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On-line electrocatalyzed luminol chemiluminescence determination of D-glucose and dissolved oxygen in simulated mammalian cell bioreactor perfusion fluid using solid phase reagent modules

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Abstract

On-line instrumentation and methods for the chemiluminescence based real-time monitoring of D-glucose and O_2 levels in mammalian cell bioreactor perfusion fluid are described. The unit processes required for the analysis include: pH adjustment using solid phase flow-through modules, immobilized enzyme catalyzed oxidation of glucose by molecular oxygen to produce hydrogen peroxide, controlled release of luminol using a solid phase flow-through module, electrocatalyzed luminescence using gold electrodes, and photodetection of chemiluminescent emissions. Calibration curves for D-glucose and dissolved O_2 in simulated bioreactor perfusion fluid have been generated using fully integrated reagentless test systems from 0–800 mg l^{-1} and 0–10 mg l^{-1}, respectively.

Introduction

Descriptions of instrumentation and methods for the *'reagentless'* electrocatalyzed chemiluminescence determinations of aqueous H_2O_2 (Atwater *et al.* 1997a), ethanol (Atwater *et al.* 1997b, Verostko *et al.* 1998), and O_2 (Atwater *et al.* 1998) have been published previously. These methods have now been extended to include the quantitation of both D-glucose and O_2 in an aqueous matrix containing substantial levels of protein and dissolved ionic species. While the research effort was directed specifically toward the requirements of the National Aeronautics and Space Administration (NASA) for a simple, reliable, and microgravity compatible analytical instrument to monitor O_2 and glucose in experimental mammalian cell bioreactors (Freed *et al.* 1997, Saltzman 1997, Begley & Kleis 2000), the methodology described herein can be of benefit in a vast array of biotechnological applications.

The basis for detection and quantitation of both dissolved O_2 and glucose is the glucose oxidase-mediated enzymatic oxidation of β-D-glucopyranose by O_2 to produce H_2O_2. The resulting H_2O_2 concentrations, which are directly proportional to analyte concentrations, are then detected by the chemiluminescence reaction with luminol. The glucose and O_2 assays are conducted separately, under conditions of excess O_2 and glucose, respectively. Thus, while the analytical methodology is substantially *'reagentless'*, glucose and O_2 sources, in addition to the contents of the sample media, must be provided. Luminol chemiluminescence-based assays in general require delivery of the luminophore in strong alkaline media, and in the presence of a catalyst to facilitate the oxidation of the luminophore by the analyte (Nieman 1989). The operational simplicity and flexibility of flow analysis instrumentation can be significantly enhanced by using in-line solid phase modules to produce reagents as they are needed. Here we describe a system in which the addition of the luminophore and pH control of the flowing stream are achieved using solid phase media, and in which the reaction is catalyzed electrochemically (Van Dyke 1986).

Materials and methods

Solid phase reagent modules

Glucose oxidase, β-D-glucose:oxygen 1-oxidoreductase (EC 1.1.3.4), from *Aspergillus niger* was immobilized on a diatomaceous earth support (Celite R-648) using the titanium activation procedure (Schussel & Atwater 1995, 1996). Solid phase basification (SPB) beds, producing an effluent pH $\approx$ 10.5 via the reaction,

$$MgO + H_2O \rightarrow Mg^{2+} + OH^-$$

were prepared using crystalline magnesium oxide. Solid phase luminophore (SPL) beds were prepared from crystalline 3-aminophthalhydrazide (luminol) using methods described elsewhere (Atwater *et al.* 1997a). In previous work these materials have been shown to produce nearly constant effluent pH and luminophore concentrations for prolonged periods of continuous flow (Atwater *et al.* 1997a).

Oxygen saturation methods

A membrane O_2 saturator was used for both the production of known dissolved O_2 concentrations for use as standards for the oxygen detection mode, and as a means of providing a constant excess oxygen concentration in the D-glucose detection mode. A tube-in-shell hollow fiber membrane oxygenator was fabricated using a bundle of nine non-porous polydimethylsiloxane hollow fibers (0.31 mm i.d. $\times$ 0.64 mm o.d.) housed within a cylindrical polycarbonate shell (26.7 cm length and 2.54 cm diam.). The liquid filled internal volume of the unit was 4.8 cm^3 and the active surface area was 124 cm^2. The hollow fibers were attached to the manifold at the inlet and outlet faces by an epoxy potting compound. To produce the desired dissolved O_2 concentration, an oxygen partial pressure is applied to the gas-filled shell side of the device. Driven by the concentration gradient, oxygen diffuses across the membrane into the aqueous phase flowing through the tubular semipermeable membranes, until equilibrium is established, as described by Henry's Law. The tube-in-shell arrangement of the hollow fiber membrane bundle promotes rapid equilibration by maximization of available surface area and minimization of diffusion distances (D'Elia *et al.* 1986, Ming-Chien & Cussler, 1986). The apparatus used for confirming the quantitative production of hydrogen peroxide as a function of dissolved O_2 concentrations is illustrated in Figure 1. As the basis for comparison, dissolved oxygen concentrations were measured amperometrically on-line using an Ingold flow-through Clark electrode with a type 170 amplifier.

