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Enzyme-Catalyzed O_2 Removal System for Electrochemical Analysis under Ambient Air: Application in an Amperometric Nitrate Biosensor

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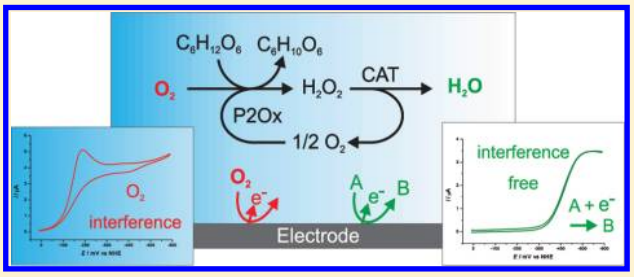
Supporting Information

ABSTRACT: Electroanalytical procedures are often subjected to oxygen interferences. However, achieving anaerobic conditions in field analytical chemistry is difficult. In this work, novel enzymatic systems were designed to maintain oxygen-free solutions in open, small volume electrochemical cells and implemented under field conditions. The oxygen removal system consists of an oxidase enzyme, an oxidase-specific substrate, and catalase for dismutation of hydrogen peroxide generated in the enzyme catalyzed oxygen removal reaction. Using cyclic voltammetry, three oxidase enzyme/substrate combinations with catalase were analyzed: glucose oxidase with glucose, galactose oxidase with galactose, and pyranose 2-oxidase with glucose. Each system completely removed oxygen for 1 h or more in unstirred open vessels. Reagents, catalysts, reaction intermediates, and products involved in the oxygen reduction reaction were not detected electrochemically. To evaluate the oxygen removal systems in a field sensing device, a model nitrate biosensor based on recombinant eukaryotic nitrate reductase was implemented in commercial screen-printed electrochemical cells with 200 μL volumes. The products of the aldohexose oxidation catalyzed by glucose oxidase and galactose oxidase deactivate nitrate reductase and must be quenched for biosensor applications. For general application, the optimum catalyst is pyranose 2-oxidase since the oxidation product does not interfere with the biorecognition element.



Many electrochemical biosensors for field analytical chemistry and point-of-care applications have been developed over the last 30 years. The common goal of this approach is to obtain quantitative information about a specific analyte from a complicated matrix without any sample pretreatment. Up until now, several oxidase-based amperometric biosensors have become medical[1−4] and environmental applications.[5−7] In contrast, field applications of biosensors based on reductases are generally limited by the need for anaerobic conditions. Indeed the cathodic reduction of the analyte catalyzed by the reductase usually occurs at a potential more negative than the cathodic oxygen reduction reaction. Moreover, biosensors are often based on mediated electrochemistry and the reduced forms of many mediators react with oxygen (e.g., methyl viologen[8]). However, the standard oxygen removal methods employing argon purging or vacuum degassing are not compatible with on-site monitoring. The precondition for any successful field application of reductase-based biosensors is therefore an effective and easy-to-use oxygen removal method.

Nitrate biosensors for on-site determination of nitrate in water, soil, or plant samples are potentially very valuable with respect to economic, environmental, and health issues.[9−16] Recently, the use of sodium sulfite as an oxygen scavenger[17,18] for a field nitrate amperometric biosensor based on recombinant eukaryotic nitrate reductase (EC 1.7.1.2; NaR) was described.[19] However, the maximum sulfite concentration, which does not affect the analysis, is a few millimolar. This is sufficient for large volumes or closed systems since the concentration of dissolved oxygen in air saturated aqueous electrolytes is below 0.4 mM[20] at typical operating temperatures for a field biosensor (5−35 °C). However, for on-site measurements very small sample volumes (<100 μL) in an open system are desired. Under these conditions, it is not possible to maintain anaerobic conditions with sulfite for the time required for electrochemical measurements due to the diffusion of oxygen from air into the electrolyte solution. Therefore, a generally applicable and more efficient oxygen scavenger that can be used at high concentration, interfering neither with the electrochemical measurements nor with the biorecognition element, is necessary.

