In vivo electron paramagnetic resonance oximetry and applications in the brain

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Abstract

Molecular oxygen (O_2) is essential to brain function and mechanisms necessary to regulate variations in delivery or utilization of O_2 are crucial to support normal brain homeostasis, physiology and energy metabolism. Any imbalance in cerebral tissue partial pressure of O_2 (pO_2) levels may lead to pathophysiological complications including increased reactive O_2 species generation leading to oxidative stress when tissue O_2 level is too high or too low. Accordingly, the need for oximetry methods, which assess cerebral pO_2 *in vivo* and in real time, is imperative to understand the role of O_2 in various metabolic and disease states, including the effects of treatment and therapy options. In this review, we provide a brief overview of the common *in vivo* oximetry methodologies for measuring cerebral pO_2. We discuss the advantages and limitations of oximetry methodologies to measure cerebral pO_2 *in vivo* followed by a more in-depth review of electron paramagnetic resonance oximetry spectroscopy and imaging using several examples of current electron paramagnetic resonance oximetry applications in the brain.

Key words: oxygen; oximetry; cerebral partial pressure of oxygen; electron paramagnetic resonance; hypoxia

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Introduction

Molecular oxygen (O_2) is essential to life, one of the most important variables in many physiological, pathological and therapeutic processes and due to its reactive nature is important to energy metabolism, particularly in the brain. Specifically, a delicate equilibrium between tissue partial pressure of O_2 (pO_2) levels and mechanisms necessary to regulate variations in delivery or utilization of O_2 are crucial to support normal brain homeostasis, physiology and energy metabolism,[1-3] and any imbalance in cerebral pO_2 levels may lead to pathophysiological complications. O_2 is required for the production of reactive O_2 species (ROS), important mediators in various physiological processes including regulation of cerebral blood flow. However, increased ROS generation leading to oxidative stress may occur when tissue O_2 level is too high or too low. For example, while it is well known that O_2 deprivation during severe hypoxia and ischemia leads to an increase in ROS, oxidative stress and brain injury, studies suggest that short durations of normobaric hyperoxia (NBO) treatment lead to neuroprotection and improved metabolic status without promoting oxidative stress despite the observations that NBO increases

cerebral pO_2 back to or above physiological levels.[4-7] Thus, NBO has been considered a logical and potential important therapy for acute ischemic stroke. Accordingly, the need for oximetry methods, which monitor cerebral pO_2 *in vivo* and in real time, is imperative to understand the role of O_2 in various metabolic and disease states in addition to the effects of treatment and therapy options.

A comprehensive evaluation of the various oximetry methods and their applications to physiological processes and diseases in different organs can be found in numerous reviews.[3,8-14] The applicability of the methods in living systems is governed by its 1) acquisition time, accuracy and capacity to make repeated measurements; 2) category of invasiveness, ease of use and accessibility to the region of interest; and 3) sensitivity and nature of O_2 parameter reported as several of these modalities, at best, provide only qualitative estimates of tissue pO_2 or estimates of vascular O_2. Nevertheless, several techniques are useful for the determination of O_2 in the brain and have enabled researchers to better understand the mechanisms and role of O_2 in the pathogenesis and treatment of various cerebral metabolic and disease states. Quantitative and repetitive measurements of O_2 levels in deeper and specific cerebral tissue, *in vivo* and in real time, remains a technical challenge and efforts have been made to improve methods for evaluating cerebral pO_2. Among the common methods, electron paramagnetic resonance (EPR), a technique that uniquely detects and characterizes molecules with unpaired electrons, can measure several different, unique and distinctive biological parameters including tissue pO_2 *in vivo* and in real time.[15-25]

In this review, we provide a brief overview of the common *in vivo* oximetry methodologies, principles and applications for measuring cerebral pO_2 with emphasis on *in vivo* EPR oximetry spectroscopy and imaging. The following sections will discuss the advantages and limitations of oximetry methodologies to measure O_2 in the brain followed by a more in-depth review of EPR oximetry, including 1) the novel advantages of EPR oximetry compared to other methods, 2) *in vivo* EPR instrumentation, 3) advancements in EPR oximetry probes and experimental applications for monitoring cerebral pO_2, and 4) future directions and clinical applications of EPR oximetry.

Oximetry Methods for Cerebral Po_2 Measurements

Taking advantage of the chemical and physical properties of O_2, common *in vivo* oximetry techniques for determination of O_2 in the brain include: 1) mass spectroscopy 2) polarography; 3) optical methods; and 4) nuclear and magnetic resonance imaging methods, such as magnetic resonance imaging (MRI), positron emission tomography (PET) imaging and EPR oximetry and imaging.

