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Technical Notes

Detection of Oxygen Consumption of Cultured Adherent Cells by Bead Injection Spectroscopy

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This paper describes a method for detecting oxygen consumption of adherent cell cultures. The sensing is based on oxygen-dependent quenching of the phosphorescence of a Pt-porphyrin complex immobilized on microcarrier beads, which are used as the cell culture substrate. Bead injection, a recent variant of the flow injection technique, is used to pack an aliquot of the beads into a small sensing layer that can be easily and rapidly renewed. The technique is tested on a model system of Chinese Hamster Ovary M1 cells grown on Cytodex-3 microcarrier beads. Cellular respiration is monitored through O_2 consumption measured across a period of 3 min. The method is validated by detecting the impairment of aerobic metabolism caused by 1.5 mM amobarbital. Further, it is shown to have enough precision to distinguish even more subtle changes, such as the increase in oxygen consumption caused by stimulation of the muscarinic m1 receptor with 100 μM carbachol.

All primary animal cells are aerobic, i.e., they are constantly consuming oxygen in their energy production. More precisely, oxygen is used as an electron acceptor at the end of the aerobic pathway of glucose oxidation. This is a conjugate pathway consisting of glycolysis, the TCA cycle, electron transport, and oxidative phosphorylation, and the net stoichiometric equation for the overall process is[1]

$$\text{glucose} + 6O_2 + \sim 30\text{ADP} + \sim 30P_i \rightarrow 6CO_2 + \sim 30\text{ATP} + \sim 36H_2O \quad (1)$$

where ADP stands for adenosine diphosphate, ATP for adenosine triphosphate, and P_i for PO_4^{3-}. Since oxygen plays such a crucial role in the aerobic energy metabolism, oxygen consumption measurements can be used to study biochemical events in which some part of the aerobic pathway is affected.[2,3]

There are several good methods for measuring oxygen consumption of cultured cells in suspension. These methods include the amperometric Clark electrode,[4,5] the use of a phosphorescent oxygen indicator in the cell suspension,[3,6] and electron paramagnetic resonance spectroscopy.[2] The phosphorescent method has also been successfully used to map oxygen consumption in a tissue sample.[7] However, there do not seem to be reports on using any of these methods on adherent cell cultures. This may be due to the fact that adherent cells are most commonly cultured as monolayers on flat solid substrates. The limited number of cells in a monolayer will be small compared with the volume of the bathing solution, which makes it difficult to detect oxygen consumption within reasonable time periods.

Since many cell types are naturally adherent, such cultures lend themselves well to studies of cellular responses to biochemical effector molecules and drugs. This is typically done by following a relevant cellular parameter, such as intracellular Ca^{2+} (or other second messenger concentration), pH, acid extrusion rate, or membrane potential, while exposing cells to the effector agent. The lack of a convenient oxygen consumption assay means that oxygen consumption cannot easily be included among the monitored parameters.

Bead injection spectroscopy (BIS) is an automated analysis method in which microbeads are used to create a small, disposable sensing layer that can be renewed for each individual assay.[8] This work presents a BIS-based optical oxygen sensor for monitoring the oxygen consumption of adherent cells. The sensing is based on oxygen-dependent quenching of the phosphorescence of a Pt-porphyrin complex immobilized on the cell culture substrate. Oxygen-dependent quenching of Pt-porphyrin phosphorescence has previously been widely utilized in pressure-sensitive paints.[9] A recent review on luminescent pressure-sensitive paints provides

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an excellent overview of both the theory and the practical aspects of phosphorescent oxygen sensing.[10]

EXPERIMENTAL SECTION

Chemicals. Carbachol, glucose, and amobarbital were purchased from Sigma (St. Louis, MO), Ham's F-12 medium (composition described in ref[11]) and G418 from Gibco BRL (Grand Island, NY) and Cytodex-3 beads from Amersham Pharmacia Biotech (Uppsala, Sweden). Fetal bovine serum was provided by Zymogenetics Inc. (Seattle, WA).

