MEMBRANE COVERED POLAROGRAPHIC OXYGEN SENSOR MANUFACTURING. PRACTICAL ASPECTS

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An oxygen sensor is an electronic device that measures the proportion of oxygen in the gas or liquid being analyzed. In this paper is presented the manufacturing process of the polarographic oxygen sensor, the most used oxygen sensor for measuring oxygen dissolved in a liquid. Using Clark type polarographic oxygen sensor mounted in a range of oxygen electrode chambers and connected to computer operated oxygen electrode control units, oxygen evolution/uptake may be measured in liquid or gas-phase samples during photosynthesis/cellular respiration studies.

1. INTRODUCTION

The electrochemical analyze of oxygen is generally achieved using Clark-type oxygen-sensitive electrode. Clark had the ingenious idea of placing very close to the surface of the platinum electrode (by trapping it physically against the electrode with a piece of dialysis membrane) an enzyme that reacted with oxygen. He reasoned that he could follow the activity of the enzyme by following the changes in the oxygen concentration around it, thus a chemosensor became a biosensor. Based on this experience and addressing his desire to expand the range of analytes that could be measured in the body, he made a landmark address in 1962 at a New York Academy of Sciences symposium in which he described how "to make electrochemical sensors (pH, polarographic, potentiometric or conductometric) more intelligent" by adding "enzyme transducers as membrane enclosed sandwiches" [1]. The concept was illustrated by an experiment in which glucose oxidase was entrapped at a Clark oxygen electrode using dialysis membrane. The decrease in measured oxygen concentration was proportional to glucose concentration. The American scientist, Prof. Leland C. Clark, is widely regarded as the “father” of the biosensor.

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2. EXPERIMENTAL

Considerations regarding the selection of membrane

The membrane type plays an important role in electrode performances especially because of the O\textsubscript{2} diffusion coefficient. Seeing the necessity of fixation on the active surface of the noble metal, the mechanic properties of the membrane must also be taken in consideration. The membrane can be made of collodion, deposited from solution; teflon, it presents good mechanical properties; polyethylene, has the advantage of a small O\textsubscript{2} diffusion coefficient; polypropylene, polystirol, cuprophan, silicon or primal derivates.

Comparative studies made on identical electrodes, covered with different types of membrane showed that the sensibility, the O\textsubscript{2} consumption, the agitation and viscosity modification artifact, are decreasing with the decrease of membrane diffusion coefficient for O\textsubscript{2} (Table 1).

Table 1. Results of comparative studies made on identical electrodes, covered with different types of membrane

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Teflon</th>
<th>Polyethylene</th>
<th>Mylar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion coefficient (cm\textsuperscript{3}/cm\textsuperscript{3}<em>h</em>760mmHg)</td>
<td>4.0·10\textsuperscript{-3}</td>
<td>2.3·10\textsuperscript{-3}</td>
<td>0.16·10\textsuperscript{-3}</td>
</tr>
<tr>
<td>Thickness (\textmu m)</td>
<td>25</td>
<td>25</td>
<td>6.25</td>
</tr>
<tr>
<td>Electrode sensibility (A/mmHg)</td>
<td>4.5·10\textsuperscript{-8}</td>
<td>0.98·10\textsuperscript{-8}</td>
<td>1.26·10\textsuperscript{-1}</td>
</tr>
<tr>
<td>O\textsubscript{2} consumption</td>
<td>1</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Effect of agitation and solution viscosity</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Response time (s)</td>
<td>20</td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td>Residual current (10\textsuperscript{-8} A)</td>
<td>0.4 - 2</td>
<td>1 - 2</td>
<td>0.6 - 1</td>
</tr>
</tbody>
</table>

From the Table 1, we can see that the electrode response time is increasing with the decrease of membrane diffusion coefficient for O\textsubscript{2}.

As shown in Ref. [2], for a fast response of a sensor, the thickness of membrane, d\textsubscript{m}, vanishes and/or the diffusion coefficient, D\textsubscript{m}, is very high. Adjusting d\textsubscript{m}, rather than D\textsubscript{m}, is more effective in adjusting the time constant, because \tau depends on the square of d\textsubscript{m}.

Steps in electrode realization

For the fabrication of a diffusion oxygen sensor (DO sensor) the following materials are needed:

**Electrode material:** 75 \textmu m (or 25 to 125 \textmu m) diameter Pt wire, 3 cm length; a short length (20 cm) of Ag wire, 0.25 mm OD; 1 mm (25.4x25.4 mm) thickness Teflon membrane; 1M KCl solution in ethylene glycol.
**Construction aids:** lead glass capillary tube (2 mm OD, 0.5 mm ID), 15 cm long; glass tube (10 mm OD, 8 mm ID), 12 cm long; a short length (20 cm) of copper wire, 0.25 mm OD; a short length (1 cm) of 7 mm ID silicon tubing; a short length (10 cm) of 2 mm ID rubber tubing (for use as a vent tube); a BNC female connector (obtainable from Radio Shack); epoxy (2 part, ribbon type).

