Measuring real levels of oxygen *in vivo*: opportunities and challenges

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Abstract

Molecular oxygen has multiple roles in cells, which can have different dependencies on the amount of oxygen at particular sites. Therefore, in order to relate oxygen to the processes under consideration, one must know the amount of oxygen present at the site of interest. The use of the various techniques generally is complementary rather than competitive. The key to their successful use requires an understanding of the capabilities of the techniques and of the nature of the process that is being investigated. This paper provides a framework for obtaining such an understanding.

Introduction

Molecular oxygen has multiple roles in cells, which usually are highly dependent on the amount of oxygen present. The physiologically most pertinent direct measurements of oxygen usually will be those made *in vivo*, because so many factors can affect the delivery and utilization of oxygen that it is very difficult to reach firm conclusions using simpler systems. Consequently, a number of methods have been developed for making measurements of oxygen *in vivo*. Each approach has particular potential advantages and limitations. If these are kept in mind, the most appropriate method or combinations of methods can be used to provide the data needed for the question that is being addressed. It then becomes possible to measure the pertinent levels of oxygen for the particular application.

The relationships between the amount of oxygen present and its effects are complex, and vary for various processes. For example, mitochondrial function is not affected directly until the oxygen level decreases below 1 μM, and radiation effects decrease when the oxygen level drops below 10 μM. Some types of oxidative damage are proportional to the amount of oxygen that is present, and some types of oxidative damage are

mediated through cell signalling that occurs at critical levels of oxygen. Therefore, in order to relate oxygen to the processes under consideration, one must know the amount of oxygen present at the site of interest.

In part because direct measurements of oxygen are difficult to make, parameters related to oxygen delivery are often measured instead. These can be quite useful, as long as one recognizes the nature of the measurement. A simple, but still effective, measurement is blood pressure. In addition to recognizing the indirect relationship of blood pressure to tissue oxygen, it also is important to know the relationship between the blood pressure that is measured (usually the systemic pressure in major peripheral arteries) and the pressure within the site of interest. Some of the differences are well known, such as the potential for differences between perfusion pressure in the central nervous system and systemic circulations. However, there can be differences in other sites as well. Especially in the presence of pathophysiology, there can be local effects due to increased interstitial pressure as well as differing patterns of the neovasculature. Measurements of perfusion are another type of indirect measurement that are extensively and effectively used. While technically challenging, the methods that use histological assessment can be quite accurate. NMR methods are widely available and are not invasive, but the resulting data are often more difficult to interpret quantitatively.

In many situations the information that is needed requires direct measurements of oxygen. The most facile measurements of oxygenation are made in the circulatory system, but unfortunately these can have considerable limitations. Some of the measurements, such as those of blood gases, which do give direct measurements of oxygenation are invasive, discontinuous and time-consuming. Others, such as those based on near-infrared (NIR) light transmission, provide data non-invasively, but the data are indirect (they reflect haemoglobin saturation and blood volume) and the methods sample multiple compartments in the circulatory system (arterioles, venules, capillaries, and perhaps some contribution from large vessels). However, the real limitation of measure-

ments in the circulatory system is that they do not report on the oxygen levels in the sites where most of the oxygen-dependent interactions take place, which are within the tissues.

Therefore there has been an increase in the development of methods to measure the partial pressure of oxygen (Po_2) in tissues (Pto_2) *in vivo* more directly. Several methods have been established, each with a different set of capabilities and limitations. The use of the various techniques generally is complementary rather than competitive. The key to their successful use requires an understanding of the capabilities of the techniques and the nature of the process that is being investigated. There are some very useful reviews of many of these techniques [1–7]. Our approach is based on EPR. This method has several potential advantages under some circumstances, especially for making repeated measurements without acute invasion. Before discussing this approach in more detail, I will first review some of the other major and complementary methods.

Polarography

Measurement of the O_2 concentration by polarography is the most frequently used method. It still is considered as the 'gold standard' for measurements of Pto_2. It provides data on the Po_2, and in versions such as the Eppendorf Histograph it provides a very useful profile of Pto_2 values along a track. Its potential limitations include: the fragility of the system; the possibility of damage to the tissue during insertion; accurate measurements are very difficult below 5 mmHg O_2; some versions may change the O_2 concentration in the region of the electrode due to consumption of O_2; and it is not suitable for making repeated measurements. The commercially available instrument that has been used in many of the studies (the Eppendorf Histograph) currently is not being marketed, but should become available in the near future. A key issue, which still needs to be clarified, is whether the physical effects of pressure and/or local damage that accompany the use of this technique perturb the measurements that are being made. If this is not the case, then this technique is probably the best one for obtaining accurate, spatially resolved, multiple measurements of Po_2 *in vivo*.

