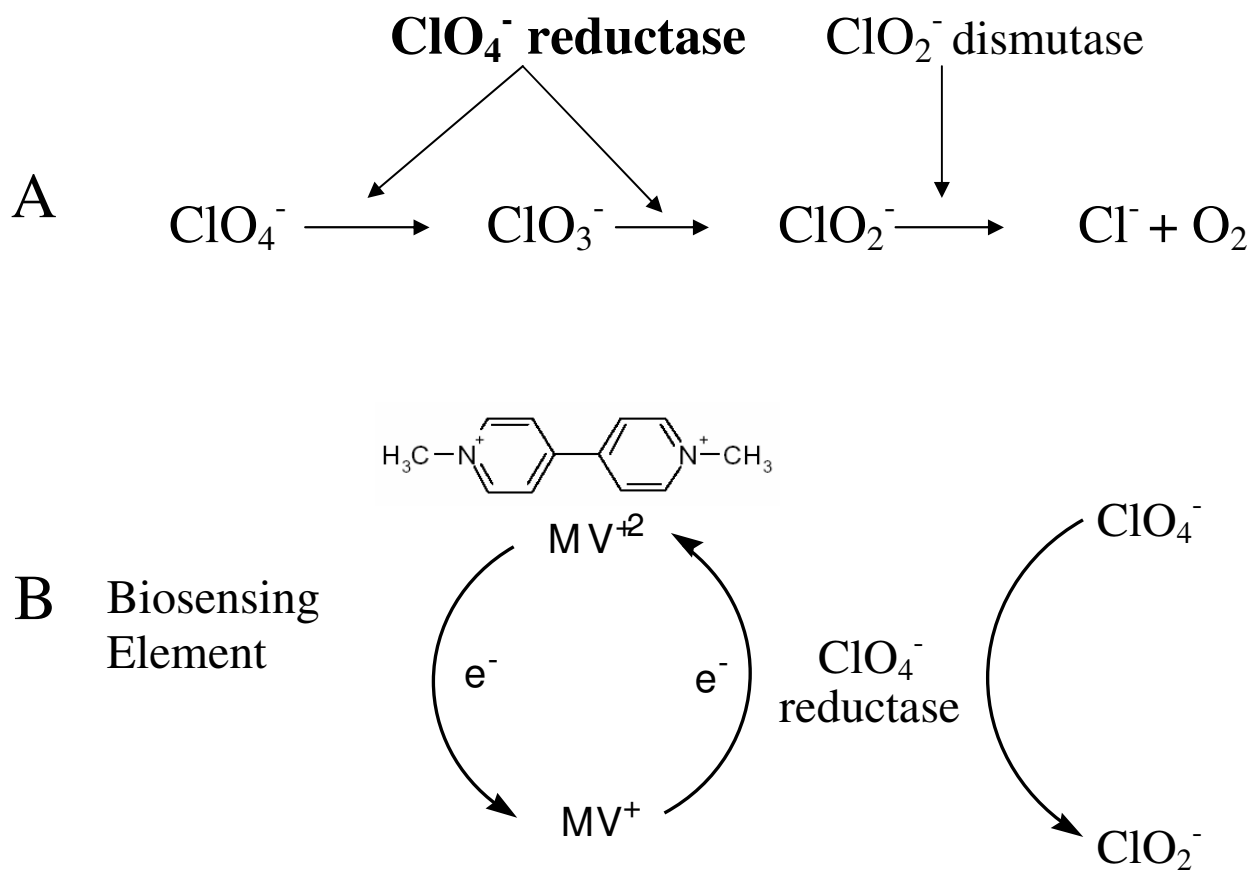
Fig. 1. Operation of the ClO_4^- biosensor. A: enzymatic pathway for ClO_4^- reduction. B: mechanism of ClO_4^- reduction at the sensing layer (GCE-Nafion-ClO_4^- reductase-MV complex).
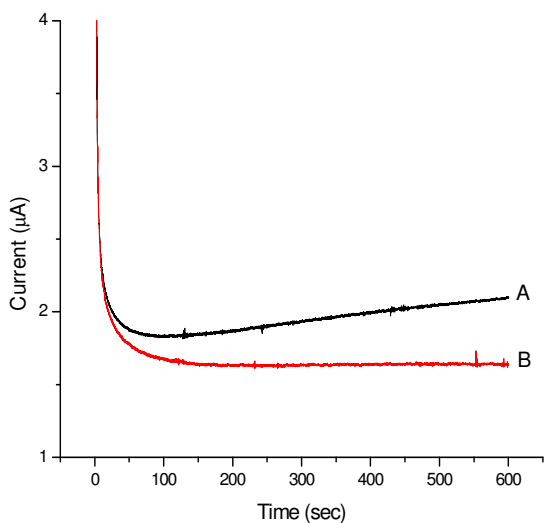
**Fig. 2. Chronoamperogram for** ClO_4^- **with** ClO_4^- **reductase (A) and without** ClO_4^- **reductase (B).**