**Chemicals for cleaning and plating:** 1M nitric acid solution; Concentrated nitric/sulphuric (1:1) solution; Methyl chloroform solution; 0.1 M HCl solution.

For obtaining a DO sensor one must follow the next steps:

1. Cut a short length of copper wire (20 cm) and attach 75 μm Pt wire by soldering (Fig. 1a).
2. Clean the Pt wire (just the Pt wire portion, not the Cu wire) by dipping in a 1:1 concentrated nitric and sulphuric acid solution. Wash with deionised water.
3. Place it in the glass capillary tube (cleaned) as shown in Fig. 1b. Flame the end until the glass melts and fuses around the Pt wire. A good wetting of the Pt wire with molten glass is necessary. Lead glass is best for this purpose because, it wets the metal well and the thermal expansion coefficient is closest to that of Pt.
4. Grind the flamed end flat (use a sand paper: start with a coarse one and then gradually use finer grit sand paper). It is important that the glass seals the metal tightly around the metal wire. The goodness of the seal may be observed under a microscope but a better way is to measure the conductivity in an electrolyte solution (by using Ag/AgCl as the counter electrode). The conductivity should remain more or less constant. If it increases with time, the seal is not good and a DO sensor made with such a leaky seal tend to be unstable.
5. Grind flat both ends of the 10 mm OD glass tube. Grind the edge of one end round (Fig. 1c).
6. Make a Ag/AgCl reference electrode by electrolytic chlorination of Ag wire. Clean Ag wire by dipping in 1 M nitric acid for 10 s. Anodise the wire in 0.1M HCl at 1.5 V and 0.4 mA/cm² current density of for 30 min. Store in 1M KCl solution overnight before use. When the chlorination is done properly, the wire will have a brownish coat on it (Fig. 1d).
7. Put the glass capillary tube in the large glass tube, and insert the chlorinated silver wire and the vent tube (Fig. 1e).
8. Connect the copper wire to the center pin of BNC connector; connect the Ag/AgCl wire to the outer body of BNC by soldering (Fig. 1f).
9. Fix the BNC connector and the vent tube by molding with epoxy (Fig. 1g).
10. Cut a 19 mm length of silicone tubing and place it in methyl chloroform for 2 min. The tubing swells. Cover the free end of the sensor assembly with a Teflon membrane and fix it tightly in place with the swelled silicone tubing. Make sure the membrane is tightly fit (Fig. 1h). Let the solvent evaporate.
11. Fill the KCl electrolyte solution with a syringe via the vent hole. After the filling, clamp the vent tube (Fig. 1i).
Figure 1. The steps one needs to follow for obtaining a DO sensor
Oxygen electrode recordings

In "coupled mitochondria", electron transport and the synthesis of ATP from ADP and Pi are mutually dependent processes, i.e., in addition to an oxidation substrate, the presence of both ADP and inorganic phosphate is required for oxygen uptake to occur [4].

The decrease in oxygen concentration, in a closed system, can be measured with a polarographic oxygen sensor. In figure 2 is presented the experimental set-up used in Clark electrode method. For measuring the concentration of oxygen in liquid medium and gases the sample is brought into contact with the membrane through which oxygen diffuses into the measurement chamber containing potassium chloride solution and the two electrodes (the reference silver/silver chloride electrode and the platinum electrode). The electric current flow between the two electrodes when polarized with a potential of -600 mV (vs. Ag/AgCl) determines the oxygen concentration in the solution.

![Figure 2. Experimental set-up used in Clark electrode method.](image)

The respiration active state, which begins with the addition of ADP, is sometimes referred to as "state 3". The slower rate respiration, after all the ADP has been phosphorylated to form ATP, is referred to as "state 4". In this state, the respiration rate is usually faster than the original rate, before the first addition of ADP, because of some ATP that is broken down by ATPase activities contaminating the preparation, the resulting ADP being re-phosphorylated by the mitochondria [5].