Mass spectroscopy

Mass spectroscopy, a technique that samples and analyzes certain quantities of gas *via* a sampling cannula or probe implanted in deep tissue, allows for molecular O_2 to be identified and measured quantitatively, and has been used for cerebral blood-gas analysis and measurements of cerebral pO_2 in animals and humans.[26-28] The method is invasive with the need for implantation of the cannula and direct connection to the analysis chamber of the mass spectrometer and it is suggested that measurements are not possible until ~2 weeks after implantation due to edema and inflammation concerns at the implant site. Nevertheless, the advantages of mass spectroscopy include simultaneous, continuous and quantitative local measurements of cerebral pO_2 by blood-gas analysis.

Polarography

Polarography, a method based on measuring electrical current resulting from the reduction of O_2 to measure tissue pO_2 in the brain, has been responsible for most of *in vivo* measurements of cerebral O_2 tension over several decades and has established a number of major findings. Even so, the microelectrodes and polarography do offer several disadvantages including invasiveness, consumption of O_2 (in most cases), and the inability to make repetitive measurements over prolonged time period (*e.g.*, days). However, advancements in the design of commercially available microelectrodes have improved the use of this technique[8,10,29,30] and this method offers several advantages, including highly quantitative direct pO_2 measurements. Moreover, while polarography normally allows for point measurements, recent improvements in planar polarographic electrodes may allow for 2D mapping of tissue pO_2[11] so that the technique is not superseded by methods which can provide images. The advantages of imaging or mapping O_2 is preferred to characterize the heterogeneity of O_2 in tissues and investigate underlying pathophysiology since O_2 is not involved in just one physiological process. Several other methods which can map or provide images are discussed in the following sections.

Optical methods

Optical methods, including fluorescence, phosphorescence and near-infrared spectroscopy (NIRS), measure vascular O_2 saturation or supply instead of direct measurements of actual O_2 content or tissue pO_2. NIRS noninvasively measures light transmitted through tissues for measurement of blood O_2 saturation or supply, and has been used extensively in clinical and animal studies to monitor cerebral O_2 utilization and saturation in traumatic brain injury in

addition to ischemic and hemorrhagic stroke.[8,14,31-35] While the measurement is mostly qualitative, imaging by NIRS is possible in addition to its ability to make repeated real time measurements.

Fluorescence and phosphorescence oximetry measures *in vivo* O_2-dependent fluorescence and phosphorescence lifetime, respectively. Both techniques are invasive or minimally invasive, but commercially available fiber optic O_2 sensors or intravenous material probes allow for assessment of O_2 distribution in the brain vascular space and phosphorescence can also provide contour maps of a selected range of O_2 distribution values.[8,36-43] Additionally, while utilization of fluorescence is limited due to the inability to perform repetitive measurements and sensitivity to movement and temperature, phosphorescence offers a significant advantage in the ability to make repeated real time measurements and permeable indicators are in development for measurements outside the vascular space.

MRI imaging

Common MRI oximetry methods such as ^{17}O nuclear magnetic resonance (NMR), blood O_2 level-dependent (BOLD) MRI and ^{19}F MRI which image O_2 are based on detection of $^{17}O_2$, the relaxation properties of water protons in the presence of deoxyhemoglobin in the blood and intravenously injected perfluorocarbons (PFCs), respectively.[8,40,42,44-46] Each technique also allows for repetitive measurements, although ^{19}F MRI is dependent on the retention of PFCs in tissue. However, several major differences exist between the methods. BOLD MRI and ^{17}O NMR are noninvasive while the toxicity of PFCs, at higher dosages, used for ^{19}F MRI has raised concerns. Also, while ^{19}F MRI and BOLD MRI follow changes in blood oxygenation, only ^{19}F MRI can report quantitative absolute values of blood oxygenation, and ^{17}O NMR only measures O_2 utilization. A major advantage of the techniques is the ability to provide anatomical information co-registered with measurements of cerebral metabolic rate of oxygen utilization, blood oxygenation or tissue pO_2.[37,44-46]

Additionally, a more recent MRI method has been proposed and developed called Mapping of O_2 By Imaging Lipids relaxation Enhancement (MOBILE), which monitors changes in R1 relaxation rate of the lipid peak rather than those of water to estimate variations in tissue pO_2.[46] The authors demonstrated, in a proof-of-concept study, that the method is sensitive to changes in tissue pO_2 in animal tumor, stroke and liver models in addition to studies on a clinical MR with healthy volunteers and brain infarcted patients.[47,48] The studies suggested that MOBILE has the potential to provide noninvasive and qualitative measurements of tissue pO_2 limited to the amount of lipids in the tissue of interest.

