



GAS-SENSING INTERNAL ENZYME FIBER OPTIC BIOSENSOR FOR HYDROGEN PEROXIDE

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Summary—Feasibility is demonstrated for a novel gas-sensing, internal enzyme biosensing scheme for the selective measurement of hydrogen peroxide. Two horseradish peroxidase catalyzed reactions are evaluated for the detection of hydrogen peroxide as it crosses a microporous Teflon membrane at 37°C. The rate at which hydrogen peroxide crosses the membrane is determined by either a fluorescence or chemiluminescence measurement and this rate is related to the concentration of hydrogen peroxide in the sample solution. Detection limits of 0.7 mM and 10 μ M are estimated for the fluorescence and chemiluminescence methods, respectively. Selectivity is demonstrated for hydrogen peroxide over ascorbic acid, uric acid and tyrosine.

Many different sensor designs have been reported for the measurement of hydrogen peroxide. Most simply, hydrogen peroxide is oxidized electrochemically and the resulting anodic current is directly proportional to the concentration of hydrogen peroxide in solution. Unfortunately, direct oxidation suffers interference from easily oxidizable species which is a particular problem with many biological samples because of the frequent presence of ascorbic and/or uric acids. Anionic¹ and gas-permeable membranes^{2,3} have been used to enhance the selectivity of this approach by restricting or eliminating the flux of interferences to the electrode surface.

Optical sensors for hydrogen peroxide have also been reported.^{4,5} In fact, the first fiber-optic biosensor was reported by Freeman and Seitz for the measurement of hydrogen peroxide. This biosensor was constructed by immobilizing a thin layer of horseradish peroxidase (HRP) at the distal tip of a bundle of optical fibers.⁴ The chemiluminescence reaction between hydrogen peroxide and luminol is catalyzed at the probe tip by the immobilized HRP and the resulting light is detected. Alternatively, Posch and Wolfbeis have reported three different sensors for hydrogen peroxide.⁵ Each is based on the catalytic decomposition of hydrogen peroxide to

form oxygen and water. The resulting change in local oxygen tension is detected by monitoring the extent of fluorescence quenching of an immobilized indicator dye.

We have examined the feasibility of a novel biosensor for hydrogen peroxide based on an internal enzyme gas-sensing measurement scheme. Sensors are constructed by placing a gas-permeable microporous Teflon membrane between the sample and a small pool of an internal enzyme solution that contains HRP. The working principle of the sensor is illustrated schematically in Fig. 1. A small fraction of hydrogen peroxide in the sample crosses the membrane and enters the internal solution where HRP catalyzes the analytical reaction. Product formation is monitored optically by a fiber-optic transducer and the resulting rate of product formation is related to the concentration of hydrogen peroxide in the sample.

Two unique biosensor configurations have been evaluated. First, homovanillic acid (HVA) is used and formation of an HVA-dimeric species⁶ is monitored by a fluorescence measurement. Second, the HRP catalyzed oxidation of luminol⁷ is monitored by a chemiluminescence (CL) measurement. In both cases, the rate of the internal enzyme reaction depends on the concentration of hydrogen peroxide in the sample solution.

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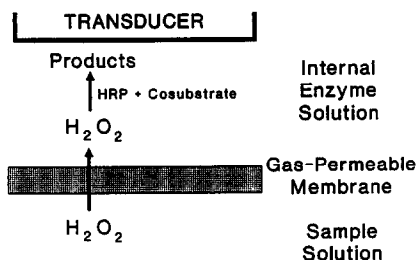


Fig. 1. Schematic of the gas-sensing internal enzyme biosensor for hydrogen peroxide.

EXPERIMENTAL

Apparatus and Reagents

The optical arrangement for the fluorescence-based sensor was the same as that described previously for our NADH-based glutamate biosensor.⁸ In this arrangement, light was brought to the sensor tip through one leg of a bifurcated fiber-optic probe. The second leg carried light emitted in the internal solution to the detector optics. A lock-in amplifier was used in conjunction with a mechanical chopper to enhance the signal-to-noise ratio of the measurement.

The optical arrangement was much simpler for the CL-based sensor. Light generated in the internal enzyme solution was collected by a bundle of quartz optical fibers and transmitted directly to an Oriel (model 77340) photomultiplier tube (PMT) operated at -650 V. The current from the PMT was measured with an Oriel (model 7070) photometer. The photometer output was digitized with an Analog Connection Jr. A/D board from Strawberry Tree, Inc., Sunnyvale, CA, U.S.A. The signal was stored on an IBM-XT computer. This arrangement does not involve filters, lock-in amplifier or light source.

