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Phase Fluorometric Optical Carbon Dioxide Gas Sensor for Fermentation Off-Gas Monitoring

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Abstract

We demonstrated an optical carbon dioxide gas sensor suitable for replacement of gas chromatographs and mass spectrometers for the measurement of carbon dioxide in the off-gas of a bioreactor for fermentation and cell culture applications. The sensor is based upon the change in lifetime of a donor fluorophore, sulforhodamine 101 (SR101), induced by fluorescence resonance energy transfer to a pH-sensitive, nonfluorescent acceptor, *m*-cresol purple (MCP). Carbon dioxide diffusing into the sensor produces carbonic acid, changing the absorbance spectrum of the MCP, and thus its spectral overlap with the SR101, changing its lifetime. This lifetime change was measured in the frequency, rather than the time domain, as a change in the phase angle of the fluorescence relative to the modulated excitation light. The sensor was calibrated by correlating the phase response to carbon dioxide concentrations. The calibration remained valid over the life of the sensor, which has been shown to be greater than 2 weeks. The sensor was most sensitive at low CO₂ concentrations and responded to concentration changes in seconds. The sensor film is very inexpensive to produce and the light source is an inexpensive light-emitting diode. Furthermore, lower cost detection electronics can be developed since only one modulation frequency is required. In addition, this sensor can potentially be used *in vivo*, with a fiber optic both delivering the excitation light and collecting the emission.

Introduction

The control of bioreactors requires accurate and continuous measurement of a number of analytes, including pH, pO₂, pCO₂, and glucose (Wolfbeis, 1990; Bishop and Lorbert, 1990; Bailey and Ollis, 1986). Carbon dioxide is usually measured with a gas chromatograph

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(GC), mass spectrometer (MS), or pH electrode modified to respond to CO₂ (a pCO₂ electrode) (Hartnett, 1994; Puhar et al., 1980). The MS is capable of producing measurements in near real time but is very expensive compared to a GC or pCO₂ electrode. The GC, while less expensive, requires 10–20 min to complete an analysis of a sample and generally must be recalibrated if turned off. The pCO₂ electrode is the least expensive of the three methods but has a response time in minutes and suffers from the same drawbacks of the pH electrode upon which it is based. These include temperature effects, slow response times, alkalinity and acidity errors, electrical resistance problems, liquid junction fouling, and reference electrode contamination (McMillan, 1991). Additionally, it must be calibrated before each use.

To overcome the drawbacks of these methods of CO₂ measurement, optical methods have been investigated (Meyerhoff, 1993; Leiner, 1991; Wolfbeis, 1991). One approach involves directly measuring the absorption of CO₂ in the infrared region (Brassington, 1990). Although this method produces quick response times, it requires bulky and/or expensive excitation sources and can be affected by substances with optical density at the excitation wavelength. All of the other methods for optical CO₂ detection measure the change in absorbance or fluorescence of a pH-sensitive dye produced by the formation of carbonic acid from water and CO₂. This absorbance change has been detected by directly measuring the absorbance at one or two wavelengths (Mills et al., 1992; DeGrandpre, 1993; Weigl et al., 1994) or by measuring the change in fluorescence of a pH-sensitive fluorophore (Mills and Chang, 1993; Uttamlal and Walt, 1995; Parker et al., 1993). We have pursued a third methodology, that of measuring the change in fluorescence lifetime induced by fluorescence resonance energy transfer (FRET) to a pH-sensitive acceptor, first demonstrated in a poly-(HEMA) (HEMA) 2-hydroxyethyl methacrylate matrix (Lakowicz et al., 1993). Fluorescence detection results in higher sensitivity and selectivity than absorbance detection (Lippitsch and Draxler, 1993). Once the CO₂ sensor has been fabricated, all of the above factors are fixed, except for the donor/acceptor spectral overlap and the extinction coefficient of the acceptor, which are both pH-dependent (Lakowicz, 1983). Since FRET eliminates the requirement that the fluorophore be sensitive to pH, a much greater range of fluorophores are available. The use of lifetime, rather than intensity measurements, circumvents the intensity-associated problems of photobleaching, probe washout, and optical absorbance, thus eliminating the need for repetitive calibrations. It should be noted that some of these measurement errors can also be reduced by intensity ratiometric measurements (Uttamlal and Walt, 1995). The sensor presented here is a variation of our previously developed CO₂ sensor (Sipior et al., 1995).