Several enzymatic oxygen scavenging systems are suitable for various polarographic,[21] biochemical,[22] and fluorescence[23,24] applications or as food antioxidants.[25] In particular, glucose oxidase (EC 1.1.3.4; GOx) has shown to be an effective catalyst

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for oxygen reduction.[21,26−28] In the present work, a similar enzymatic method was developed for an amperometric nitrate biosensor for on-site monitoring. In this approach, oxygen is reduced by catalytic oxidation of an aldohexose by its corresponding oxidase (eq 1). Then, in a second step, the hydrogen peroxide resulting from this reaction is disproportionnated into water and O_2 catalyzed by a catalase (EC 1.11.1.6; CAT) (eq 2). The oxygen molecules regenerated by the catalase are further reduced by the oxidase. In the net reaction, two aldohexose molecules are necessary for the reduction of one oxygen molecule (eq 3).

$$\text{oxidase reaction:} \quad O_2 + C_6H_{12}O_6 \rightarrow C_6H_{10}O_6 + H_2O_2 \tag{1}$$

$$\text{catalase reaction:} \quad H_2O_2 \rightarrow \frac{1}{2}O_2 + H_2O \tag{2}$$

$$\text{net reaction:} \quad O_2 + 2C_6H_{12}O_6 \rightarrow 2C_6H_{10}O_6 + 2H_2O \tag{3}$$

The use of relatively inert aldohexoses as the reducing agents for oxygen opens the possibility to introduce high concentrations of the aldohexoses for achieving oxygen depletion in small volumes for extended time periods under ambient air without affecting the biorecognition component of the reductase biosensor. Since the main issue with this approach is the reactivity of the oxidation product ($C_6H_{10}O_6$) of the aldohexoses, two novel strategies for biocatalyzed oxygen reduction based on galactose oxidase (EC 1.1.3.9; GOase) and pyranose 2-oxidase (EC 1.1.3.10; P2Ox) were investigated besides the GOx based approach. Each of these oxidases catalyzes the oxidation of a different hydroxyl group of the aldohexoses (Figure 1). GOx catalyzes the oxidation of the

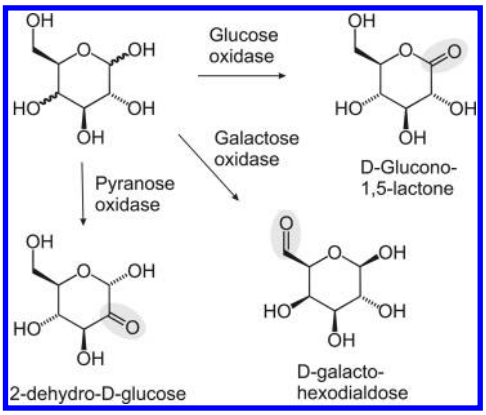

Figure 1. Glucose oxidase, galactose oxidase, and pyranose 2-oxidase catalyzed reactions starting from their respective aldohexose substrate. Only partial reactions are shown here omitting oxygen and hydrogen peroxide.

hemiacetal of glucose to the corresponding lactone.[25] GOase catalyzes the reaction of the primary alcohol in galactose to the corresponding aldehyde.[29] P2Ox catalyzes the oxidation of a secondary alcohol of glucose to the respective ketone.[30] Besides their efficiency in oxygen reduction, the possible interferences of the products from the three oxidase-catalyzed reactions with the electrochemical reaction as well as with the catalytic reaction of the biorecognition element were studied.

EXPERIMENTAL SECTION

Instrumentation and Chemicals. All electrochemical experiments were conducted with the BAS CV 50W interface and software (Bioanalytical Systems) except for the measurement of plant extract samples 7 to 9 (see Results and Discussion) performed with a custom-made field potentiostat. The electrochemical cell consisted of a conventional 3 electrode set with 2 or 2.5 mL of electrolyte. The working electrode was a highly oriented pyrolytic graphite edge-plane disk electrode (PGE) from Pine Instruments (geometric surface area A = 0.196 cm^2) and from ALS (A = 0.071 cm^2). The reference electrode was Ag/AgCl in 3 M KCl purchased from Bioanalytical Systems. A platinum wire (1.0 mm in diameter) served as the counter electrode. The air−electrolyte interface surface area was about 0.8 cm^2. Screen printed carbon electrode (SPCE) microwell arrays (Figure S1 in the Supporting Information) were used as received from PalmSens. They consist of strips of 40 mm × 84 mm in dimension on which 8 sets of 3 electrodes are screen printed. The working and counter electrodes were made of carbon, and the pseudoreference electrode was made of silver. An 8-hole methacrylate box (L × W × H = 14 mm × 84 mm × 5 mm) was fixed onto the sensorstrip using a double layer adhesive, allowing one to produce an 8-cell electrochemical array. The maximum volume for each well is 300 μL. The electrolyte volume was either 100 or 200 μL. The surface area of the interface with air was of about 0.2 cm^2. The strips were used with a PalmSens CH8 multiplexer by means of a 24-pin connector as well as a custom-made 24-pin connector. All experiments were performed at RT (~22 °C).