Effect of applied potential and matrix pH on sensor response to ClO_4^-

The effects of applied potential and matrix pH on sensor response to ClO_4^- were tested. The effect of applied potential on sensor response to ClO_4^- was examined at –0.4, -0.6, -0.8, -1.0 and –1.2 V (Fig. 3). Biosensor response to ClO_4^- was maximal in the range –0.6 to –1.0 V. Slightly lower reduction current was observed at –1.2 V and the lowest reduction current was recorded at a potential of –0.4. Biosensor response at –0.8 V and –1.0 V was significantly (p<0.05) higher than the response recorded at –0.4 V. The influence of sample pH was tested at pH 6.0, 6.4, 6.8, 7.0, 7.2, 7.6 and 8.0 (Fig. 4). ClO_4^- reduction current increased with increasing pH and was maximal in the range 7.6 to 8.0. Neutral pH displayed the lowest change in current. The sensor response at pH 6.8 to 7.0 was significantly (p<0.05) less than the response recorded at pH 7.6 to 8.
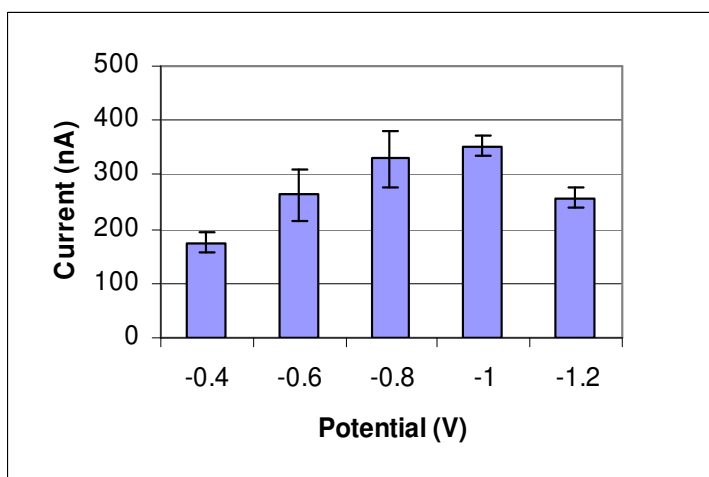
Fig. 3. Effect of applied potential (E) on biosensor response to ClO_4^-. **Results are means of two independent experiments.**
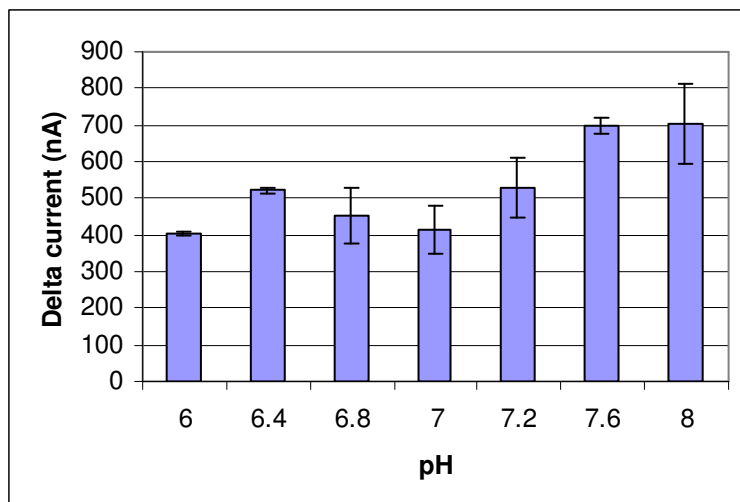
Fig. 4. Effect of matrix pH on biosensor response to ClO_4^-. Results are means of two independent experiments.