PET imaging

PET imaging measures O_2 by detecting positron emitting radionuclides of a hypoxia biomarker, and has been used extensively and noninvasively in various *in vivo* brain and ischemic stroke studies.[49-54] Disadvantages to PET imaging is availability and the cost of cyclotrons needed to produce short-lived radionuclides, various interferences in the interpretation of PET imaging data, and PET measures O_2 consumption but not tissue pO_2. However, PET can be combined with computed X-ray tomography (PET/CT) and MRI (PET/MRI) to obtain anatomical information along with PET data.

EPR spectroscopy and imaging

A detailed description of EPR oximetry spectroscopy and imaging is available in literature.[10,24,25,55-57] Briefly, EPR cannot directly detect O_2 at 37°C even though O_2 has two unpaired electrons. The detection of O_2 is based on the principle that the interaction, but not consumption, with molecular O_2 can change the EPR spectrum of certain stable paramagnetic materials or O_2 sensitive spin probes, *e.g.*, the O_2-dependent broadening of EPR spectral linewidth can be calibrated and used to determine O_2 concentrations, quantitatively. EPR oximetry in combination with spin probes have several desirable features for measuring tissue pO_2 *in vivo*, including: 1) good accuracy, reproducibility and repetitive measurements; 2) high sensitivity to detect pO_2 values as low as 0.5 mmHg or 0.067 kPa; and 3) quick response to the change of tissue pO_2, typically less than 1 minute. Additionally, the development of appropriate spin probes and low-frequency EPR spectroscopy with imaging capability allows for reliably mapping of interstitial cerebral pO_2 in living animals in real time.[58]

Despite these potential advantages, the application of low frequency, *in vivo* EPR in biomedical and brain research has been very limited, partly because of the availability of highly specialized instrumentation (about a dozen exist around the world, as of today), and implantation of particulate spin probes or injection of soluble spin probes is minimally invasive. While temporal and spatial resolution in EPR imaging (EPRI) needs improvement, the use of soluble spin probes allows for mapping O_2 distribution *via* EPRI, achieved through the conversion of line width at each single pixel. Yet, overlay of EPRI with MRI is required to allow for anatomical confirmation of O_2 distribution since EPRI, alone, offers no anatomical designations. Moreover, a relatively new imaging modality, Overhauser-Enhanced MRI (OMRI) or proton-electron double-resonance imaging, based on the overhauser effect combines

the advantages of MRI with the sensitivity of EPR.[9,10,59] In the presence of a spin probe, MRI is recorded both with and without radiofrequency (RF) irradiation and the map of the difference of the two OMRI reconstructions is related to the linewidth of the probe which can used to evaluate tissue pO_2. High RF power and long RF irradiation times are required to obtain good signal-to-noise ratio (SNR), yet the method has been used to study redox status in the brain[60-63] and O_2 imaging in tissues,[57,61] suggesting the potential for cerebral pO_2 measurements.

Instrumentation for *in vivo* EPR Oximetry Studies

A detailed description of EPR instrumentation is available in literature.[10,19,21,64] Briefly, the most common EPR instrumentation operates at either the conventional EPR frequency (X-band, 9–10 GHz) or at low frequency EPR (L-band, 250 MHz–1.5 GHz) depending on the size of aqueous samples to measure. For X-band (9.8 GHz) EPR, the sample size (thickness) is limited to approximately 1 mm for aqueous (biological) samples, as otherwise it impinges upon areas in the cavity of higher electric field strength. However, a vast majority of "*in vivo*," *i.e.*, *ex vivo* detection of biological samples, such as blood, tissue extracts, and frozen thin tissue slices in liquid nitrogen, have been carried out using this type instrumentation since X-band EPR spectrometers are widely available and have excellent detection sensitivity.[64] Nevertheless, it is difficult to study living animals at X-band, and these spectrometers are not suitable to measure deep tissue pO_2, particularly in living animals and in real time.

EPR at lower frequencies, in the range of 250 MHz to 1.5 GHz, allows significantly larger aqueous samples to be studied with a depth of penetration of up to 30 mm, but at the expense of the detection sensitivity. The advantage to measurements in living animals includes: 1) real time and *in vivo* measurements; 2) the elimination of sample processing and handling procedures, avoiding the introduction of artifacts; and 3) more effective determinations of the specific pathway and role of ROS or tissue pO_2 than *in vitro* model systems.[64] Therefore, even with decreased detection sensitivity, low frequency *in vivo* EPR with direct detection in isolated organs, whole bodies of small animals, such as mice and rats, and larger animals, such as rabbits, is possible[17,65-68] and has provided direct information regarding the pathophysiology of many diseases in which free radicals and other paramagnetic species are involved. More importantly, the use of *in vivo* EPR to monitor various biologically important parameters, including highly accurate, minimally invasive and repeated measurements of deep tissue pO_2 would otherwise be very difficult, if possible at all, to achieve with any other existing methods.