Flow analysis apparatus

The complete flow analysis apparatus is illustrated schematically in Figure 2. Two flowing streams are combined immediately upstream of the electrogenerated chemiluminescence cell. The first stream carries the sample solution through an A/G Technology model UFP-30-E-1A polysulfone hollow fiber, ultrafiltration module with a nominal cut-off of 30 000 Da, then through the O_2-saturation sub-system and into a 1 ml immobilized glucose oxidase bed. The test apparatus incorporates the means to prepare samples of known glucose and dissolved oxygen content. In the oxygen detection mode, the sample stream carries a 300 mg glucose l^{-1} solution through the oxygen saturation system, where the sample of known oxygen content is prepared. In the glucose detection mode, the sample stream carries deionized (DI) water through the O_2 saturation system. Downstream of the O_2 saturator, glucose is injected into the flowing stream via a syringe pump.

The second stream carries a luminol solution with pH $\approx$ 10.5. This is produced by the sequential flow through 2.5 cm^3 solid phase basification (SPB), 0.5 cm^3 solid phase luminophore (SPL), and second 2.5 cm^3 SPB beds. This latter bed is necessitated by the pH drop which occurs as a consequence of the dissolution of the luminophore. The first SPB bed is required to achieve sufficiently high pH that luminol will dissolve in a controlled manner. The two streams mix at the inlet to the chemiluminescence observation cell. The chemiluminescent reactions are initiated electrically within the cell using gold foil and gold mesh electrodes biased at +0.8 V DC. A Hammamatsu R878 head-on type photomultiplier tube is optically coupled to the gold mesh bearing side of the cell. The cell and detection scheme have been described in detail elsewhere (Atwater *et al.* 1997a).

Results

A series of experiments, in which each step increased in complexity, led to the flow analysis analyzer configuration illustrated in Figure 2. The quantitative nature of the glucose oxidase catalyzed reaction between D-glucose and O_2 was demonstrated by collection and

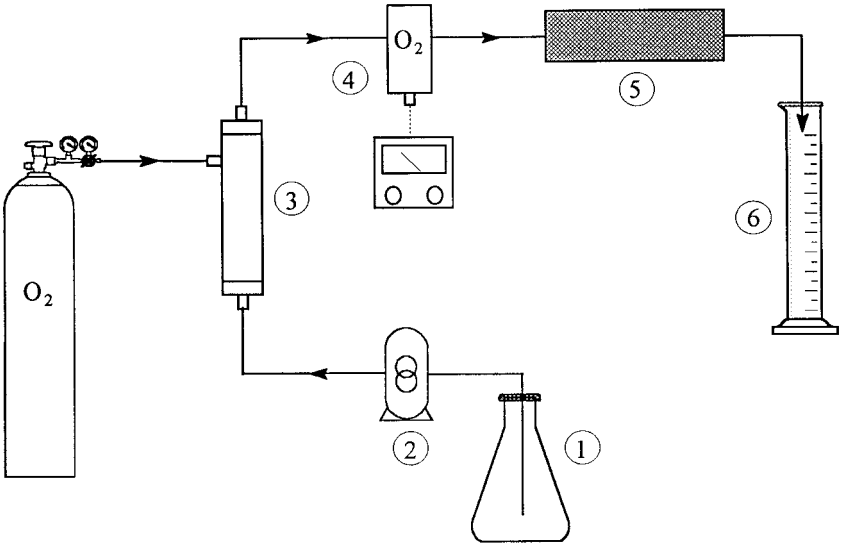

Fig. 1. Oxygen saturation subsystem. (1) D-glucose solution, (2) peristaltic pump, (3) membrane oxygen saturator, (4) in-line dissolved oxygen electrode and meter, (5) immobilized glucose oxidase bed, (6) sample collection vessel.