Calibration of the ClO_4^- biosensor

The ClO_4^- biosensor construct was tested at different concentrations (1 to 200 µg/L). The ClO_4^- biosensor responded to ClO_4^- at as low as 1 µg/L ClO_4^-. A linear response was obtained in the range 25 to 100 µg/L ClO_4^- (Fig. 5). Kinetic evaluation of the sensor response to ClO_4^- at 25 to 100 µg/L ClO_4^- revealed a zero order reaction ($r^2 > 99\%$) with k values of 10.3, 24.2, 33.9 and 48.2 at 25, 50, 75 and 100 µg/L ClO_4^-, respectively (Fig. 6). Correlation between the biosensor response (change in current) and ion-chromatography conductivity readings in the linear domain of the biosensor (25 to 100 µg/L) is presented in Fig. 7. A very strong correlation between the responses of the two instruments with a coefficient of determination (r^2) of 0.997 was observed. The closeness of this curve fit is an indication that the ClO_4^- biosensor produced a reliable set of data. The sensitivity of the biosensor was dependent upon the concentration of ClO_4^- reductase immobilized on the sensing layer. Reduction of enzyme concentration decreased the sensitivity.

$$y = 3.736x - 4.5$$
$$r^2 = 0.99$$
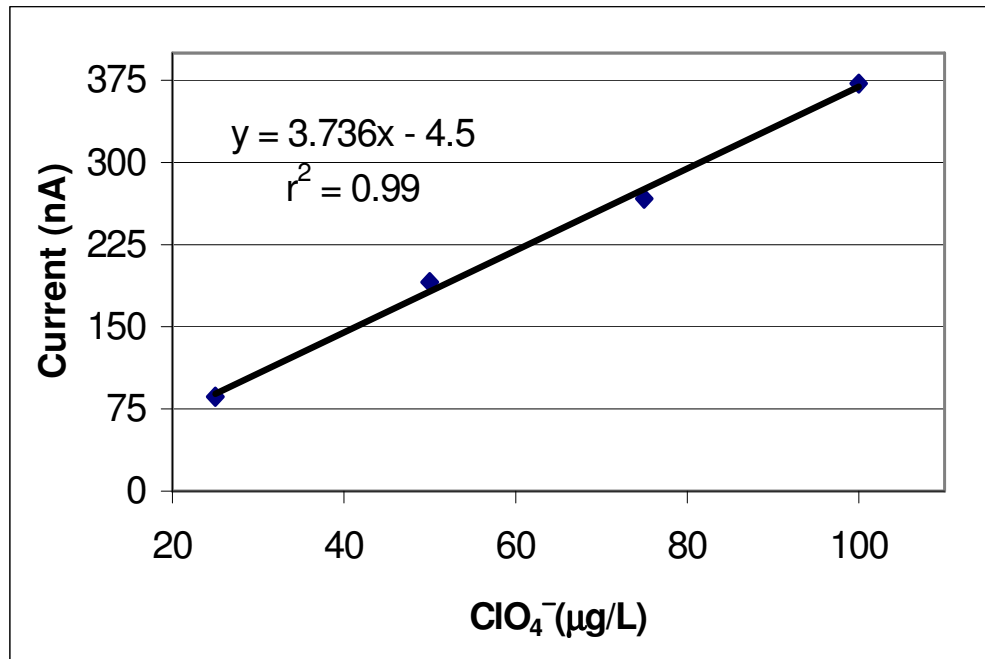
Fig. 5. Standard curve for ClO_4^-. Concentrations tested were in increments of 25 μg/L (1, 25, 50, 75, 100, 125, 150, 175, 200 μg/L). Linear range for this sensor was 25 to 100ppb and was used for the calibration.
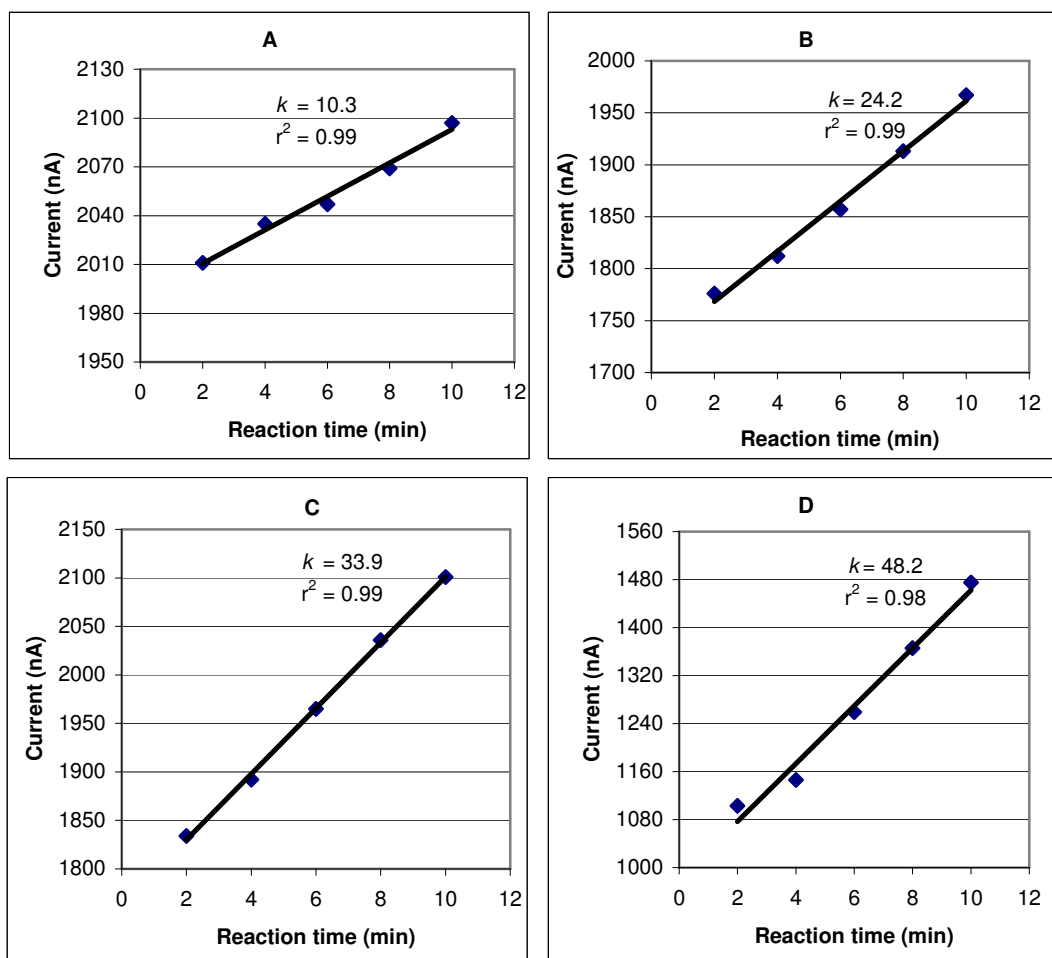
Fig. 6. Kinetics of biosensor response to ClO_4^- at concentrations of 25 µg/L (A), 50 µg/L (B), 75 µg/L (C) and 100 µg/L (D) in 10 min. $k = di/dt$ and was calculated from the curve ($i = kt + c$).
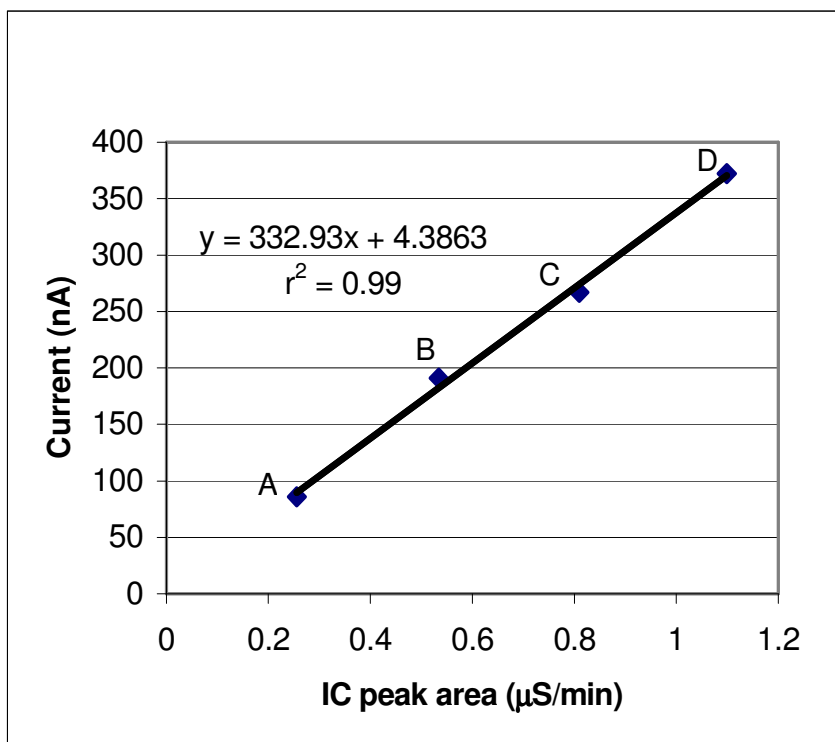
Fig. 7. Correlation between biosensor response (nA) and ion chromatography (IC) response (µS) at 25 µg L^{-1} (A), 50 µg L^{-1} (B), 75 µg L^{-1} (C) and 100 µg L^{-1} (D).