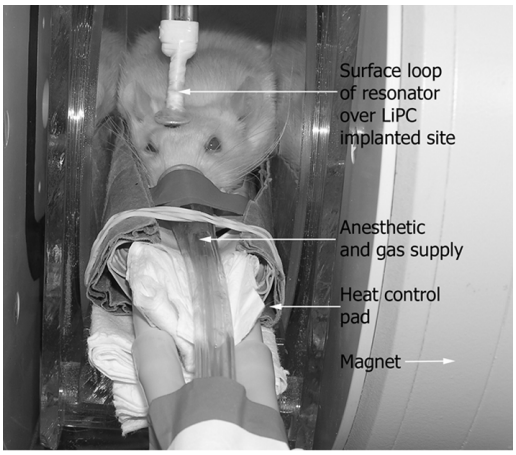


Figure 1: A picture of a rat placed between the magnets with a surface loop of resonator over its head for measurement of cerebral tissue oxygen (O_2) level.

Note: The electron paramagnetic resonance (EPR) oximetry probe, lithium phthalocyanine (LiPc), has been implant into the rat brain earlier. This EPR spectrometer operates at a microwave frequency of 1.2 GHz. Reproduced from Liu et al.[15]

While local availability or accessibility to low frequency EPR instrumentation has dictated or limited the use of EPR to measure tissue pO_2 *in vivo*, with advances in the past decade, more low frequency EPR spectrometers and imagers have become commercially available to allow for *in vivo* EPR oximetry. **Figure 1** shows a characteristic *in vivo* low frequency EPR spectrometer set up for EPR oximetry in a rat after implantation of the paramagnetic O_2-sensitive particulate probe, lithium phthalocyanine (LiPc). In a typical *in vivo* low frequency EPR oximetry experiment, the anaesthetised animals are administered spin probes *via* intraperitoneal (i.p.)/intravenous (i.v.) injection or *via* surgical implantation, followed by surgical procedure or challenge to the animal by drug or stimulus, *etc*. The animal is then inserted into a whole body resonator or under a surface coil in the cavity of the EPR, and EPR spectra are recorded. The majority of low frequency EPR spectrometers operate at about 1 GHz, which provides a good compromise between detection sensitivity and depth of microwave penetration.[64] However, a few laboratories have successfully used spectrometers operating around 250 MHz for *in vivo* EPR experiments.[67,69,70] Moreover, the first custom-designed clinical EPR spectrometer for human study was built at Dartmouth Medical School, Hanover, NH, USA and the initial results have since been reported.[71,72]

EPR Oximetry Probes and Applications in Brain Research

Advancements in low frequency EPR instruments com-

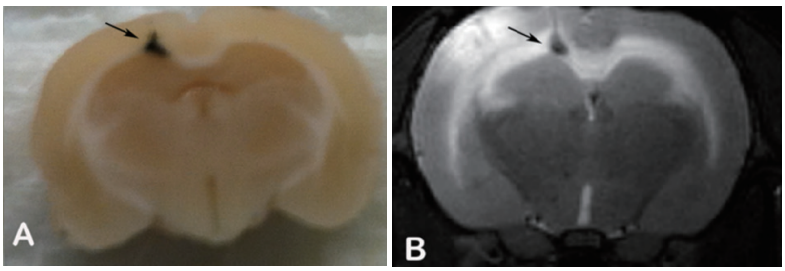
bined with recent progress in the development of novel O_2 sensitive EPR oximetry probes have established EPR oximetry as a versatile technique for sensitive, repetitive, reproducible, and relatively minimally invasive monitoring of cerebral pO_2, *in vivo*. For example, O_2-sensitive paramagnetic particulate or soluble spin probes can provide a method to monitor cerebral pO_2 at single sites or for mapping and imaging of cerebral pO_2, respectively. In the following sections, we will describe the use of EPR oximetry spin probes in *in vivo* EPR oximetry applications, particularly in the brain, using examples mostly from the author's laboratory.