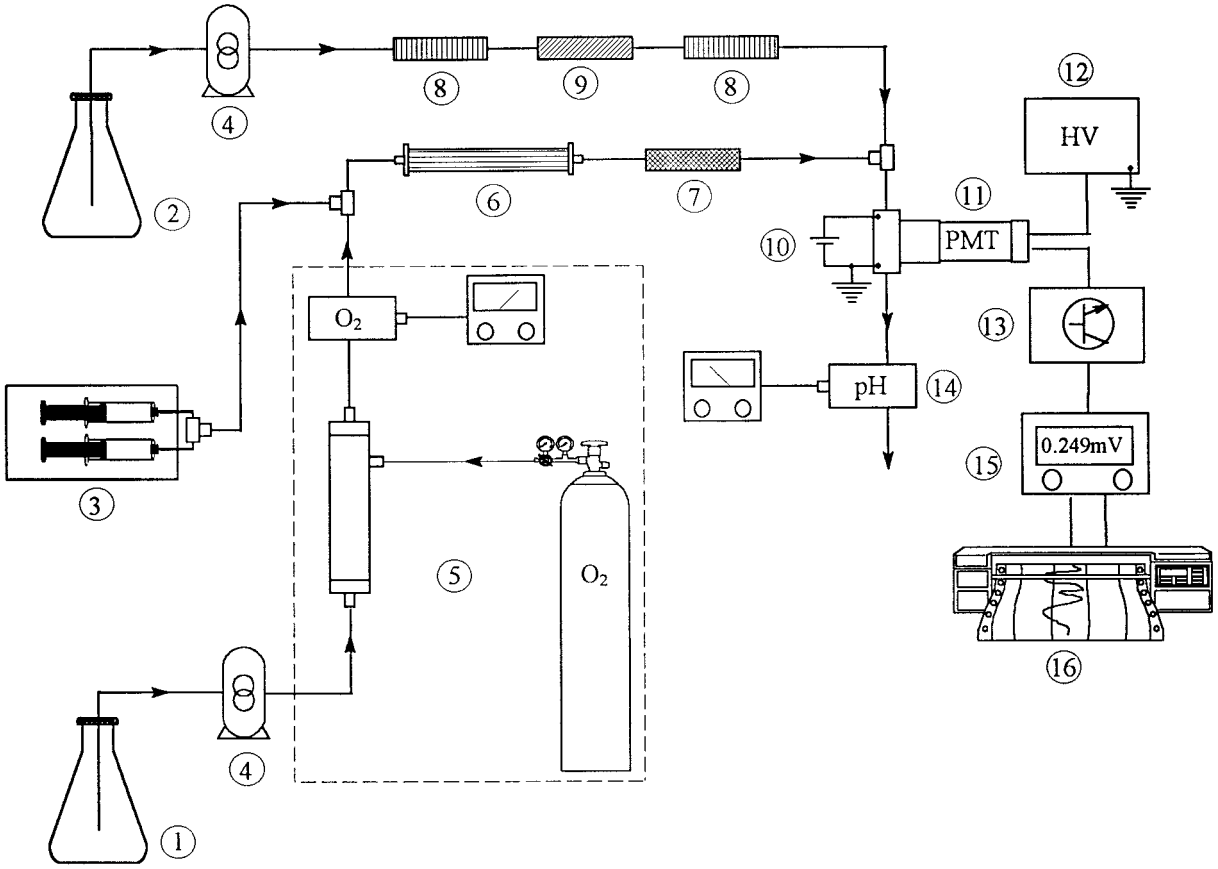

Fig. 2. Reagentless glucose and O_2 chemiluminescence experimental apparatus: (1) D-glucose solution (O_2 detection mode), DI water (glucose detection mode), (2) DI water, (3) syringe pump, (4) peristaltic pump, (5) O_2 saturation subsystem, (6) ultrafiltration membrane, (7) glucose oxidase bed, (8) SPB, (9) SPL, (10) ECL observation cell, (11) PMT, (12) power supply, (13) signal conditioning, (14) pH probe, (15) digital voltmeter, (16) recorder.

off-line analysis of the effluent from the immobilized enzyme bed under conditions of both constant O_2 and varying D-glucose levels, and of constant D-glucose with varying O_2 levels. This also confirmed the adequacy of the O_2 saturation sub-system. To establish the correct operation of the observation cell and associated electronics, detection of D-glucose and O_2 was then undertaken using a glucose oxidase bed, O_2 saturator, aqueous phase reagents, and chemical catalysis by Cu(II). Building upon this, the conditions required to establish the 'reagentless' chemiluminescence reaction chemistry were investigated using SPB and SPL beds in conjunction with electrical catalysis of the reactions.