Pt-tetra(paracarboxy-phenyl)porphyrin tetraethyl ester (Pt-(*p*-COOEt)$_4$PP) was provided by Professor Martin Gouterman (University of Washington, Department of Chemistry). It was converted to free acid form as described in the literature.[12] Briefly, 5.5 mg of Pt-(*p*-COOEt)$_4$PP was added to 10 mL of dimethylformamide and refluxed under stirring until all the solid was dissolved. Addition of 0.8 mL of aqueous 1 M NaOH under reduced heat resulted in formation of the free acid (Pt-(*p*-COOH)$_4$PP) as a precipitate. Further addition of water dissolved most of the precipitate, and the resulting solution was kept on gentle heat for 2 h to ensure complete reaction. The solvent was removed using a rotovap, and the obtained solid was redissolved in water. Pt-(*p*-COOH$_4$PP was reprecipitated by addition of 1.5 mL of 2 M HCl. Again, the solvent was removed by a rotovap, and the solid Pt-(*p*-COOH$_4$PP was finally dissolved in 6 mL 0.010 M aqueous NaOH to yield the Pt-(*p*-COOH$_4$PP stock solution used for preparing the oxygen indicator beads.

Cell Culture. Cytodex-3 microcarrier beads were prepared by letting dry bead powder swell overnight in balanced salt solution. The beads were then sterilized by soaking in 95% ethanol for 2−3 h, were washed repeatedly with balanced salt solution, and finally were suspended in cell culture medium.

The cells used in the experiments were Chinese Hamster Ovary cells (CHO M1 WT3, American Type Culture Collection) transfected with the gene for the rat m1 muscarinic receptor. The growth medium was Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum and 50 μg/mL of G418. A wet volume of 80 μL of Cytodex-3 beads was suspended in 6 mL of growth medium and inoculated with $\sim 6 \times 10^5$ cells. The cells were then allowed to grow to full confluence. One day prior to experiments, the nutrient medium was switched to Ham's F-12 + 5% fetal bovine serum + 50 μg/mL of G418.

The working buffer used in the measurements was a phosphate-buffered balanced salt solution (0.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.5 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 3 mM KCl, 0.13 M NaCl) with 10 mM glucose and 10 mM HEPES. The pH was adjusted to 7.4.

The phosphorescent oxygen probe was attached to Cytodex-3 microcarrier beads noncovalently by adding 40 μL of Pt-(*p*-COOH$_4$-PP stock solution to a (wet) volume of 80 μL of Cytodex-3 beads suspended in 1 mL of phosphate buffer. The suspension was gently shaken periodically, and all the Pt-(*p*-COOH$_4$PP was taken up by the beads in a matter of minutes.

Table 1. Bead Injection Protocol Used in the Oxygen Consumption Assays

BI step	illumination	recorded event
1. pack beads	OFF	
2. wash beads	OFF	
3. aspirate blank and deliver onto cells	OFF→ON	baseline
4. stop flow for 180 s:		
a. 174 s	OFF	
b. 6 s	ON	O_2 consumption in absence of drug
5. wash beads	ON→OFF	
6. aspirate drug and deliver onto cells	OFF→ON	baseline
7. stop flow for 180 s:		
a. 174 s	OFF	
b. 6 s	ON	O_2 consumption in presence of drug
8. wash beads	ON→OFF	
9. discard beads	OFF	

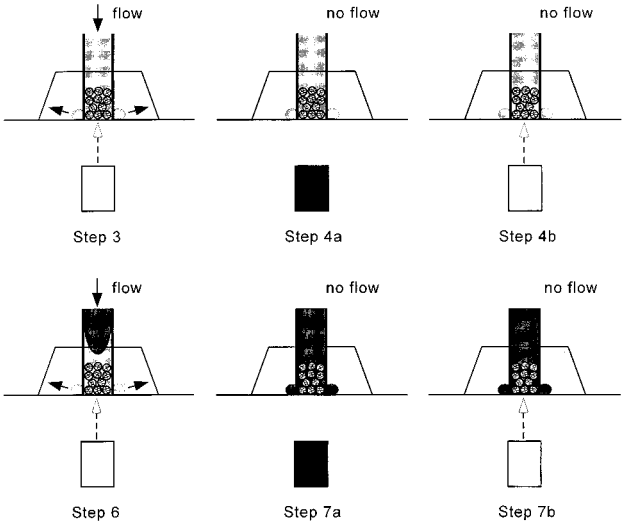

Figure 1. The central steps in the bead injection protocol (steps 3, 4a, 4b and 6, 7a, 7b in Table 1).