Materials and Methods

Sensor Preparation.

Unless otherwise specified, all chemicals were obtained from the Aldrich Chemical Co. (Milwaukee, WI) or the Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Glass substrates for the sensor slides were DESAG catalog number AF 45, obtained from Abrisa Industrial Glass (Ventura, CA). USP grade carbon dioxide and N.F. grade nitrogen were obtained from Potomac Airgas (Linthicum, MD). Mixtures of defined

percentage carbon dioxide composition in compressed air were obtained by metering with a Series 150 two-tube gas blender from Advanced Specialty Gas Equipment Corp. (South Plainfield, NJ). Standard (5% calibration charts were used to determine flow rates.

The carbon dioxide sensors were prepared by mixing donor and acceptor dyes, along with a phase transfer agent and a plasticizer, with an ethyl cellulose (EC) solution and then coating a thin film on the glass substrate. The sensor was based on an absorbance CO₂ sensor (Mills et al., 1992), and the sensor preparation was adapted from our previously developed CO₂ sensor (Sipior et al., 1995). A stock EC solution was prepared by dissolving 10 g of EC in 20 mL of ethanol and 80 mL of toluene, giving a viscous solution with a density of 0.82 g/mL. Quantities of the EC solution were determined by weight rather than volume due to its viscous nature. The tetraoctylammonium hydroxide (TOAH) phase transfer agent solution was prepared as needed by adding 164 mg of tetraoctylammonium bromide and 139 mg of silver-(I) oxide to 0.6 mL of methanol and 35 μ L of water. This solution was agitated for 30 min and then centrifuged to remove residual silver oxide. The acceptor dye solution was prepared by dissolving 12.0 mg of *m*-cresol purple (MCP) in 1.250 mL of methanol and 500 μ L of the previously prepared TOAH solution. The donor dye solution was prepared by dissolving 3.0 mg of SR101 in 1.000 mL of methanol. The mixture used to prepare the sensor film consisted of 658 mg of the EC solution, 40 μ L of the donor dye solution, 65.5 μ L of the plasticizer tributyl phosphate (TBP), 65.5 μ L of the phase transfer agent solution, and 35.0 μ L of the acceptor solution. These mixtures were coated onto the 0.559 mm thick glass substrates by placing an excess of the mixture on the substrate and drawing the slide under a utility knife blade held fixed above the slide with appropriate spacers (a stack of three cover slips with a total thickness of about 0.686 mm), producing a wet film which dried to a thickness of approximately 0.025 mm, as determined by the difference in the thickness of the substrate and the coated substrate. Above thickness measurements were obtained with a micrometer reading in thousandths of an inch and converted to millimeters. Films produced by this method were thicker toward the edges of the substrate, as indicated by the darker color. All measurements were taken at the center of the film, away from the edges. The sensor slides were allowed to dry for about 20 min before storage in a humidior. The humidior consisted of a desiccator with damp cotton replacing the desiccant and provided 100% relative humidity.

Cultivation.

Luria-Bertani (LB) medium consisting of 5 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract (Difco Laboratories, Detroit, MI) was inoculated with *Escherichia coli* and incubated overnight in shake flasks at 250 rpm and 37 °C in a New Brunswick Scientific model G24 environmental incubator shaker (New Brunswick, NJ). The fermentation was carried out in a 4 L stainless steel fermentor (Applikon B. V., The Netherlands) containing 2.2 L of LB medium, 0.5 g/L KH₂PO₄, 2.0 g/L K₂HPO₄, and 5 g/L glucose. The fermentor was inoculated with 100 mL of the culture, with a second inoculation of 80 mL 11 min later, to obtain a higher initial optical density. Agitation was controlled at 300 rpm. Temperature was maintained at 37 °C with a circulating water bath. House compressed air was sparged through the fermentation broth at 1 L/min. An additional 2.08 and 9.17 g of glucose was added at 5:11 and 6:11 h, respectively. Foaming was controlled with the addition of 50 μ L of

Pluronic F-61 surfactant (BASF Corp., Parsippany, NJ) when required (4:11 h). The pH was controlled with the addition of 1.5 mL of 1.0 M NaOH at 3:00 h and 3.0 mL at 4:07 h.