GOx (EC 1.1.3.4; type II from *Aspergillus niger*, 17.3 U mg^{-1}), P2Ox (EC 1.1.3.10 from *Coriolus sp.*, 10.4 U mg^{-1}), and CAT (EC 1.11.1.6 from bovine liver, 2−5 kU mg^{-1}) were purchased as lyophilized powders from Sigma and used as received. Purified NaR (YNaR1, freeze-dried with sucrose and EDTA) and GOase were from NECi (see nitrate.com). All other chemicals were of reagent grade. Deionized water (18 mΩ) was used in all experiments.

Oxygen Removal. The efficiency of the oxygen removal system to achieve and maintain anaerobic conditions was tested in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (50 mM, pH 7.0) with ethylenediaminetetraacetic acid (EDTA, 20 μM) at various glucose concentrations. Cyclic voltammetry (CV) was performed at 2 mV s^{-1} from 0 to −800 mV vs Ag/AgCl for the cell with 2 mL of electrolyte and from −200 to −1000 mV vs Ag pseudo reference electrode for the carbon SPCE cell with 100 μL of electrolyte. The CAT and oxidases (at various concentrations) were added directly to the electrolyte as the lyophilized powder or as a solution in the same buffer.

NaR Activity. To investigate the effect of the oxygen removal system on the NaR activity and stability, MOPS buffers (pH 7.0) at various concentrations were mixed with methyl viologen (MV, 50 μM), KNO_3 (2.5 mM), EDTA (20 μM), NaR (330 nM, 1.2 U mL^{-1}), the oxidase at various concentrations, the respective aldohexose at 50 mM, and the CAT at various concentrations. In all cases, the CAT was added before the oxidase to prevent build-up of hydrogen peroxide in the electrolyte. The total volumes for the unstirred, open electrochemical cells with the PGE and the SPCE were of 2.5 mL and 100 μL, respectively. Multiple CVs at 2 mV s^{-1} from 0 to −800 mV vs Ag/AgCl were performed at specific times after

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mixing the components for O_2 removal and nitrate reduction. In some experiments, gluconolactone (D-(+)-gluconic acid δ-lactone) was added to simulate the full oxidation of glucose. The gluconolactone was added as the pure compound to achieve a concentration of 50 mM in the electrolyte.

pH-Dependent Measurements. For the pH-dependent activity and stability tests of the P2Ox and the GOx based oxygen removal systems, buffered glucose (50 mM) solutions were prepared in a pH range of 3.0 to 9.1. The buffer solutions for pH values 3.0, 4.0, 5.0, 6.0, and 7.0 were prepared by mixing adequate amounts of a citric acid (0.1 M) solution with a Na_2HPO_4 (0.2 M) solution. For pH 8.0, a NaH_2PO_4/Na_2HPO_4 buffer (0.2 M), and for pH 9.1, a $NaHCO_3/Na_2CO_3$ buffer (0.2 M) was used. A CV was recorded at 20 mV s^{-1} in the 2 mL electrochemical cells (from 0 to −800 mV vs Ag/AgCl) containing the buffered glucose solution (1.8 mL) of the respective pH value. The maximum current of the oxygen reduction signal was determined without subtraction of the background current. Then, 100 μL of aqueous solutions of CAT (167 μM) and GOx (125 μM) or of CAT (167 μM) and P2Ox (150 μM) were added. After 1 min of stirring, another CV was recorded and the maximum current was determined. Further CVs were recorded every hour (5 h overall) while purging the system with about 2 mL of air using a pipet each time 1 min before a measurement. After the last CV was recorded, the pH of each solution was determined.