Instrumentation. The instrumental setup was nearly identical to that described in ref 13. It consisted of a FIAlab-3000 sequential injection system (Alitea USA, Medina, WA) for manipulation of solutions, a jet ring flow cell for trapping beads, and a fluorescence microscope for detecting fluorescent signals from the trapped beads and cells.

The fluorescence detection system consisted of a Zeiss Axiovert 100 inverted microscope equipped with a 40×/1.30 NA objective (Carl Zeiss, Oberkochen, Germany) and a SE-510 photometer head (Photon Technology International, South Brunswick, NJ). The microscope was connected to a Delta Scan illuminator (Photon Technology International) to provide the excitation of the phosphorescent oxygen indicator. The measurements were performed with the excitation wavelength set at 509 nm. The fluorescence filter system consisted of a 560-nm dichroic mirror and a 550-nm long-pass emission filter.

Oxygen Consumption Assay Protocol. The bead injection protocol for drug assays is described in Table 1. In addition, graphical representation of the steps that are directly associated with signal detection is shown in Figure 1. Each assay was

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repeated four times. Each assay was followed by measurement of the sensor signal in a deoxygenated solution. This was done to improve the reliability of sensor output, as discussed in Data Processing.

The sensor response was calibrated by perfusing the sensor with buffer solutions containing different levels of dissolved O_2. The O_2 concentrations were adjusted by purging the solutions with air and N_2 at controlled flow rates. VCD-1000 flow controllers (Porter Instrument Co., Hatfield, PA) were used to achieve steady gas flow rates. Dissolved O_2 was expressed as the partial O_2 pressure in the purging gas (taking partial O_2 pressure in air to be 0.21 atm).

Data Processing. The dependence of the sensor luminescence intensity on dissolved oxygen concentration is governed by the Stern-Volmer equation[14]

$$\frac{I_0}{I} = 1 + K_{SV}[O_2]$$

where I is the luminescence intensity corresponding to a particular O_2 concentration, I_0 is the luminescence intensity in the absence of O_2, and K_{SV} is the Stern-Volmer quenching constant.

The equation can be rearranged to obtain an expression that is directly proportional to O_2 concentration

$$\frac{I_0}{I} - 1 = K_{SV}[O_2]$$

Raw luminescence intensity data (I) from all experiments were converted into $[(I_0/I) - 1]$ for further data analysis and/or presentation. In addition to achieving direct proportionality to O_2 concentration, the transformation also makes the data independent of indicator concentration on the beads and the number of beads seen by the detector in any particular experiment.

To evaluate the effect of a drug on the cellular aerobic metabolism, the O_2 consumption in the presence of a drug (step 7 in Table 1) was divided by the O_2 consumption of the same cells in the absence of the drug (step 4 in Table 1):

$$\text{rel } O_2 \text{ consumption} = \frac{\Delta O_2(\text{drug})}{\Delta O_2(\text{no drug})} = \frac{[(I_0/I) - 1]_{\text{drug}}}{[(I_0/I) - 1]_{\text{no drug}}}$$

This treatment makes the data independent of the number of cells packed into the flow cell in any particular experiment. Thus, it allows reliable comparison of data from different runs.

RESULTS AND DISCUSSION

Detection Optimization. In order for the cellular metabolism to consume oxygen to a detectable degree, it was necessary to stop the flow for a period of 3 min. It was observed, however, that the sensing itself also consumed oxygen at a high enough rate to dramatically decrease the O_2 concentration in the monitored volume in stopped-flow conditions. Such effects have been observed earlier (the so-called induction effect in pressure-sensitive paints) and can most likely be attributed to the singlet oxygen produced in the sensing event.[10] Since singlet oxygen is very reactive, it will react with components of the surrounding matrix or solution, thus removing itself from the O_2 pool. The behavior is analogous to O_2 consumption by the amperometric Clark electrode.