Particulate probe EPR Oximetry

Paramagnetic O_2-sensitive particulates probes for *in vivo* EPR oximetry include the tiny solid crystal, LiPc, activated charcoal, bubinga char, india ink, LiPc derivatives of naththalocyanine, and more recently implantable resonators with multiple sensor loops loaded with LiPc. These insoluble particulate spin probes can be inserted surgically, injected as a powder, or fed to the animal;[25,56,57,73-76] however, LiPc has mainly been used to monitor cerebral pO_2. There remain some biocompatible concerns with prolonged exposure to particulates, but LiPc and other particulate probes are resistance to chemical reactions, have a high degree of inertness in biological systems, and are relatively non-toxic. Additionally, limited mobility of the probes makes it possible for highly accurate, minimally invasive and repeated single site measurements over both short and long periods of time (*e.g.*, days and weeks).[10,76] These desirable advantages are particularly beneficial when working with cerebral tissue; thus, it is possible to make repeated measurements without compromising the integrity of neural systems. Adequate cerebral pO_2 level is critical to neuron survival, and local changes in O_2 concentration can therefore markedly impact brain physiology, thus for various animal model studies of localized cerebral pO_2 at a single site, a stereotactic technique is used to implant LiPc at the predefined sites. Our lab has demonstrated that LiPc can make reliable cerebral pO_2 measurements in chronically implanted animals over a longitudinal study (up to 2 months) with no inflammation, tissue damage or adverse reaction to LiPc.[76] **Figure 2** demonstrates the ability to implant LiPc in the brain of small animals. The EPR spectrum is then obtained, and the line width of the spectrum is converted to tissue pO_2 through a linear calibration curve.

Recently, EPR oximetry and LiPc brain implantation has been used to directly investigate *in vivo* white matter (WM) cerebral pO_2 in a vascular cognitive impairment rat model for studying hypertensive and WM damage. Spontaneously hypertensive-stroke prone (SHR/SP) rats fed a high salt, low protein diet (Japanese permissive diet) along with unilateral carotid artery occlusion (JPD/UCAO) display WM injury pathology which resembles that seen in vascular cognitive impairment patients with small vessel disease, making the SHR/SP a relevant model for studying the human disease found in the elderly patients with hypertension-related WM hyperintensities.[77] However, in light of the significance of O_2 in brain physiology, cerebral pO_2 specifically in WM and related to WM injury had not been explored *in vivo*, due to the technical challenge of cerebral pO_2 measurements in the WM. EPR oximetry and LiPc brain implantation in the WM provided a unique and novel method for this measurement. We measured cerebral pO_2 in the WM of SHR/SP rats given JPD/UCAO at 12 weeks of age compared to SHR/SP rats fed a regular diet (**Figure 3**).[76] SHR/SP rats given JPD/UCAO demonstrated a significant reduction deep WM pO_2 to hypoxic levels up to 16 weeks when they died (**Figure 3B**). The continued decrease in pO_2 in the JPD/UCAO group was associated with vascular damage and death by 16 weeks, which contrasted with the normal histology in untreated rats. These findings are significant because the presence of hypoxia in the deep WM of SHR/SP rats may contribute to selective WM injury unrelated to frank ischemic infracts, suggesting that the vulnerability of patients with limited reserve capacity may have chronic or intermittent hypoxia secondary to the hypertensive vascular disease.

Additionally, EPR oximetry with LiPc implantation provides a unique opportunity to investigate *in vivo* and real time cerebral pO_2 in specific regions associated with



Note: (A) Coronal brain section showing the location of the LiPc crystal (arrow) in the white matter. (B) A representative *in vivo* MRI of an anesthetized rat implanted with LiPc crystal (arrow) in the white matter. Reproduced from Weaver et al.[76]

Figure 2: Representative 4.7T magnetic resonance imaging (MRI) (T2-weighted 2D RARE, rapid acquisition with relaxation enhancement) image and brain section of rat brain implanted with lithium phthalocyanine (LiPc) crystal.

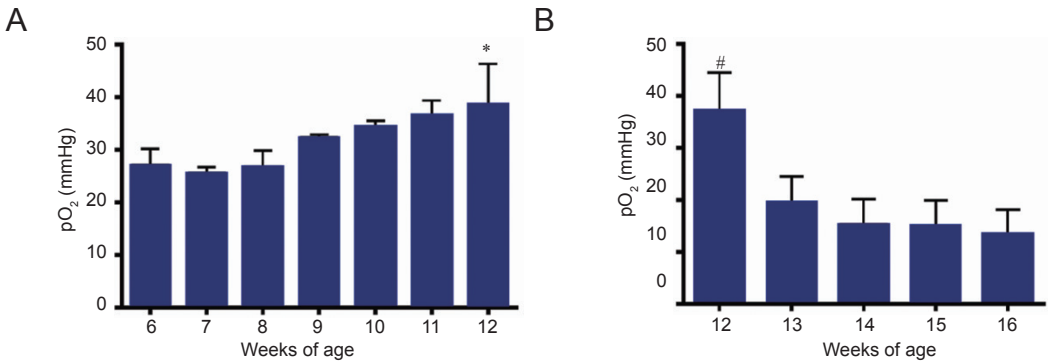


Figure 3: The change in interstitial pO_2 levels in the WM of SHR/SP rats post implantation from 6 to 12 weeks of age and JPD/UCAO from 12 to 16 weeks of age.