Determination of $c(NO_3^-)$ in Extracts from Plant Samples. The plant extract samples were prepared according to the literature.[11] The nitrate determination via the Griess assay was performed after the enzymatic reduction of nitrate to nitrite[31] as well as after the reduction with cadmium,[11] followed by the spectroscopic determination of nitrite after reacting with the Griess reagent.

The electrochemical nitrate determination with the model biosensor was performed as follows: For a set of measurements, 10 mL of a buffer containing EDTA (40 μM), D-glucose (100 mM), MV (100 μM), and MOPS (400 mM) at pH 7.0 (adjusted with KOH) in deionized H_2O was prepared (solution A). NaR (1.67 nmol, 6 U) was added to 2.5 mL of solution A to give solution B. A 250 μL solution with GOx (150 μM), CAT (330 μM), EDTA (40 μM), MV (100 μM), and MOPS (400 mM) at pH 7.0 in deionized H_2O was prepared (solution C).

The background currents of the samples (in absence of NaR) were first measured. For this, 100 μL of solution A was added to 125 μL of pure sample in a test tube. A volume of 25 μL of solution C was then added and mixed by gently shaking the test tube. Then, 200 μL of the resulting solution was immediately added to a SPCE well and, after a 1 min waiting time, the current was set for 3 min at −1000 mV vs the Ag screen printed pseudo reference electrode.

To obtain the catalytic current, the same procedure as for the background current was repeated in a second SPCE well, except that solution B (with NaR) was used instead of solution A. Each sample measurement was done twice. The background current was then subtracted from the current obtained for the plant extract sample in the presence of NaR. In order to obtain the net current due to the reduction of nitrate in the sample, the background current due to the presence of NaR and its stabilizing agent (sucrose) must be subtracted as well. The latter was obtained by repeating the chronoamperometric experiment for 3 min at −1000 mV vs the Ag screen printed pseudo reference electrode in MOPS (200 mM pH 7.0), EDTA (20 μM), D-glucose (50 mM), MV (50 μM), GOx (3.7 μM), and CAT (8.3 μM) in the presence and absence of NaR (330 nM, 1.2 U mL^{-1}). The difference in current (four repetitions for the experiments with and without NaR) was 1.7×10^{-8} A (±2.5%).

The calibration curve was obtained from the measurement under the same conditions but with nitrate standard solutions instead of sample solutions. A modified Michaelis−Menten equation (eq 4) was used to fit the experimental data by nonlinear regression:

$$i_{cat} = i_{cat,max} c(NO_3^-)/[K_M + c(NO_3^-)] \quad (4)$$

where the catalytic current (i_{cat}) is used instead of the enzymatic reaction rate. $c(NO_3^-)$ is the nitrate concentration, $i_{cat,max}$ is the maximum catalytic current, and K_M is the Michaelis−Menten constant.

The nitrate concentration of the plant extract was obtained by applying eq 4 to the catalytic current from the sample measurements using the parameters extracted from the calibration curves (K_M and $i_{cat,max}$ values).

RESULTS AND DISCUSSION

Initially, the glucose-GOx-CAT system was tested with respect to its ability to remove oxygen and maintain anaerobic conditions at pH 7.0 in unstirred electrochemical cells open to ambient air. The standard three electrode cell (PGE as working electrode) with 2.0 mL of electrolyte solution was used for initial testing. For model field applications, commercially available single-use screen printed carbon electrodes (SPCE) were used with wells filled with 100 μL of electrolyte. For both electrochemical cells, the presence of oxygen was monitored at the carbon based working electrodes by CV.

In absence of GOx and CAT, the cathodic oxygen reduction signal appears at potentials lower than 50 mV vs NHE at the PGE whether glucose is present in solution or not (Figure 2).