Analysis.

CO₂ sensor lifetime measurements were performed on an ISS K2 multifrequency phase fluorometer (Champaign, IL). The excitation source was a Nichia America Corp. (Lancaster, PA) model NLPB500 blue light-emitting diode (LED) biased with 5.0 mA from an ILX Lightwave (Bozeman, MT) model LDX-3412 precision current source. Modulation (6.0 dBm at 25 MHz) was supplied by the Marconi Instruments (Allendale, NJ) model 2022A signal generator provided with the ISS K2 fluorometer. The bias and modulation currents were applied to the LED through a Picosecond Pulse Labs (Boulder, CO) model 5580 bias tee. The excitation light from the LED was passed through a Spindler & Hoyer Inc. (Milford, MA) SWP-25–490 short-wave pass filter and Andover (Salem, NH) 500FL07–50S and 600FL07–50S short-wave pass edge filters. Glass substrates coated with the sensor film were oriented in the cuvette holder at a 45° angle away from the detector. Off-gas from the fermentor was directed into the cuvette holder through a square rubber stopper. Sensor fluorescence was collected through two Andover 550FH90–50S long-wave pass edge filters. For comparison, some of the off-gas was periodically directed into a Shimadzu (Kyoto, Japan) GC-8A gas chromatograph with a thermal conductivity detector, fitted with a C-R3A Chromatopac integrator and 60/80 mesh Carboxen-1000 15 m × 1/8 in. column (Supelco, Bellefonte, PA). The helium carrier gas flow rate was 7.5 mL/s, with an injection temperature of 110 °C and a column temperature of 180 °C. Dissolved oxygen (DO) was measured with an Ingold (Wilmington, MA) polarographic dissolved oxygen electrode, while off-gas oxygen was measured with an optical oxygen sensor developed in this lab (Bambot et al., 1994). Data from both oxygen probes were collected with a Strawberry Tree (Sunnyvale, CA) Workbench data acquisition system installed in a Macintosh SE computer. Samples (4 mL) of the fermentation broth were withdrawn from the fermentor for analysis. Culture optical density at 600 nm (OD) was measured with a Milton Roy (Rochester, NY) Spectronic 401 spectrophotometer, and pH was measured with an Orion Research (Cambridge, MA) model 611 pH meter fitted with a Fisher Scientific (Pittsburgh, PA) Accumet combination pH probe. Centrifuged samples were used to measure supernatant glucose with a Model 2700 Select enzymatic glucose analyzer (Yellow Springs Instrument Co., Inc.).

Sensor Calibration.

The sensor was calibrated in the gas phase at 25 °C. The sensor was mounted in the fluorometer in the same fashion used during the analysis of CO₂ in the fermentation off-gas. Phase measurements were performed at a series of known CO₂ concentrations in house compressed air. The house compressed air was sparged through water before mixing with the CO₂. The percentage of CO₂ in the mixture was obtained by metering with a Series 150 two-tube gas blender from Advanced Specialty Gas Equipment Corp. (South Plainfield, NJ). Standard (5% calibration charts were used to determine flow rates. Phase readings were obtained at a modulation frequency of 25 MHz.