Note: LiPc crystal was implanted in the rat brain 48 hours before the first EPR measurement. EPR spectra were collected from anesthetized rats breathing 30% oxygen and converted to pO_2 (mmHg). (A) Interstitial pO_2 levels in WM of SHR/SP without surgery or diet from 6 to 12 weeks of age. (B) Interstitial pO_2 levels in the WM of JPD/UCAO rats after UCAO surgery with JPD from 12 to 16 weeks of age. Data are expressed as the mean ± standard error (SEM) (n = 8). *P < 0.05, *vs*. weeks 6, 7, and 8; #P < 0.05, *vs*. weeks 13 to 16. Reproduced from Weaver et al.[76] pO_2: Partial pressure of oxygen; SHR/SP: spontaneously hypertensive-stroke prone; JPD: Japanese permissive diet; UCAO: unilateral carotid artery occlusion; LiPc: lithium phthalocyanine; EPR: electron paramagnetic resonance; WM: white matter.

methamphetamine (METH) neurotoxicity in drug abuse mouse model. The mechanisms underlying METH-induced neurotoxicity are not fully understood, but several clinical and animal studies suggest that cerebral pO_2 may contribute. Thus, changes in cerebral pO_2 may play an important role and contribute to METH-induced oxidative stress and neurotoxicity.[78,79] Using EPR oximetry to measure cerebral pO_2 in the striatum *in vivo* and in real time, we demonstrated, for first time, that 20 minutes after a single injection of METH, striatal pO_2 was reduced significantly and consecutive acute METH exposure for 3 days further attenuated striatal pO_2, significantly. More importantly, striatal pO_2 did not fully recover even 24 hours after a single dose of METH and continual exposure to METH exacerbates the condition leading to a sustained and significant decrease in striatal pO_2.[80] Our findings are significant because results suggest that even one-time exposure to METH can attenuate cerebral pO_2, thus promoting ischemic insults and further supporting findings of potentially deleterious consequences to brain function and the development of stroke in young adults.

Moreover, unique capability of *in vivo* EPR oximetry and LiPc to measure localized interstitial pO_2, both absolute values and temporal changes of pO_2 in ischemic penumbra and core during ischemia and reperfusion highlight the importance of monitoring tissue pO_2 during acute ischemia stroke and NBO treatment.[74,75,81-85] For example, available data from relevant animal models demonstrate that NBO treatment, if applied for short duration, restores penumbra tissue back to or above preischemic levels without an increase ROS or oxidative stress, thus improving metabolic status and the survival of tissue, leading to neuroprotection against brain injury and blood-brain barrier (BBB) disruption.[81-83] Therefore, the potential that NBO is a viable neuroprotective strategy for acute ischemic stroke is compelling.[4] Furthermore, if two LiPc crystals are implanted in the ischemic core and the contralateral hemisphere respectively, the pO_2 can be followed at both the ischemic and non-ischemic (contralateral) sides simultaneously,[84] and more recently, an implantable resonator with multiple sensor loops loaded with LiPc at varying depths and positions allow for multisite determination of cerebral pO_2 at depths > 1 cm by EPR.[74,75,85]

These results show that EPR oximetry with LiPc can make accurate and repeated measurements of cerebral pO_2 in several neurological models, providing an important tool to study the role of O_2 in the pathophysiology of the brain. While, we have detailed the use of LiPc in the brain, the same approach has been used to study altered tissue oxygenation associated other diseases in other organs such as the kidney, liver and skeletal muscle,[57,86,87] highlighting the importance of investigating the role of O_2 in various tissues.

Soluble probe EPR oximetry

EPR oximetry can also be performed with soluble spin probes, which allow for mapping of O_2 using EPRI. While *in vivo* EPRI can be performed with particulates, such as imaging O_2 gradients in the gastrointestinal tract of a living mouse using charcoal,[10,66,88] soluble spin probes, such as nitroxides or trityls, are preferred for mapping O_2 in tissues and been used effectively for *ex vivo* and minimally invasive *in vivo* EPRI O_2 mapping applications, including the brain.[20-23,58,89,90] For example, EPRI of tumor-bearing legs of mice showed O_2 concentration images with a high spatial resolution (1 mm) and pO_2 (~3 mmHg), EPRI of

rat abdominal regions has revealed O_2 difference in the liver and kidneys, and EPRI oximetry has been used *ex vivo*, particularly for monitoring ischemic hearts. However, a major obstacle to the development of minimally invasive EPRI to quantitate cerebral pO_2 is the difficulty in delivering O_2-sensitive probes across the BBB into cerebral tissue. Trityl radicals are not particularly useful for measurement of cerebral pO_2 by EPRI because delivery to the cerebral tissue at sufficiently high concentrations is difficult. Therefore, BBB permeable labile-ester-containing nitroxides, with variable lipophilic, pharmacokinetic and pharmacodynamic properties offer the unique capability to be used to measure cerebral pO_2.[90-93] Esterase hydrolysis of BBB permeable nitroxide leads to the corresponding carboxy-nitroxide, and the anionic character of the carboxy-nitroxide, at physiologic pH, facilitates their entrapment and accumulation in cerebral tissue, and confers resistance to bioreduction. Thus, these probes: 1) cross the BBB; 2) have high O_2 sensitivity; 3) have low toxicity; 4) are retained at high concentration; and 5) are resistant to bio-reduction.