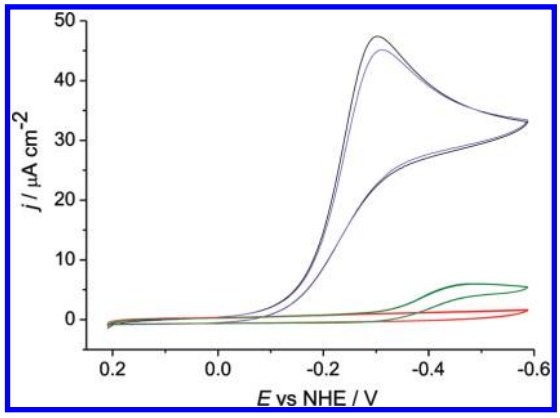

Figure 2. Cyclic voltammogram with PGE at 2 mV s^{-1} in 2 mL of MOPS buffer (50 mM, pH 7.0) (black line), upon addition of glucose (50 mM) (blue line), and upon addition of GOx (12.5 μM, 15 U mL^{-1}) and CAT (16.6 μM, 2 kU mL^{-1}) (red lines, 9 CVs recorded over 5 h). CV with PGE at 2 mV s^{-1} in 2.5 mL of MOPS buffer (200 mM, pH 7.0) with MV (20 μM), KNO_3 (10^{-4} M), EDTA (20 μM), glucose (50 mM), GOx (12.5 μM, 15 U mL^{-1}), CAT (16.6 μM, 2 kU mL^{-1}), and NaR (330 nM, 1.2 U mL^{-1}) (green lines, 8 successive CVs).

After addition of GOx and CAT, oxygen is efficiently removed from the glucose containing electrolyte (Figure 2). The remaining background current is comparable or lower to the one obtained after purging the electrolyte with inert gas for

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30 min (Figure S2 in the Supporting Information). Similar results were obtained with the SPCE system. With about 10 U mL^{-1} of the oxidase and at least 1 kU mL^{-1} of the catalase, no waiting time was necessary to run a CV without an O_2 signal.

Besides the parameters controlling the diffusion kinetics of oxygen into the electrolyte,[32] the cell geometry (electrolyte volume and air−liquid interface surface area) and the glucose concentration defines the time period over which the anaerobic conditions can be maintained. In the 2 mL electrochemical cell, 20 mM glucose was sufficient to keep the solution oxygen-free for at least 2 h. With 50 mM glucose, the anaerobic conditions were maintained at least 5 h (Figure 2). In the case of the SPCE cell with 100 μL of electrolyte, higher glucose concentrations (50 mM) were necessary to keep anaerobic conditions for at least 90 min. For field biosensor applications, smaller sample volumes are preferred and 50 mM glucose was used in the following experiments.

The components (i.e, aldohexoses and biocatalysts) as well as the final products of the oxygen removal system are all redox-inactive at carbon based electrodes in the electrochemical window of interest for reductase biosensors. Only the electrochemical reduction of hydrogen peroxide produced from the oxygen reduction may theoretically occur at the electrode surface. However, the catalase ensures that hydrogen peroxide concentration remains low enough to avoid any electrochemical detection of this reaction intermediate. Thus, the absence of electrochemical interferences due to the GOx-based oxygen removal system was demonstrated.

The second requirement for the oxygen removal system for field biosensors comprises the absence of interactions with the biorecognition element involved in the catalytic reaction for analyte detection. To investigate this aspect, a model nitrate biosensor system consisting of recombinant eukaryotic nitrate reductase[31] (NaR) and methyl viologen (MV) in solution as the electron mediator[33] was tested in the presence of the enzyme-based oxygen removal system. The NaR activity in the presence of a constant, saturating nitrate concentration was monitored by CV. The catalytic current due to the reduction of nitrate to nitrite was used as a measure of the NaR activity.

When the measurement was carried out in the 2.5 mL cell with glucose in the presence of GOx and CAT, the cyclic voltammogram displayed the sigmoidal shape typical for NaR catalyzed reduction of nitrate mediated by MV (Figure S3 in the Supporting Information). The activity of the enzyme is comparable to the one obtained when the anaerobic conditions are achieved by argon purging, showing that the enzymatic oxygen removal system does not affect the initial activity of NaR. However, the loss of activity over 4 h was 11.3% (Figure S3 in the Supporting Information). After the last CV was recorded, the pH of the solution had decreased to 6.0. In the low volume open SPCE wells (100 μL), an even faster decrease in activity was observed (45% in 60 min, Figure S4 in the Supporting Information). Since the ratio of electrolyte volume to air−electrolyte interface surface area is much lower in the case of the 100 μL wells, more glucose is oxidized per volume and time unit as compared to the 2.5 mL cell. This results in a faster increase in the concentration of the reaction products. The oxidation of glucose yields a gluconolactone (D-(+)-gluconic acid δ-lactone), which is rapidly hydrolyzed to gluconic acid.[25] The latter is responsible for the observed pH drop and decrease in NaR activity (Supporting Information). The reaction intermediate, hydrogen peroxide, is not likely to participate in the NaR inhibition, since the absence of a redox signal for H_2O_2 demonstrates that it is quickly disproportionated by the catalase. Apart from the gluconolactone, the only other product of the oxygen removal system is water.