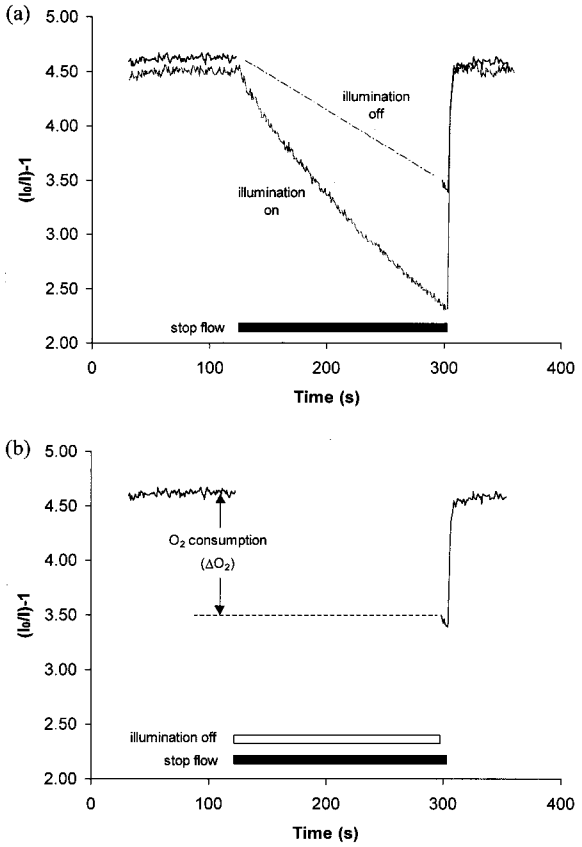

Figure 2. (a) Oxygen depletion during stopped-flow period with illumination on (consumption due to cells and sensing, gray trace) and illumination off (consumption due to cells only, black trace). The dashed line represents the approximate oxygen consumption trend during the illumination off period. (b) Measurement of cellular oxygen consumption as the difference between the initial baseline (average of 10 data points before switching off illumination) and the level at the end of the stopped-flow period (average of the first two data points after switching on illumination).

The sensing-related decrease in O_2 was strongly dependent on illumination intensity, and choosing the minimal intensity that still yielded a good signal-to-noise ratio helped bring down this undesired effect considerably. The magnitude of the effect in optimal conditions is shown in Figure 2a: the decrease in $[(I_0/I) - 1]$ with illumination off is purely due to cellular oxygen consumption, whereas the decrease observed with illumination on represents oxygen consumption by the cells and the sensing. It can be seen that even in optimized conditions the sensing-related consumption is comparable to the cellular consumption. Therefore, it was necessary to keep illumination switched off for most of the stopped-flow period. Illumination was only switched on for the last 6 s of the stopped-flow period to record the final level of O_2 around the cells. Oxygen consumption (ΔO_2) was measured as shown in Figure 2b.

Sensor Calibration. The sensor response showed good agreement with the Stern-Volmer equation in the range 0−0.17 atm of O_2 and some deviation from it in the range 0.17−0.21 atm. Fitting five data points in the 0−0.17 atm range to the Stern-Volmer

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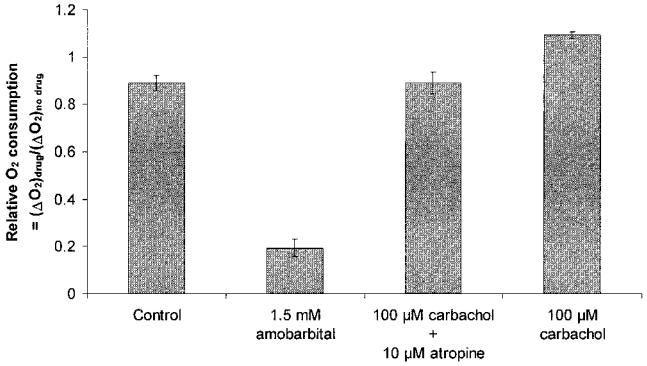

Figure 3. Oxygen consumption by the CHO M1 WT3 cells exposed to 1.5 mM amobarbital, 100 μM carbachol + 10 μM atropine and 100 μM carbachol. The control refers to basal oxygen consumption measured in plain phosphate buffer. The reported standard deviations are based on four experiments ($n = 4$).

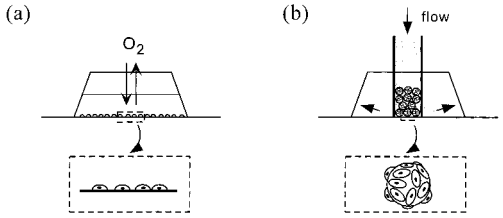

Figure 4. (a) Conventional setup for inspection of adherent cells by microscope, where cells are grown as a monolayer on the glass bottom of a microscope chamber. The open architecture causes consumed O_2 to be replenished from surrounding air. (b) Bead injection flow through chamber has microcarrier beads packed inside a steel tube pressed against the glass bottom of a microscope chamber. Pumped liquid can trickle through the junction between steel and glass, but exchange of O_2 between the bead layer and air is negligible.

equation yielded a calibration equation $I_0/I = 0.87 + 23(p(O_2))$, with $r^2 = 0.998$.