ClO$_4$$^-$ biosensor stability

The ClO$_4$$^-$ biosensor displayed excellent stability after repeated use (24 times on day 1 over an 8-h period at room temperature). Upon further storage at 4^{o}C for 7 days and subsequent analysis of 18 water samples for ClO$_4$$^-$, 30% loss of activity (ClO$_4$$^-$ reduction current) was observed (Fig. 8). The sensor lost approximately 95% of its activity after further storage at 4^{o}C for 7 days.
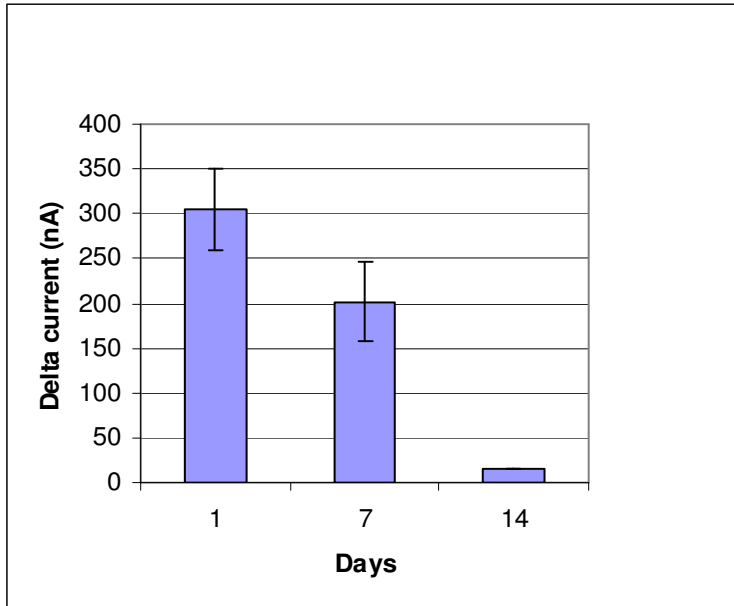
Fig. 8. Stability of the ClO$_4$$^-$ biosensor over 14 days of its construction. Sensor was stored at 4 ^{o}C after each day of analysis.