To circumvent the pH drop due to gluconic acid formation, higher buffer concentration within the limits of ionic strength not affecting the sensing enzyme activity may be used. In the case of NaR, the MOPS buffer with a concentration of 200 mM is the best compromise with respect to both maximum catalytic activity and stability (Figure S3 in the Supporting Information). Stable catalytic currents are observed at both saturating (Figure 3) and low nitrate concentrations (Figure 2 and Figure S5 in the Supporting Information). Moreover, the increased buffer concentration does not affect the efficiency of the GOx based oxygen removal system (Figure S6 in the Supporting Information).

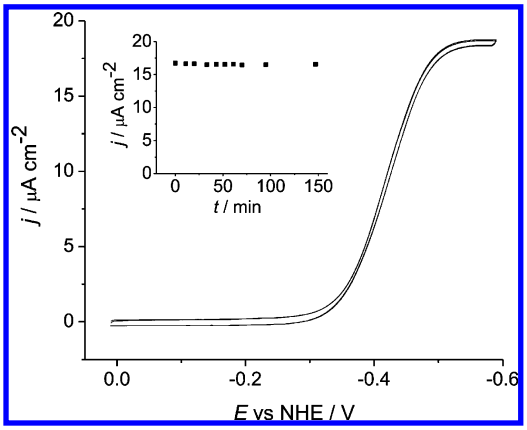

Figure 3. Cyclic voltammograms with PGE at 2 mV s^{-1} in 2 mL of MOPS buffer (200 mM, pH 7.0) with MV (50 μM), KNO_3 (2.5 mM), EDTA (20 μM), glucose (50 mM), GOx (12.5 μM, 15 U mL^{-1}), CAT (16.6 μM, 2 kU mL^{-1}), and NaR (330 nM, 1.2 U mL^{-1}). The inset shows the change in catalytic current density vs time.

Although a suitable buffer concentration was found to limit the effect of gluconic acid in the case of nitrate reductase, an adequate buffer concentration may not be available for all reductases. Furthermore, the time over which the anaerobic conditions can be maintained exceed the time over which the sensing enzyme activity is stable due to the acid production and buffer strength limitations (Figure S7 and Table S1 in the Supporting Information). An alternative strategy to fully exploit the potential of the oxygen removal system for general applications in biosensors was to avoid the production of gluconic acid. For this purpose, other oxidases that involve the production of a pH-neutral product were tested as oxygen reduction catalysts.

The oxidation of galactose with galactose oxidase (GOase) involves the oxidation of a primary alcohol to an aldehyde (Figure 1). The product, D-galacto-hexodialdose, is not expected to affect the pH of the solution. The galactose-GOase-CAT system showed the same ability to remove oxygen from a MOPS buffered solution (pH 7.0) as the glucose-GOx-CAT system in the 2 mL electrochemical cell (Figure S8 in the Supporting Information).

However, in the biosensor model system, the activity of NaR decreased quickly when GOase and CAT in the presence of galactose were used for oxygen removal. Although the pH value of the electrolyte remained stable at 7.0, the loss of NaR activity over 47 min was 58.6% (Figure S9 in the Supporting Information).

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In this case, it is suspected that the aldehyde group of the oxidation product reacts with primary amine residues of NaR resulting in the enzyme deactivation. Therefore, GOase cannot be used to maintain anaerobic conditions for biosensors based on NaR. It may only be applied for electrochemical biosensors based on redox enzymes that are not affected by the aldehyde functionality. The addition of another primary amine to quench the aldehyde product was not attempted in the present work.