Results and Discussion

The CO₂ sensor presented here was composed of a fluorescent donor, SR101, a pH-sensitive acceptor, MCP, a phase transfer agent, TOAH, and a plasticizer, TBP. The phase transfer agent allowed the incorporation of the donor and acceptor molecules, along with water, into the hydrophobic EC matrix. Additionally, the phase transfer agent provided the initially basic environment, which was then made more acidic as CO₂ diffused into the sensor. The plasticizer increased the permeability of the sensor film to CO₂. Our previous SR101/MCP CO₂ sensor exhibited a maximum phase change with CO₂ concentration at a frequency of 95 MHz and used an externally modulated 10 mW helium-cadmium (HeCd) laser for excitation. This required a large laser and power supply, 25 W rf amplifier, a Pockels cell modulator, and associated optics, along with sensitive alignment of the light through the modulator. This modulation methodology is expensive, bulky, and inefficient, producing a modulated light output power less than 0.2% of the input power. Additionally, detection at 95 MHz required expensive detection electronics, due to the high modulation frequency. By reducing the amount of acceptor in the sensor, the maximum phase change with CO₂ concentration was reduced to a frequency of 25 MHz, while reducing the total phase change (0–5% CO₂) from a maximum of about 30° to about 17.5°. Since we can measure the phase to one-tenth of a degree, the reduction in total phase change does not present any problems in the CO₂ measurements presented here. This relationship between acceptor concentration, maximum phase change, and frequency is shown in Figure 1. It can be seen that, as the acceptor concentration is reduced, both the maximum phase change and the frequency at which it occurs are reduced.

The reduction of the modulation frequency to 25 MHz allowed the use of an inexpensive blue LED to provide the required modulated excitation light, eliminating the 10 mW output laser, 25 W rf amplifier, Pockels cell modulator, associated optics, and the sensitive alignment. In addition, since the amount of quenching was reduced, the emission was increased, and thus the signal to noise was also increased. The depth of modulation with frequency, under conditions used in this experiment, is shown in Figure 2. Although the modulation decreases above about 1 MHz, the depth of modulation was still about 14% at 25 MHz. The amount of light modulation could be increased by supplying more modulation signal through the bias tee but was not required for these measurements. The modulation falls off at lower frequencies due to the bandwidth of the bias tee used and the bandwidth of the rf amplifier modulating the detection photomultiplier tube (pmt). If required, the modulation at lower frequencies could be increased with a suitable bias tee. At higher frequencies, the roll-off is due to packaging capacitance of the LED leads, rather than any inherent lifetime limitation of the LED material. Since the LED is directly modulated, potentially all of the output light is available for sensor excitation. In the configuration used in the ISS K2 multifrequency phase fluorometer, approximately 100 μ W of light was delivered to the sensor, which was 3–4 times the amount produced by the externally modulated HeCd laser, which had an output of 10 mW. In a commercial version of this sensor, the LED would be mounted much closer to the sensor, and maximum current would be supplied to the LED, producing approximately 1 mW of modulated light (Nakamura et al., 1994).

The spectral output of the blue LED, after passing through the excitation filters, is shown in Figure 3. Filters were required to remove the small amount of red light present in the LED output. The figure shows the small absorbance of the SR101 over the spectral output of the blue LED. Although currently available green LED's, with output maximums at about 560 nm, would provide a better overlap with the absorbance of the SR101, the output power and modulation depth would be much lower. Additionally, the filters necessary to exclude excitation light from the emission detector would result in the loss of some of the sensor emission. The transmission profile of the emission detector filter used in the present study is also shown in the figure, demonstrating the low loss of emission light due to the filter. The absorbance of MCP in buffered water solutions at low (6.0) and high (10.6) pH is indicated by the dotted lines in Figure 3. At low pH, the spectral overlap with the emission of SR101 is small and results in very little FRET to the acceptor. At high pH, the spectral overlap with the emission of SR101 is large and FRET to the acceptor is very efficient.

In the absence of CO₂, modulated excitation light from the blue LED excites the fluorescent donor SR101. Among other mechanisms, the excited state is depopulated by both emission of light and FRET to the MCP acceptor. Interpretation of the decay curve of the emitted light determines the fluorescence lifetime. The acceptor is deprotonated by the basic phase transfer agent, producing a large spectral overlap with the emission of the SR101, thus allowing efficient FRET. The water associated with the phase transfer agent permits the deprotonation of the acceptor by lowering the energy required. Upon diffusion of CO₂ into the sensor, the water in the sensor combines with the CO₂, producing carbonic acid, which then reacts with and neutralizes the hydroxide of the phase transfer agent, lowering the local pH. This allows the protonation of the acceptor dye, changing its spectral overlap with the emission of the donor, thus reducing the rate of FRET. Since the excited state of the donor is not depopulated by FRET, its fluorescence lifetime is greater. The longer lifetime produces a relatively larger phase lag between the modulated excitation and emission, along with the relatively larger amount of demodulation of the emission.