Effect of NO$_3$$^-$ on ClO$_4$$^-$ sensor response

We examined the effects of different concentrations of nitrate on the biosensor response to ClO$_4$$^-$ (Table 1). Addition of nitrate to the 100 µg/L ClO$_4$$^-$ solution did not cause any substantial interference to the biosensor response readings and kinetic constant. At 0.1 and 50 mg/L NO$_3$$^-$, the biosensor response and k values were 681±163 and 710±96, respectively and the k values were 170±38 and 182±22, respectively. Addition of high concentration of NO$_3$$^-$ (100 mg/L), however, substantially ($p < 0.05$) interfered with the sensor response to NO$_3$$^-$.

Table 1. NO$_3^-$ interference with ClO$_4^-$ measurements in buffer at 100 µg/L ClO$_4^-$.

NO$_3^-$ (mg/L)	Response (Δi, nA)	k	r^2
0	681±116	170±27	0.97±0.01
0.1	671±59	168±15	0.98±0.01
1	652±21	163± 4	0.97±0.02
25	699±95	172±15	0.96±0.02
50	710±69	182±16	0.99±0.01
100	389±31	139±36	0.99±0.01

Kinetic constant (k) = di/dt and was calculated from the curve (i = kt + c).

Discussion

Enzymatic electrodes integrated with nitrate reductase as the recognizing element and redox colorants, particularly methyl viologen (dication 1,1'-dimethyl-4,4'-bipyridinium) (MV) as artificial regenerators have been reported as successful biosensors (Moretto et al., 1998; Ferreya et al., 2000). The reduced form of the methyl viologen (MV^{2+}) is used as a redox regenerator mainly due to its redox potential, which is one of the most negative found in electrochemically reversible organic systems (Moretto et al., 1998). Enzymes and organic molecules that are not themselves electroactive can be reduced by mediators. In our study, sequential coating of the GCE with a nafion layer followed with a ClO$_4^-$ reductase layer and saturation with electrochemically reduced MV as the enzyme regenerator produced an efficient, sensitive and stable biosensor for ClO$_4^-$. With the initiation of the reaction, the background current decayed rapidly reaching a steady state baseline in < 2 min. A nitrate reductase based nitrate biosensor reached a steady state baseline current within 3-4 min (Glazier, 1998). In our study, the magnitude of the background current varied between measurements with the ClO$_4^-$ biosensor. This was, however, normalized by calculating the change in current (2-10 min) and a linear response with respect to time and concentration was observed. The linear increase in ClO$_4^-$ reduction current over the reaction time as well as the linear relationship at different concentrations demonstrates that the enzyme was active and to specifically ClO$_4^-$. Moreover, the bare electrode (without the enzyme immobilized) displayed no response to ClO$_4^-$ as no increases in current was observed. Attempts to immobilize the enzyme by mixing the nafion-ethanol solution before casting it on the GCE surface resulted in precipitation and the sensor made with this procedure retained a weak response to ClO$_4^-$.

Based on response time, the ClO$_4^-$ biosensor was similar to the amperometric nitrate biosensor developed by Glazier et al. (1998), which displayed a response time of approximately 240-300 seconds (including the background current decay time). Higher response time (10-60 min) was reported for a urease-based biosensor (Krawczynski vel Krawczyk et al., 2000). Methods for the determination of low concentrations of ClO$_4^-$ such as ion chromatography (IC) and capillary electrophoresis (CE) are time consuming due to sample processing, run time and equipment breakdown. Interestingly, the response time of the ClO$_4^-$ biosensor was remarkably very short. Although a linear response was not achieved at 1 to 25 µg/L, current data strongly indicates that ultra-low concentrations of ClO$_4^-$ can be at least qualitatively measured using the ClO$_4^-$ reductase-based biosensor. Further efforts will be directed towards obtaining a linear response at ultra low concentrations of ClO$_4^-$. This can be achieved by improving the electrochemical device, the signal/noise ratio and enzyme loading.