The third enzyme, pyranose oxidase (P2Ox), was chosen to circumvent the drawbacks of both glucose oxidase and galactose oxidase. As for the two other oxidases, fast oxygen removal was achieved and the anaerobic conditions were maintained for at least 5 h in the 2 mL electrochemical cell (Figure S10 in the Supporting Information). However, in contrast to the other systems, the glucose-P2Ox-CAT system showed virtually no effect on the NaR activity. At saturating nitrate concentration, the catalytic current decreased by only 0.9% over 4 h (Figure 4). Even at low nitrate concentration, the

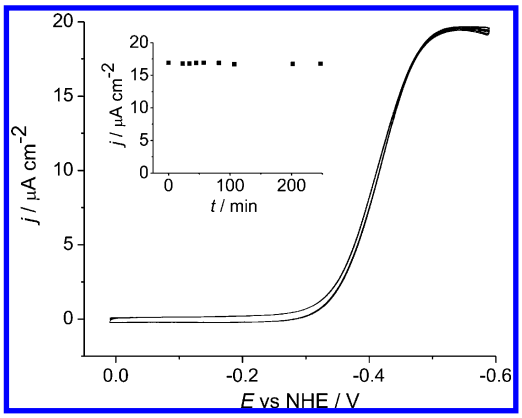

Figure 4. Cyclic voltammograms with PGE at 2 mV s^{-1} in 2.5 mL of MOPS buffer (50 mM, pH 7.0) with MV (50 μM), KNO_3 (2.5 mM), EDTA (20 μM), glucose (50 mM), P2Ox (7.3 μM, 5 U mL^{-1}), CAT (8.3 μM, 1 kU mL^{-1}), and NaR (330 nM, 1.2 U mL^{-1}). The inset shows the change in catalytic current density vs time.

fluctuations in current density (±28 nA cm^{-2}) are within the one observed for the background current (±37 nA cm^{-2}) (Figure S11 in the Supporting Information). This demonstrates that the ketone (Figure 1) produced from the oxidation of glucose with P2Ox as catalyst does not inhibit NaR under the measurement conditions. The pH of the solution is also not affected by this reaction, making an adjustment of the buffering strength unnecessary. Therefore, the maximum operating time of a nitrate biosensor coupled to the P2Ox based oxygen removal system is only limited by the time period over which anaerobic conditions are maintained.

In order to investigate the usability of the P2Ox and the GOx based oxygen removal systems for a broader application area, the activity and stability of the systems were tested over a pH range of 3.0 to 9.1 (Figure S12 in the Supporting Information). At both pH 3.0 and pH 9.1, it was not possible to fully remove all redox-active species either with the GOx or with the P2Ox based system. By contrast, a complete removal of any redox-active species in the desired potential range was achieved for pH values from 4.0 to 8.0 for both oxidases. In all these cases, the depletion was extremely fast, except for P2Ox at pH 8.0, where full removal was only observed in the system 1 h after the enzyme addition. No significant change in current was observed in the ensuing measurements for pH 5.0 to 8.0 even after 5 h. At pH 4.0, a slight increase in the maximum current was observed four hours after the addition of enzymes. The use of higher enzyme concentrations might reduce these limitations at very low and very high pH values. This was shown for the P2Ox system at pH 8.0, where the use of 37 μM of P2Ox resulted in a complete oxygen removal directly after the enzyme addition. It should be noted that, at pH 4.0, the addition of CAT and/or P2Ox leads to the formation of a precipitate. Still complete oxygen removal is achieved at this pH, at least for measurements that do not exceed 3 h.

The enzyme based oxygen removal system was tested under field conditions, using the model nitrate biosensor with the SPCE wells. The samples consisted of plant leaf tissue extracts prepared in the laboratory in a manner similar to field analysis samples.[11] The complicated plant extract matrix was a good test for the proposed oxygen removal system for nitrate biosensors. The general procedure consisted in measuring the current due to the reduction of the nitrate in the samples by NaR in solution with MV as the electron mediator, as seen previously. In addition to the standard lab bench potentiostat, a custom-made hand-held potentiostat was used to simulate the field conditions. In the particular case of NaR, both GOx (with increased buffer strength) and P2Ox may be used as the oxygen reduction catalyst at pH 7.0. Since GOx is currently less expensive than P2Ox, the former was chosen for the field test.