Administration of a soluble nitroxide to the animal for O_2 mapping of the brain can provide information over larger regions instead of a single site. For example, focal cerebral ischemia causes heterogeneous changes in tissue oxygenation with a region of decreased blood flow, the penumbra, surrounding a severely damaged ischemic core. To demonstrate the O_2 distribution in the ischemic brain, L-band EPRI, coupled with a blood-soluble BBB permeable nitroxide as the pro-imaging agent, is used to obtain a 2D spectral-spatial EPR image and map O_2 distribution in a focal cerebral ischemic mouse model (**Figure 4**).[58] **Figure 4A**, **B** shows O_2 concentration in the head before and after induction of focal cerebral ischemia and by comparing EPR and MR images (**Figure 4C**), the low-O_2 or hypoxic region in the EPR image matched well with the corresponding infarction area identified with the MR diffusion image. The spatial pO_2 profiles highlight the heterogeneous change in pO_2 values across the brain section and the distribution of tissue pO_2 values was centered at ~40 mmHg in the non-ischemic brain, whereas several loci in the ischemic brain had pO_2 < 20 mmHg (**Figure 4D**, **E**). These findings are significant, suggesting that the anoxic regions are smaller than the infarct area, perhaps offering a window of opportunity for therapeutic intervention aimed at correcting these intensely hypoxic foci.