Catalytic properties of enzymes are pH dependent. As the pH of environmental sample matrices are likely to vary, we investigated the performance of the biosensor at different pH values. The optimal pH profile of the biosensor mirrors the pH profile of the purified enzyme (Okeke and Frankenberger, 2003) with maximal activity at pH 7.5 – 8.0. The high maximal pH would be an advantage in that chlorite dismutase characterized from a similar organism was optimally active at pH 6.0 and with minimal activity in the range 7.5 to 8.0 (van Ginkel et al., 1996). Thus it is possible to contruct a microbial biosensor for ClO_4^- without the potential interference from chlorite dismutase. The biosensor responded to ClO_4^- at an applied potential as low as 0.4V. The low operating potential of this biosensor is useful in that interferences from electroactive contaminants common in ground water can be greatly reduced when operated at low potentials (Lei et al., 2004; Mulchandani et al., 2001).

Oxygen can create a serious interference through increases in background current. Glazier et al. (1998) reported that the reduction of oxygen at the electrode surface can cause large amounts of background currents. We successfully eliminated this potential interference from residual oxygen by spiking the analytical buffer solution with L-cysteine hydrochloride to a final concentration of 0.25 mg/ml. The capacity of L-cysteine•HCl to remove oxygen had been reported by Atlas (1993). Logan et al. (2004) similarly showed that 0.5 mg/L of L-cysteine.HCl can create and sustain anoxic conditions.

ClO_4^- reductase has been reported to reduce nitrate (Kengen et al., 1999, Okeke and Frankenberger, 2003). Nitrate is a common ground water contaminant and the presence of high concentrations of nitrate in the sample matrix would be expected to substantially interfere with ClO_4^- detection using the ClO_4^- reductase-based biosensor. Thus we investigated the possible interference of nitrate with ClO_4^- measurements. Surprisingly, the addition of nitrate in the range 0.1 to 50 mg/L to a 100 µg/L ClO_4^- solution did not significantly affect the rate constant (Table 1). Thus, water samples that meet the nitrate regulatory limit (<45 mg/L nitrate) apparently may not pose serious interference problems to the ClO_4^- reductase- based biosensor. A higher concentration of nitrate (100 mg/L), however, caused significant interference. Nitrate interference could be alleviated by preatreatment with a nitrate specific nitrate reductase. A nitrate reductase based biosensor (Glazier et al., 1998) did not reduce ClO_4^-, a higher oxidation analogue. Halide analogues of nitrate such as chlorate and bromate are all susbstrates for nitrate reductase (Glazier et al., 1998) and thus, can be removed by pretreatment with nitrate reductase.

Studies on the stability of our ClO_4^- biosensor showed substantial decreases in the degree or magnitude of the biosensor response with use and aging of the enzyme electrode. This can be attributed to decay in enzyme activity over time (Glazier et al., 1998). It was, however, interesting that the ClO_4^- biosensor constructed in this study was substantially stable after several measurements at room temperature (24 $^{\circ}$C) and upon storage at 4°C for 1 week.

Conclusion

In conclusion, enzyme-based biosensors are currently emerging as important environmental analytical tools. A microbial enzyme-based biosensor has been developed for amperometric detection of ClO_4^- in water. A ClO_4^- reductase-based biosensor will be suitable for rapid quantification of ClO_4^- in the environment with the possibility of on-line monitoring of ClO_4^- in automated bioreactor systems. The amperometric sensor reported is portable, inexpensive, easy to operate and responds in real time. High concentrations of NO_3^- showed some degree of interference in the measurement. Future studies will study ClO_4^- biosensing in natural ground water from different sites impacted with ClO_4^-, examine pre-treatment of water samples with NO_3^- reductases that are not active on ClO_4^- and improve on sensor design to allow for analysis of ultra low concentrations of ClO_4^- in the presence of nitrate.

List of Publications

1. **Okeke Benedict C.,** Guangyu Ma, Quan Cheng, Mark E. Losi and William T. Frankenberger Jr Development of a Perchlorate Reductase Based Biosensor for Real Time Analysis of Perchlorate in Water. *J. Microbiol. Method.*
2. **Okeke B. C.**, Guangyu Ma, Quan Cheng, Mark E. Losi and William T. Frankenberger Jr. (2006). Perchlorate Detection in Water by a Microbial Enzyme-Based Biosensor. Abstracts of the 106th General Meeting of the American Society for Microbiology, Orlando, FL USA.

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