The respiratory control index (RCI), given by the ratio [state 3 rate]:[state 4 rate], indicates the tightness of the coupling between respiration and phosphorylation. In vitro, the case of isolated mitochondria, the coupling is not perfect, probably as a result of mechanical damage during the isolation procedure. Typical RCI values range from 3 to 10, varying with the substrate and the quality of the preparation. In vivo, the coupling is thought to be better but, may still not achieve 100%.
The ATP:O ratio, the relationship between ATP synthesis and oxygen consumption can be calculated by measuring the decrease in oxygen concentration from state 3, after adding a known amount of ADP. Figure 3 is a simplified representation of a mitochondria respiration experiment from which we eliminated the basal respiration due to imperfect coupling and the recycling of ATP. The change in concentration must be multiplied by 2 (factor corresponding to the 2 atoms in an oxygen molecule) and by the chamber volume, so that the answer (in micro-atoms of oxygen) can be related to the quantity of ADP added. The quantity of oxygen in the chamber is calculated from published oxygen solubility data at the appropriate temperature.

Example:
Considering a decrease in \([O_2]\) = 0.135 mM; \(V_{\text{chamber}} = 2.5\) ml; Oxygen atoms consumed in state 3 = \(0.135 \times 2 \times 2.5 = 0.68\) micro-atoms; Injected ADP (20 microlitres of a 50mM solution) = ATP formed = 1 micromole; ATP:O = [ATP formed] : [oxygen consumed] = 1.48 (for succinate oxidation).

NAD-linked substrates give consistently higher values for the ATP:O ratio (about 2.5) compared with succinate (about 1.5). These results indicate that electrons from relatively poor reducing agents such as succinate (also acyl CoA and glycerol phosphate) enter part of the way along the respiratory chain, by-passing the first coupling site where energy is captured for ATP synthesis.

The sharp changes in slope after exhaustion of ADP and again after all the oxygen has been used up imply that mitochondria must have very high affinities for ADP and oxygen. The concentration of both these compounds is very low in most healthy cells, since they are efficiently scavenged by the mitochondria.

The addition of an uncoupling agent (such as dinitrophenol or CCCP) leads to a permanently high rate of respiration in the absence of ADP, until all the oxygen has been consumed. Many natural and synthetic poisons block mitochondrial respiration.
The interactions between inhibitors and uncouplers allow two major types of inhibition to be distinguished: those that prevent respiration by blocking the respiratory chain itself, and those that inhibit the ADP phosphorylation system, so it only blocks respiration in coupled mitochondria.

**Determination of ADP:O quotient and respiratory control index**

The mitochondria isolation was achieved by differential centrifugation of a homogenate, containing 6 g chicken liver in sucrose buffer as shown in reference [6].

The amount of consumed oxygen (QO₂) linked to substrate (succinate) oxidation was determined, at normal pressure and laboratory temperature, following the next protocol [7, 8]:

1. A calculated amount of incubation medium (3ml) was added in the reaction vessel, so that this will fill, when all the other substances will be added
2. After about 5 minutes, a mitochondria suspension (0.1ml) was added and the graph was followed for 1-2 minutes.
3. The substrate (20 µl of 3.3 mM succinate) was introduced in the vessel and after 1-2 minutes registration was made.
4. The ADP was injected (0.01ml of a 90mM solution) and the O₂ concentration was recorded until all ADP has been consumed. The final diagram is illustrated in figure 4.
5. After about 2 minutes, another addition of ADP is made (0.15 mM).

As an uncoupling agent of oxidative phosphorylation, in the case of succinate, we can use 10 µl DNP.

![Figure 4. Diagram of mitochondria respiration experiment.](image)
The QO$_2$ was calculated with the following relation:

\[
QO_2 = \frac{(O_2 \text{ contained in the medium/ml}/X \text{ units}) \times Y \text{ units} \times ml \text{ reaction medium} \times 2}{(0.240 \text{ \(\mu\)moles O}_2/\text{ml}/86 \text{ divisions}) \times 31 \text{ divisions} \times 3\text{ml} \times 2} = 0.520 \text{ \(\mu\)atoms O}_2
\]

Injected ADP: 0.01ml of a 90mM solution which means 0.900 \(\mu\)atoms ADP;
The ADP:O quotient: 0.900 \(\mu\)atoms/0.520 \(\mu\)atoms = 1.7;
The respiratory control index: the slope with ADP/ the slope without ADP = 5.9.

4. CONCLUSIONS

The whole process of measuring oxygen evolution/uptake in liquid or gas-phase samples using a membrane covered polarographic oxygen sensor is totally dependent on the source of oxygen. The rate of oxygen diffusion to the cathode and, the current output of the electrode depend on the oxygen concentration in the main incubation chamber. It also depends on several other factors: temperature, membrane thickness and permeability, sample viscosity and stirring speed. The control of all this factors is very important for the precision of the results. Oxygen electrodes measure the velocity of a physical-chemical process that is far from equilibrium and the instrument must be calibrated against a known standard, usually air.

5. REFERENCES