Fluorescence quenching

Recently a method based on fluorescence quenching has been used in a commercial system that involves the insertion of a fibre-optic probe directly into the tissue. The system, with the brand name 'OxyLite', has a ruthenium compound embedded in a rubber matrix at the end of the fibre-optic probe. Photodiodes stimulate the ruthenium, and the fluorescence rise time is digitized. The method is temperature-sensitive and so quantification requires temperature correction, which is accomplished by thermocouples attached to the fibre-optic probes. The calibrations are stable and the sensitivity is highest at lower Po_2 values [<4 kPa (<30 Torr)] [8,9]. There are plans for adding the capability to make measurements along a track, similar to the Eppendorf Histograph. It has many of the same advantages and potential limitations as the Eppendorf.

NMR methods

NMR methods to measure Pto_2 have appeal because of the widespread availability of NMR instruments and the potential for making non-invasive measurements. A technique based on NMR spectroscopy of myoglobin is useful in tissues that contain myoglobin, but has limited sensitivity [10]. The range over which it has the most sensitivity is approx. 0.13–1.33 kPa (0–10 Torr), which is very useful for investigating conditions with low oxygen levels such as ischaemia/reperfusion injury and the radiobiological oxygen effect. BOLD, or blood oxygen level dependent imaging, is sensitive to deoxy-haemoglobin and has been used extensively to measure changes in local oxygenation. The advantages of this method are that it is sensitive over the observed range of haemoglobin saturation and it is potentially useful in all tissues, but measurements directly reflect only changes in haemoglobin saturation, and therefore additional information is needed to infer absolute haemoglobin saturation. Therefore additional information is needed to infer Pto_2 values [11,12].

NMR can be used to make direct measurements of local Pto_2, by measuring the oxygen-sensitive T_1 relaxation rate of perfluorocarbons [13–17]. These compounds have to be introduced into the region of interest, either by direct injection [18] or by vascular infusion [19]. In the latter case, the localization of the perfluorocarbons is dependent on an as yet unknown cellular uptake pathway, and deposition is limited to well perfused regions [20]. The injection method allows for direct placement of the perfluorocarbons. The

compounds also wash out at variable rates, although they are usually present for long enough that measurements can be made hours after direct injection [18], or after several days when taken up by the cells [19,20]. The sensitivity of the method is a limiting feature, but with improved chemical composition (more fluorine atoms of identical chemical shift to improve the signal) and T_1 measurements, the method can provide data within ± 2 mmHg in approx. 7 min [18]. It has been shown to give similar values for Po_2 as the Eppendorf microelectrode.

NIR spectroscopy

NIR spectroscopy methods have potential advantages, especially when used in combination with techniques that can measure Po_2 directly in tissues, such as polarography or EPR oximetry. NIR measurements of haemoglobin can provide quantifiable data on intravascular haemoglobin content and saturation, thereby providing complementary data on the supply of oxygen to the tissue from the vascular system and the total oxygen capacity. This method has been especially useful since newer systems were developed in the early 1990s that use time-resolved or frequency-domain light measurement. These systems can be used to quantify the exact haemoglobin concentration and oxygen saturation within bulk tissue [21]. Advantages of NIR methods include the ability to provide data from relatively deep tissue sites, and the potential for imaging haemoglobin with modest spatial resolution [22]. Combining data about haemoglobin and Pto_2 provides a complementary set of information to determine supply and demand for oxygen within a tissue. This can be particularly important in determining the response of a tissue to damage or the viability of a tissue.

Phosphors

While the phosphorescence technique is capable of detecting O_2 concentrations as low as 0.2 μM, for a long time it has been considered to be a method for surface O_2 detection, with a sampling depth of less than 1 mm under normal conditions [23]. Recent work in this area has focused on the development of phosphors with longer wavelengths, which should greatly expand the applications *in vivo*. The phosphors are usually in the vascular system, so this method provides complementary data to measurements of tissue Po_2 obtained with EPR oximetry or polarography. It

seems likely that this method will be used increasingly as it is developed further [24–29].

Biopsy-based techniques

Some very useful methods [1,4,6,7] based on biopsies have been developed, such as the 'comet assay' and drugs that are localized selectively in hypoxic tissues. While not directly providing measurements of Po_2 or $[O_2]$, these methods do provide information on the occurrence of hypoxia and, at least to some extent, may be able to provide quantitative information that can be related to $[O_2]$.