The graph of percent CO₂ vs phase is shown in Figure 4 as the curved solid line. The calibration was performed under the same conditions as the fermentation, accounting for any cross-sensitivity to oxygen. Since the sensor functions by measuring a pH change, it cannot be used when acidic or basic vapors are present. Actual measurements are indicated with solid circles, with a fit to these data points indicated with the line through the data points. The phase measurements obtained during the fermentation were converted to CO₂ concentrations using this fit. It can be seen that the sensor is most sensitive at lower values of CO₂ concentration. If a different measurement range is required, the acceptor can easily be changed either to provide a sensor more sensitive to small CO₂ concentrations or to provide measurements over an extended range. Although not necessary for a commercial sensor, this calibration curve can easily be linearized by adding 55.5° to the phase reading and taking the tangent of the sum, shown as the empty circles in Figure 4. The tangent function was employed since the tangent of the phase angle is proportional to the fluorescence lifetime. The addition of 55.5° was empirically determined to produce the straightest line. The best linear fit to these data points is also shown in the figure. The GC was also calibrated at a series of known CO₂ concentrations provided by mixing CO₂ and house compressed air in a gas blender. Each data point was normalized by dividing the area

of the CO₂ peak by the total peak area for that sample. This procedure was performed at a series of increasing CO₂ concentrations. These data points, along with the best straight line fit, are shown in Figure 4 as the solid circles with the dotted line. The GC peak area measurements obtained during the fermentation were converted to CO₂ concentrations using this linear fit.

The CO₂ measurements taken during the fermentation are shown in Figure 5. The solid line represents measurements taken every 6 s by the optical CO₂ sensor. The phase data were converted to %CO₂ using the calibration curve shown in Figure 4. For comparison, fermentation off-gas samples were introduced into a calibrated GC, and the resulting peak areas were converted to %CO₂ using the calibration curve shown in Figure 4. These data are shown as the circles in Figure 5. Since each GC sample required 20 min, samples were taken every half-hour. The optical sensor data agree with those obtained from the GC within a few tenths of a percent, with the greatest disparity occurring at the highest CO₂ concentrations. Since the optical CO₂ sensor obtained readings every 6 s, variations in %CO₂ caused by variation in flow rate through the fermentor can be seen as a noiselike variation. The changes in %CO₂ sometimes induced by connection of the GC to the fermentor off-gas flow tube are also apparent as large dips. It can be seen that there is very good agreement between measurements obtained with the optical CO₂ sensor and the GC.

Figure 5 also illustrates the effectiveness of %CO₂ as an indicator of low glucose levels. Within minutes of glucose depletion, the %CO₂ in the fermentor off-gas fell precipitously. Addition of glucose caused a rapid increase in %CO₂, again within minutes. This can be seen in Figure 5, where 2.08 g of glucose was added at just after 5 h and 9.17 g was added at 6 h. Glucose was measured off-line and thus involved withdrawal of samples, with subsequent remote measurement, a time and labor intensive procedure that increases the chance of contamination of the fermentor. Although the drop in CO₂ levels in the off-gas could be detected with a GC, the long processing time can introduce significant delays between depletion of glucose and detection of that depletion. This is dramatically illustrated in Figure 5, where a GC measurement taken at 4 h indicated the greatest %CO₂ measured in this fermentation, while the next GC measurement taken approximately one-half hour later indicated a CO₂ level below 0.5%. When the 20 min required for the GC to analyze the sample is added to the half-hour between sampling, almost 1 h passed before the fall in CO₂ levels, and thus the detection of glucose depletion. The optical CO₂ sensor provides CO₂ level detection within seconds and can thus detect glucose depletion within minutes of its occurrence.