Once the basic operating conditions were identified, analysis of bioreactor perfusion fluid simulants containing 0.9% (w/w) NaCl, 10% (w/w) bovine serum albumin, and variable concentrations of D-glucose and dissolved O_2 began. Preliminary experiments indicated that the perfusion fluid simulant matrix had a very significant effect on the pH of the mixed solution flowing into the observation cell. When a 3 ml min^{-1} carrier stream flow through SPB, SPL, and secondary SPB beds was combined with a 0.2 ml min^{-1} flow of perfusion fluid, a pH of 6.86 resulted for the mixture, due to the buffering capacity of the bovine serum albumin. Because of the requirement for alkaline conditions to promote the oxidation of the luminophore, a hollow fiber polysulfone ultrafiltration membrane module was installed upstream of the glucose oxidase bed to remove this interference.

D-*Glucose*

For the detection and quantification of D-glucose, a carrier stream flow of 3 ml min^{-1} of de-gassed DI water was established through the SPL and SPB beds. A 165 μl min^{-1} sample stream flow of DI water containing 20 mg dissolved O_2 l^{-1} was routed through the immobilized glucose oxidase bed. The sample and carrier streams were mixed at the inlet to the electrocatalyzed chemiluminescence (ECL) cell. Injection of a small volume of perfusion fluid simulant solution into the sample stream was achieved using a 0.035 ml min^{-1} flow introduced down-stream of the O_2 saturator by the syringe pump. A blank and samples containing D-glucose concentrations of 300, 550, and 800 mg l^{-1} were analyzed. For each sample, an electric potential of +0.8 V was applied to the electrodes after 5 ml of flow through the glucose oxidase bed. Output from the analyzer was recorded 15 seconds following the application of voltage. The results are shown in Figure 3. Good linearity is indicated over this concentration range.

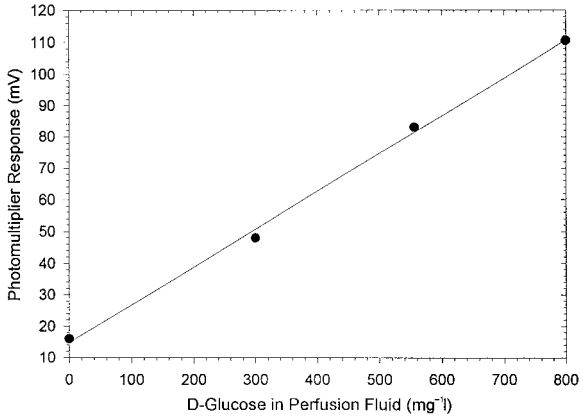

Fig. 3. Quantitation of D-glucose in perfusion fluid simulant.

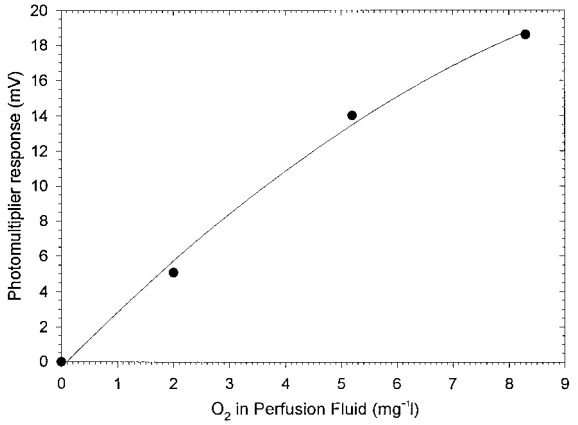

Fig. 4. Quantitation of O_2 in perfusion fluid simulant.

Dissolved oxygen

The O_2 detection mode of operation differed slightly from the above. The injection of sample into the analysis stream was not simulated because of difficulties arising from the method in which the dissolved oxygen samples were prepared. The sample stream consisted of DI water containing 300 mg D-glucose l^{-1}, corresponding to a minimum fivefold stoichiometric excess. The carrier stream and sample streams were operated at flow rates of 3.0 and 0.2 ml min^{-1} respectively. A blank and samples containing 2.2, 5.2, and 8.3 mg O_2 l^{-1} in DI water were analyzed. The results are presented in Figure 4.

Conclusions

The feasibility of the on-line quantitation of dissolved oxygen and D-glucose in bioreactor perfusion fluids by electrocatalyzed chemiluminescence, using solid phase modules to minimize reagent requirements, has been established. Current instrumentation for the detection of these important analytes, such as amperometric biosensors for glucose and Clark electrodes for dissolved O_2 suffer from short shelf lives, short active lives, high rates of signal drift and the consequent requirement for frequent re-calibration. The 'reagentless' chemiluminescence based analytical methodology offers an attractive alternative for real-time bioprocess monitoring, with several potentially beneficial features. Since both analytes are detected and quantified using minor variations of the same basic reaction scheme, instrument design requirements are greatly simplified. Other advantages inherent to the technique are accuracy, sensitivity, and strong potential for both automation and miniaturization.

Acknowledgement

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