Absorbance measurements were made with a Perkin-Elmer Lambda 1 single beam spectrometer. All pH measurements were made with a Beckman (model 71) pH/mV meter in conjunction with a Ross Sure-Flow combination pH electrode (Orion model 8172).

HRP (type II), hydrogen peroxide, homovanillic acid, luminol, tyrosine, ascorbic acid, and uric acid were used as received from the Sigma Chemical Co., St. Louis, MO, U.S.A. Stock solutions of hydrogen peroxide were prepared by diluting a 30% solution. All hydrogen peroxide solutions were standardized by titration with sodium thiosulfate.⁹ Microporous Teflon membrane was purchased from Gore & Associates, Elkton, MD, U.S.A.

Procedures

Sensors were fabricated by stretching a square of the microporous Teflon membrane over one end of a plastic tube. An O-ring was used to hold this membrane in place. Unless stated otherwise, the Teflon membranes used throughout had an average pore size of $1.0 \mu\text{m}$ and an overall porosity of 91%. These membranes were laminated with a polyethylene scrim for mechanical strength. A specific volume of the internal enzyme solution was then added and the fiber-optic probe was positioned within the sensor cavity. For the fluorescence-based sensors, $30 \mu\text{l}$ of the internal enzyme solution was used. This internal enzyme solution contained 0.763 mg/ml (167.9 units/ml) HRP and 13.7 mM homovanillic acid dissolved in a pH 8.5, 0.10 M Tris-HCl buffer. For the CL-based sensors, $70 \mu\text{l}$ of the internal enzyme solution was used. This internal enzyme solution was composed of $10.9 \mu\text{g/ml}$ (2.18 units/ml) HRP and 0.968 mM luminol dissolved in a pH 9.0, 0.01 M borate buffer.

Sensor responses were measured by placing the sensor tip in a fresh aliquot of working buffer with no hydrogen peroxide. A pH 7.0, 0.05 M phosphate buffer was used as the working buffer for both the fluorescence and CL-based sensors. After a steady-state background signal was established, a microliter volume of a hydrogen peroxide standard was added to give the desired concentration. The sensor signal was monitored as a function of time and the intensity-time data were analyzed after the experiment was completed.

RESULTS AND DISCUSSION

The vapor pressure of hydrogen peroxide is critical for the success of this approach because it will dictate both the sensitivity and detection limit for hydrogen peroxide measurements. In fact, the feasibility of this approach depends solely on the vapor pressure of hydrogen peroxide under the experimental conditions. A significant amount of hydrogen peroxide must be present in the vapor phase to allow detection in the internal solution.

An experiment was performed to determine the amount of hydrogen peroxide that crosses the microporous Teflon membrane as a function of time. In this experiment, a $0.02 \mu\text{m}$ microporous Teflon membrane with a 50% porosity separated two buffer solutions composed of 0.05 M phosphate adjusted to pH 7.0. Hydrogen peroxide was added to the outer solution and

fractions of the inner solution were collected for analysis at specific time intervals. The outer solution was stirred continuously while the inner solution was not stirred at all. The volume of the inner solution was 0.4 ml and the inner diameter of the chamber holding the inner solution was 8.5 mm. The amount of hydrogen peroxide in the inner solution was determined by the well known *o*-dianisidine/HRP reaction.¹⁰

Initially, no measurable hydrogen peroxide was detected in the inner solution when this experiment was performed at 25°C. At 37°C, however, significant amounts of hydrogen peroxide were found. Figure 2 summarizes the results from one such experiment where two membranes were tested. In this experiment, the concentration of hydrogen peroxide in the outer solution was 465 μM . In both cases, the concentration of hydrogen peroxide in the inner solution increases as a function of time with approximately 2 μM hydrogen peroxide in the inner solution after 3 min. Considering the active surface area of the membrane, the mean flux of hydrogen peroxide into the internal solution is 0.514 $\text{nmol}\cdot\text{cm}^{-2}\cdot\text{min}^{-1}$ under these conditions.