Other parameters monitored during the fermentation, including DO, pH, and OD, are shown in Figure 6. The amount of oxygen in the off-gas was measured with an optical oxygen sensor developed in this laboratory (Bambot et al., 1994). These data are not included in Figure 6, since the oxygen levels in the off-gas remained within the range of 99.5–100% (with the atmospheric concentration defined as 100%). This is possibly because DO in the fermentor reached 0% in less than 1 h after inoculation and stayed there throughout the remainder of the fermentation, indicating growth in a microaerobic regime with little consumption of O₂ from the gas phase. The cell density was tracked by measuring OD. During the initial growth phase, OD increased quickly, then increased much more slowly

after the glucose was depleted. The pH quickly decreased during the same period and then somewhat mirrored the CO₂ concentration, although this effect is small. Of the parameters measured during the fermentation, glucose and CO₂ levels are the most important indicators of cell growth, since both are directly associated with cell respiration. We have shown that a real-time measurement of CO₂ concentration can promptly and decisively indicate depletion of glucose, while other measurements display little or no change.

Conclusion

We believe that a commercial version of the optical CO₂ sensor could be produced for under \$1000. The LED source is available for under \$30, and since only a single modulation frequency is required, the excitation source including driver electronics should be available for under \$100. The sensor film itself can be produced for pennies a sensor, since it is a mixture of off-the-shelf chemicals. Fluorescence levels are sufficient for detection to be achieved with a high-speed amplified silicon detector, currently available for about \$300 in single quantities. A phase detection integrated circuit can be used in the detection electronics, along with a microprocessor for converting phase readings to CO₂ concentrations. If fiber optic bundles are used to convey excitation and fluorescence, the sensors could be multiplexed with one excitation per detection unit. The sensor should compete with electrode sensors and could replace expensive and bulky GC's and MS's. Our current research has produced a version of this sensor that works at sub-megahertz modulation frequencies, which we are attempting to produce in an autoclavable version suitable for making dissolved as well as off-gas CO₂ measurements. The potential availability of an online, real time CO₂ monitor at a cost of only a few hundred dollars is expected to be a significant advance for practitioners of fermentation and cell culture.

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Figure 1.

Effect of acceptor concentration on the phase at 0 and 10% CO₂ concentrations. The dashed line shows the frequency response of the CO₂ sensor employed in the off-gas measurements. The solid line shows the frequency response of a CO₂ sensor with approximately twice the acceptor concentration. The modulation measurements are depicted with squares; the phase measurements are depicted with circles. Note the increased phase change between 0 and 10% CO₂ of the solid line at high frequencies.

Figure 2.

Depth of modulation as a function of frequency of the blue LED used to excite the CO₂ sensor. A dc bias of 5.0 mA and 6.0 dBm of modulation were applied to the LED through a bias tee. Total light output, measured at the tip of the LED, was approximately 0.5 mW. In the present study, a modulation frequency of 25 MHz was used, resulting in a modulation of 14%.

Figure 3. Spectral characteristics of the filtered output of the LED (dot-dash line), the absorbance (in solution) and emission (in EC) of the SR101 donor (solid lines), the absorbance of the MCP acceptor in water buffered at pH 6.0 and 10.6 (dotted lines), and the cutoff filter used on the detector (dashed line).

Figure 4.

Calibration of the optical CO₂ sensor, showing phase angle data (solid circles) with a fit line (solid), and data points linearized by taking the tangent of the phase angle plus 55.5° (empty circles) with a fit straight line (solid). The gas chromatograph calibration data points are indicated by solid circles on a fit straight line (dotted).

Figure 5. Fermentation measurements of CO₂ off-gas obtained with the CO₂ optical sensor (solid line) and GC (empty circles) and glucose levels (empty squares on dashed line). Note close agreement between optical and GC measurements.

Figure 6. Fermentation measurements of CO₂ off-gas obtained with the CO₂ optical sensor (solid line), pH (solid line with solid circles), optical density at 600 nm (solid line with empty circles), and dissolved oxygen (dashed line).