EPR methods

In addition to the EPR methods described below, within the last few years there has been a very significant amount of progress in another type of EPR method for the measurement of oxygen, based on the use of soluble free radicals combined with new capabilities for the use of EPR *in vivo* [30–32]. Eventually this approach could be capable of providing detailed maps of Po_2. The current limitations of spin label (nitroxide) oximetry include low sensitivity at O_2 concentrations of < 10 μM, especially at the frequencies needed to obtain images over larger volumes, and the fact that the nitroxides can undergo bioreduction to EPR-inactive species. The low toxicity of nitroxides makes them very suitable for studies in animals if the problems of sensitivity can be overcome. The very recent development of the triphenyl radicals may significantly expand the potential for using oxygen-sensitive free radicals for imaging.

Our approach has emphasized the use of *in vivo* EPR spectroscopy with stable solid particulates as the oxygen sensors. The potentially useful features of the EPR oximetry techniques that we use include the following [33–35].

Non-invasiveness

Most approaches do require an initial placement of the paramagnetic materials into the tissues, but after that the measurements are made non-invasively from the surface. With 1 GHz EPR, measurements from the surface have a depth of sensitivity of up to 10 mm under most circumstances. In the near future this will be extended to lower frequency, with a consequent increase in the depth that can be probed. We also sometimes use an invasive variant based on the implantable microresonator or needle-catheter resonator.

Repeatability

Measurements can be made as frequently as desired over a period of 1 year or more.

Sensitivity

Measurements can, at low Po_2, resolve differences of less than 0.13 kPa (1 Torr).

Accuracy

Repeat measurements have low variability, and values obtained correlate closely with measurements of Po_2 by other methods.

Provision of localized measurements

The spatial resolution is the same as the size of the paramagnetic particles, which can be as small as a single particle of less than 0.2 mm in diameter [the Po_2 that is measured is the average Po_2 in the tissues that are in immediate equilibrium with the surface of the paramagnetic particle(s)].

Little effect of chemical and physical conditions

The range of chemical and physical conditions that are likely to be encountered in viable biological systems have little or no effect on the measurements. These include pH, oxidants, reductants and the presence of other paramagnetic materials.

Little or no toxicity

The paramagnetic materials are very inert in biological systems as assayed in both cell cultures and *in vivo*.

Capability of making several measurements simultaneously

This is accomplished by inserting multiple discrete solid particles and applying a magnetic field gradient so that sites less than 1 mm apart can be resolved [36].

Time resolution

The method has the capability of following changes in Po_2 with a time resolution of seconds or less.

Responds to Po_2 (rather than to the concentration of O_2)

This is in contrast with nitroxides and other oxygen-sensitive materials, which may respond to the concentration of oxygen or a product of concentration and the rate of diffusion of oxygen.

Opportunities for measuring oxygen *in vivo*

Using the approaches described briefly in this paper, for most experimental needs there now is the possibility of accurately measuring oxygen levels *in vivo*. In order to do this most efficiently and effectively, however, the investigator needs to select and use the appropriate methods. The variables that should be considered include the following: (1) the site for which the information is needed; (2) the levels of oxygen that are likely to be present and the accuracy with which these need to be known; (3) the time resolution that is needed; (4) whether there will be a need for repeated measurements; (5) what other physiological parameters need to be known in order to draw appropriate conclusions from the measurements of oxygen; and (6) the degree of invasiveness that can occur without compromising the experiment. With this information at hand, it then should be possible to choose the set of techniques that will be used and how they will be employed. In most cases, more than one type of measurement will be needed. It is unlikely that one technique, no matter how well utilized, will be optimal for most measurements.

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Mitochondrial respiration at low levels of oxygen and cytochrome c

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Abstract

In the intracellular microenvironment of active muscle tissue, high rates of respiration are maintained at near-limiting oxygen concentrations. The respiration of isolated heart mitochondria is a hyperbolic function of oxygen concentration and half-maximal rates were obtained at 0.4 and 0.7 μM O_2 with substrates for the respiratory chain (succinate) and cytochrome c oxidase [N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) + ascorbate] respectively at 30 °C and with maximum ADP stimulation (State 3). The respiratory response of cytochrome c-depleted mitoplasts to external cytochrome c was biphasic with TMPD, but showed a monophasic hyperbolic function with succinate. Half-maximal stimulation of respiration was obtained at 0.4 μM cytochrome c, which was nearly identical to the high-affinity K'_m for cytochrome c of cytochrome c oxidase supplied with TMPD. The capacity of cytochrome c oxidase in the presence of TMPD was 2-fold higher than the capacity of the respiratory chain with succinate, measured at environmental normoxic levels. This apparent excess capacity, however, is significantly