Although the detection of hydrogen peroxide in the inner solution indicates that the internal enzyme approach is feasible, the low flux of

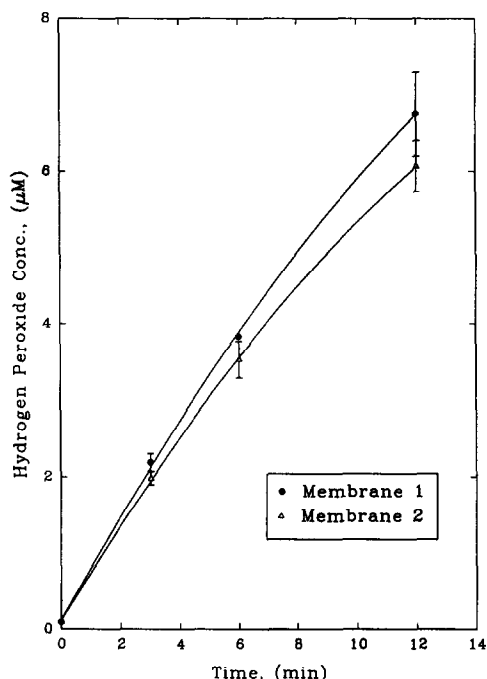


Fig. 2. Mass transport of hydrogen peroxide across a microporous Teflon membrane at 37°C.

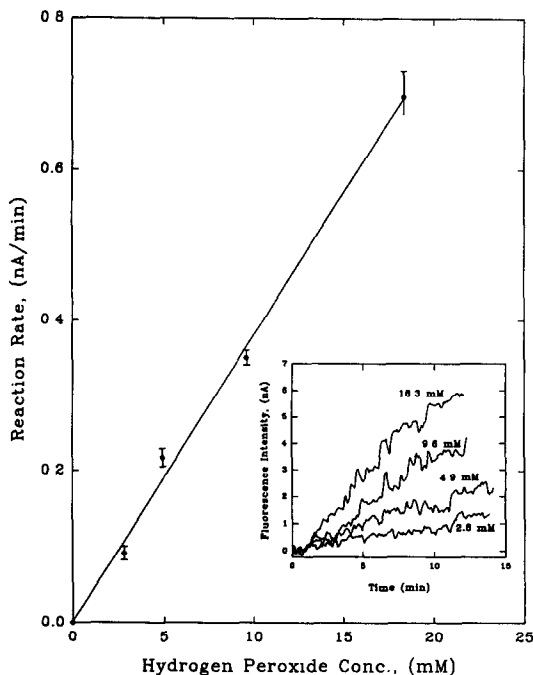


Fig. 3. Response curve for the fluorescence-based sensor. Inset shows sample intensity-time curves for the fluorescence-based sensor at the indicated concentrations of hydrogen peroxide.

hydrogen peroxide across the membrane indicates that the detection chemistry must be quite sensitive with a low limit of detection. For this reason, fluorescence and CL detection schemes have been explored.

The fluorescence detection scheme is characterized by a linear increase in fluorescence intensity as a function of time over the first 5 min of the experiment. In this case, the HVA-dimer accumulates according to the HRP catalyzed reaction as hydrogen peroxide continues to cross the membrane. Reaction rates have been calculated by linear least squares analysis of the intensity-time profiles over the first 5 min. Figure 3 shows a plot of reaction rate versus hydrogen peroxide concentration which is linear over the tested concentration range from 3 to 18 mM . From this plot, the estimated limit of detection ($S/N = 3$) is 0.7 mM . Sample intensity *vs.* time plots are presented for several hydrogen peroxide concentrations in the Fig. 3 inset.

Intensity versus time curves look considerably different for the CL-based sensor. The Fig. 4 inset presents examples of such curves for 1.0, 2.0 and 3.96 mM hydrogen peroxide. In the CL case, photons are the monitored reaction product and the rate of photon production corresponds directly to the measured light intensity (photon flux). Unlike the fluorescence-based

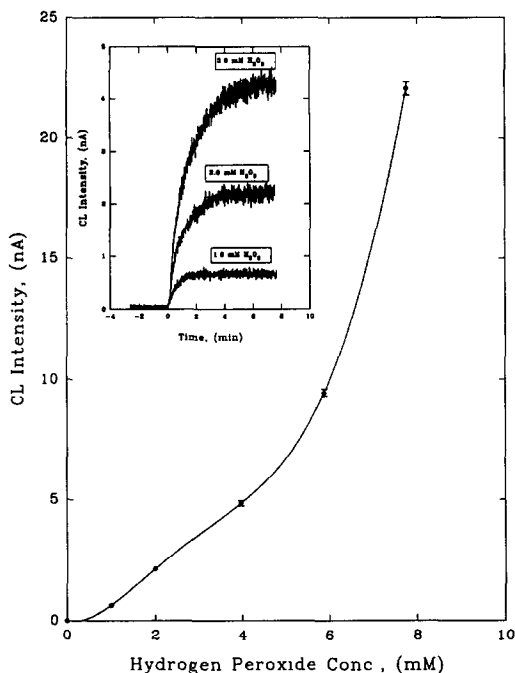


Fig. 4. Response curve for the CL-based sensor. Inset shows sample intensity-time curves for the CL-based sensor at the indicated concentrations of hydrogen peroxide.