The reproducibility of the current responses from the SPCE at constant nitrate concentration in the presence of the oxygen removal system were tested for 200 μL of total volume. The relative standard deviation from the measurements of eight different SPCEs for the current values is 2.95% (Figure S13 in the Supporting Information). The model nitrate biosensor was calibrated with standard nitrate solutions by measuring the catalytic current (Figure S14 in the Supporting Information). The modified Michaelis–Menten equation (eq 4) closely fits the experimental data. In contrast, catalytic currents from calibration performed in absence of oxygen removal system are not exploitable for nitrate determination (Figure S15 in the Supporting Information) because of the large and fluctuating background current due to oxygen reduction.

The nitrate content in the plant samples found with the model nitrate biosensor in the presence of the oxygen removal system (Table 1) correlate with those obtained in the Griess

Table 1. Nitrate Analysis of Plant Samples Comparing Griess Assay (Nitrate Reduction by Cd and NaR) and the Electrochemical Method with the Model Nitrate Biosensor

	$c(NO_3^-)$/ppm		
	Griess assay	Griess assay	
sample	Cd	NaR	biosensor
1	1.22	1.17	1.23
2	1.21	1.52	1.58
3	4.18	4.26	4.04
4	3.96	3.83	4.92
5	1.18	1.19	0.96[a]
6	3.65	4.00	3.37[a]
7	4.36	4.90	4.48[a]
8	1.61	1.75	1.70[b]

[a]Electrochemical measurement performed with a custom-made field potentiostat. [b]Spiked sample measurement with three different standard concentrations.

assay[12] based on an automated cadmium reduction analyzer[11] as well as with one based on NaR[31] for the nitrate reduction to nitrite.

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The measurements performed with the hand-held custom-made potentiostat to fully simulate field conditions (Table 1, entries 5−7) also correlate with the results from the laboratory-based method. Therefore, the model nitrate biosensor coupled to the GOx based oxygen removal system enables on-site nitrate quantification. Moreover, it also offers a complementary method to the Griess assay. Indeed, the Griess assay based on spectrophotometric detection is affected by highly colored and heterogeneous matrixes as well as by mild reducing agents and nucleophiles that interfere with the diazonium intermediate or the azo chromophore.[12] Both types of interferences can be present in the complex matrixes of a plant extract. The nitrate biosensor based nitrate determination is mainly affected by compounds deactivating the NaR resulting in underestimation of the nitrate concentration. The latter issue is easily overcome with spiked samples (Table 1, entry 8) resulting in excellent agreement between the Griess assay and the electrochemical biosensor results.

CONCLUSIONS

New oxygen removal systems based on oxidases in the presence of their aldohexose substrate and a catalase were developed for field biosensor applications. Electrochemical measurements under ambient air were free of oxygen interferences even for electrolyte volumes as low as 100 μL. By targeting the oxidation of the hemiacetal in glucose with glucose oxidase for oxygen reduction, the production of gluconic acid needs to be compensated by a high buffer concentration to maintain the pH at the optimum value for the biorecognition element. Quantification of nitrate in an open electrochemical biosensor based on nitrate reductase as the biorecognition element and GOx as O_2 reduction catalyst was demonstrated. However, the stability of the biosensor response is limited by the capability of the buffer to neutralize the acidic product and by the maximum buffer strength tolerated by the enzymes involved. Therefore, although this approach is successful to maintain anaerobic conditions in the model nitrate biosensor described here, it may not be suitable for all biorecognition elements. This issue was addressed with oxidases that catalyze the oxidation of alcohol groups from the aldohexose. With pyranose 2-oxidase as a catalyst, a secondary alcohol in glucose is oxidized to a ketone. This product is pH-neutral and does not affect the activity of nitrate reductase. The time period over which the anaerobic conditions are maintained are only limited by the maximum glucose concentration that can be used. This makes the novel pyranose 2-oxidase based system most promising for oxygen removal for general applications of electrochemical biosensors based on a wide range of reductases, cell geometries, and sample volumes.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on February 10, 2012. A misspelled word was corrected throughout the manuscript, and the corrected version was reposted on February 15, 2012.