Drug Responses. The effects of exposing the cells to different drugs are shown in Figure 3. Relative O_2 consumption values of >1 and <1 indicate increased and decreased consumption, respectively, induced by the drug. Amobarbital, an agent that blocks a step (Complex 1) in the aerobic electron transport chain,[1,15] is seen to strongly diminish the cellular O_2 consumption when administered to the cells in a 1.5 mM dose. This can be taken as proof that the sensor is truly probing the aerobic O_2 consumption of the cells.

Exposing the cells to 100 μM carbachol results in an increase in O_2 consumption. Carbachol is a drug that binds to the M1 muscarinic receptors expressed in the CHO M1 WT3 cells and elicits a response in them.[16] The carbachol action, however, is not directly linked to the aerobic energy metabolism, as opposed to the amobarbital action above. The carbachol-induced increase in aerobic turnover must therefore be a more general effect of cell stimulation on the aerobic metabolism. Exposure of the cells to carbachol in the presence of 10 μM atropine does not result in such an increase. Atropine prevents binding of carbachol to M1 receptors; therefore, this experiment confirms that the previously observed increase in O_2 consumption must be due to cell stimulation caused by carbachol binding to the M1 receptors.

CONCLUSIONS

The paper describes a method for detection of O_2 consumption by adherent cell cultures within a time frame of minutes. Its operation is based on the following advantages given by the bead injection-based reagent handling and optical O_2 sensing:

(1) Bead injection allows packing a relatively large number of adherent cells (on the order of 10^4) in a small ($\sim$3 μL) volume, in a closed environment. This is an absolute requirement for being able to detect O_2 consumption within the minute time scale. A graphical comparison of the open and closed setups is shown in Figure 4.

Bead injection also allows rapid renewal of inspected cells. Thus, each assay can be conducted on a fresh set of cells. (2) Optical O_2 sensing allows placement of the sensing element right next to the cells. Consequently, O_2 consumption can be measured on the spot rather than downstream, resulting in maximal sensitivity because the deoxygenated solution is not diluted. (3) In this case, optical and electrochemical detection share the disadvantage that detection itself consumes oxygen. However, the optical method still retains the advantage that it can be switched on without a charging current that initially obscures the signal.

The 3 min period needed for reliable quantitation of O_2 consumption compares favorably to existing measurement techniques for suspended cell cultures.[2,3] It should be further pointed out that the cultured cells, both in this work and in the above references, were obtained from immortal cell lines, which typically derive most of their energy through the anaerobic pathway. Application of any of these techniques to primary cell cultures, which are fully aerobic and thus consume far greater amounts of O_2, should necessitate even shorter measurement periods.

It was demonstrated that stimulation of the muscarinic M1 receptors on the CHO M1 WT3 cells increases their oxygen consumption. These results are especially interesting since the rate of the overall (i.e., aerobic + anaerobic) cellular energy metabolism has been shown to reflect a broad array of perturbations in a cells' environment caused by various biochemical effector agents.[17] Presently, the overall metabolic rate is quantitated by measuring the rate of CO_2 and lactic acid extrusion from the cells with the commercial Cytosensor Microphysiometer. The results presented in this paper indicate that O_2 measurements might be used as an alternative, or complementary, indicator of stimulation for aerobic cells.

In addition to the specific application of measuring cellular oxygen consumption, oxygen sensing by BIS has potential for even more general use. The greatest advantage of the technique is the renewability of the sensor surface, which eliminates one of the most serious drawbacks of oxygen optrodes (or other optrodes): gradual degradation due to photobleaching or wash-out.[18] Further, since the indicator is adsorbed on the surface of beads rather than entrapped inside a solid polymer matrix, mass transfer effects

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should be considerably smaller than in most existing optosensors. Consequently, this technique could be successfully applied to monitoring oxygen for chemical and bioprocess control. The above general advantages are, of course, not limited to oxygen sensing. Indeed, bead injection spectroscopy has already found several applications as a microanalytical technique.[8]

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