system, a steady-state intensity is expected for a constant reaction rate. The intensity versus time plots in the Fig. 4 inset reveal that steady-state intensities are not established until several minutes after hydrogen peroxide is added to the outer solution. An acceleration of the reaction rate is indicated during the early stages of the measurement under these reaction conditions. The data in this figure also demonstrate a larger intensity, or faster reaction rate, for higher sample concentrations of hydrogen peroxide. A plot of the magnitude of the steady-state intensity *vs.* hydrogen peroxide concentration is presented in Fig. 4. This plot reveals a nonlinear response curve for the CL-based sensor over the concentration range from 1 to 18 mM. The limit of detection is less than 0.2 mM (see Fig. 5) and it is estimated to be 10 μ M.

Selectivity has been established for hydrogen peroxide over common interferences. Responses for 0.196 mM hydrogen peroxide and 98 mM ascorbic acid are presented together in Fig. 5 for comparison. No response was obtained for ascorbic acid under these conditions while a relatively large response was measured for hydrogen peroxide. Similarly, no responses were detected for 98 mM uric acid or 3 mM tyrosine. As predicted, the selectivity is excellent for

hydrogen peroxide over these ionic compounds. Selectively over volatile substances will be governed by the specificity of HRP and by the buffer capacity of the internal solution. Acidic or basic gases, such as carbon dioxide or ammonia, could potentially interfere if they cause a change in the pH of the internal solution which can alter the rate of the internal enzyme reaction.

The internal enzyme biosensor configuration offers several important features from a sensing standpoint.¹¹ First, internal enzyme biosensors do not contaminate the sample during operation because the analytical reaction does not occur in the sample solution. Conventional biosensor configurations can significantly alter the sample solution composition depending on the experimental conditions.¹² In addition, there is no need to alter the sample by adding reagents. By physically separating the internal solution from the sample solution, internal enzyme biosensors are insensitive to sample turbidity although an optical measurement is made. Finally, the combination of a selective membrane barrier with an enzymatic reaction enhances the selectivity of the measurement. The major disadvantage of this approach is the need to replace the internal solution frequently because reagents are consumed.

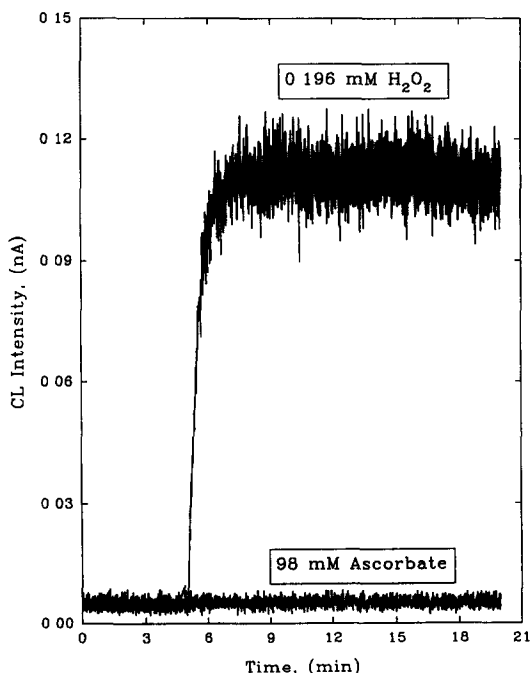


Fig. 5. Responses of the CL-based sensor for 0.196 mM hydrogen peroxide and 98 mM ascorbic acid.

CONCLUSION

Although more detailed experiments are needed to fully characterize the analytical utility of this gas-sensing internal enzyme fiber-optic biosensor for hydrogen peroxide, our results indicate that CL detection is clearly superior to fluorescence detection. Concentrations for all species in the internal solution and pH of the internal solution must be optimized for the CL-based biosensor. The effects of temperature and sample solution pH must also be evaluated. Finally, the capacity to make repeated measurements must be maximized by considering reagent concentrations and internal solution volume. Ultimately, it will be interesting to compare the response properties of an optimized CL-based internal enzyme biosensor to those reported for analogous electrochemical sensors.^{2,3}

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