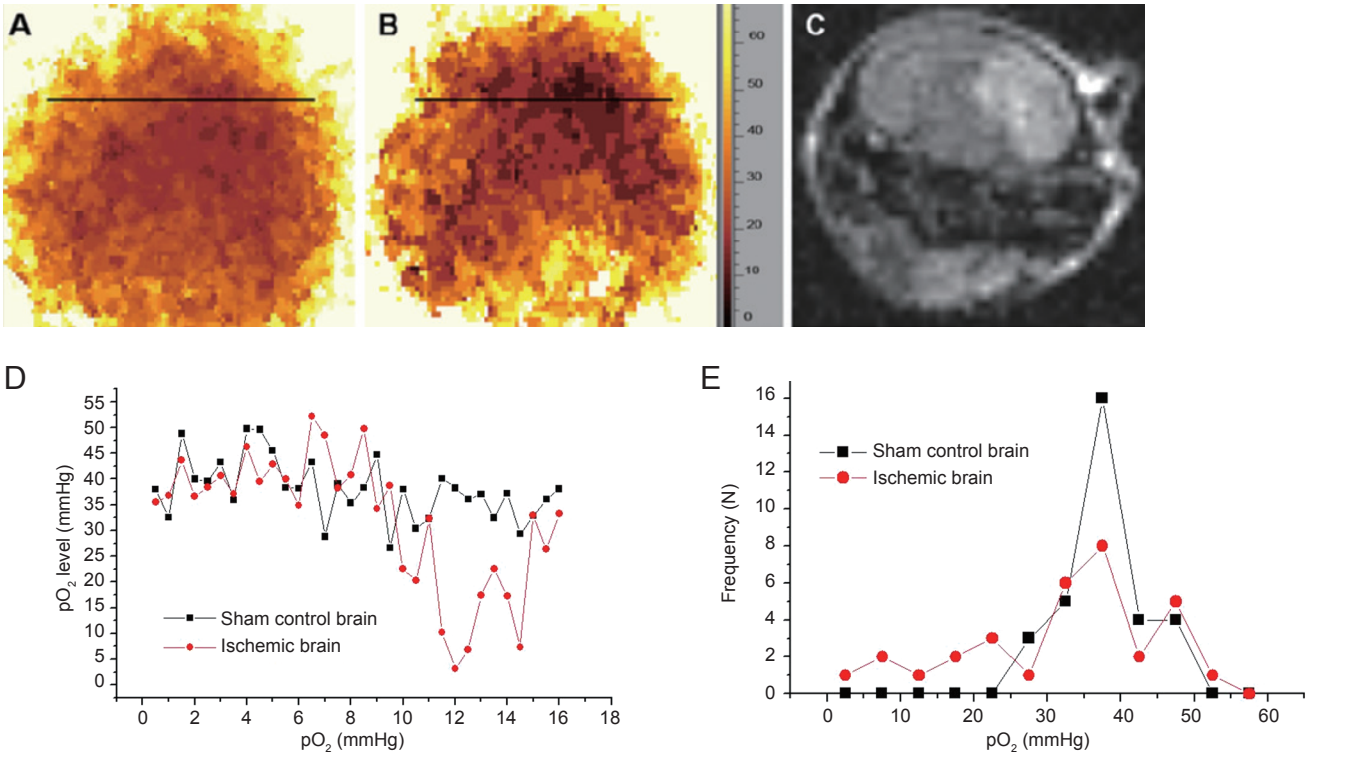


Figure 4: pO_2 distribution in sham control and ischemic mouse brain obtained by EPR 2D spectral-spatial linewidth imaging.
Note: (A) pO_2 map in the head of a sham control mouse head obtained by electron paramagnetic resonance imaging of the nitroxide. (B) pO_2 map in the head of the same mouse after ischemia. (C) MR diffusion image of the focal ischemic mouse head. (D) Representative spatial profiles of pO_2 in sham control and ischemic mouse brain. The pO_2 values were measured along the black lines shown in A and B. (E) Frequency of pO_2 values along the black lines shown in A and B. The results demonstrate that the hypoxic region of the pO_2 image matched well with the infarction area of the MR diffusion image. Reproduced from Shen et al.[58] pO_2: Partial pressure of oxygen; MR: magnetic resonance.

Additionally, these results demonstrate the usefulness of EPRI for O_2 mapping of brain, and that unique information is very difficult, if possible at all, to be obtained with any other current methods.

Furthermore, in an effort to improve O_2 quantitation by EPRI, ^2H,^{15}N-substituted nitroxide probes have been proven to be ~2.5-fold more sensitive for measuring pO_2 than their ^1H,^{14}N counterparts.[89,94-100] Complete isotopic substitution of ^1H,^{14}N (spin I = 1) with ^2H,^{15}N (spin I = 1/2) significantly reduces EPR signal linewidth; thereby, increasing the spectral peak intensity and sensitivity for measuring and mapping O_2. Proof-of-concept *in vitro* phantom spectral spatial EPR images demonstrate that ^2H,^{15}N-substituted nitroxides offers significant improvement in spectral resolution and reconstruction made possible by the higher SNR and ~2-fold narrower linewidth.[89,94,99] Additionally, similar to non-isotopic nitroxides, labile-ester-containing ^2H,^{15}N-substituted nitroxides can diffuse across the BBB to enter brain tissue, where it is entrapped for EPRI,[58,94] and we have demonstrated that *in vivo* O_2-dependent line broadening of the corresponding carboxy-^2H,^{15}N-substituted nitroxide after injection of the labile-ester-containing ^2H,^{15}N-substituted nitroxide is greater than that of its ^1H,^{14}N-nitroxides counterpart. At 1 GHz, the *in vivo* peak amplitude of the carboxy-^2H,^{15}N-substituted nitroxide is ~2-fold higher than non-isotopic version in the brain and this ~2-fold increase of *in vivo* peak amplitude significantly improves the sensitivity, resolution, reconstruction and spectral response to cerebral pO_2; thus, improving cerebral pO_2 mapping by EPRI.[94] These results establish that *in vivo* EPRI with ^2H,^{15}N-substituted nitroxide provides images with sufficiently short acquisition time and sufficiently high spatial resolution for *in vivo* O_2 quantification in the brain and motivates their use for superior and higher-resolution O_2 mapping in pathological states in the brain where O_2 levels are compromised brain compared to ^1H,^{14}N-nitroxides.

Future development of *in vivo* EPR oximetry

Studies discussed, herein, highlight the importance of investigating the role of O_2 in the brain, and continuous technical advancements in EPR instrumentation and development of O_2 sensitive spin probes which improve the spatial resolution and reduce data acquisition time in EPRI and promise more oximetry applications will follow. Currently, EPR oximetry images of the brain are acquired using 2D spectral-spatial imaging along a designated image plane which represents integral averages of pO_2 along the spatial axis (**Figure 4**) and not the absolute values in a particular spatial location. In order to obtain the complete spectral-spatial information *in vivo*, it is necessary to perform 3D spectral-spatial imaging. While, 3D spectral-spatial oximetry imaging has been demonstrated in isolated rat heart[21,101] and tumor oxygenation studies,[102,103] 3D spectral-spatial imaging is still challenging using commercially available EPR spectrometers due to poor image resolution and long acquisition times of several hours or more. However, recent developments in pulse and rapid scan techniques for EPR and EPRI have the potential to greatly reduce acquisition time and improvements in SNR ratio per unit time compared to more common EPR methods.[67,69,70,99,104,105] With future developments in rapid-scan techniques, fast magnetic field gradients and pulsed EPR methods, advancements in 3D functional EPR imaging may be feasible. Another potential shortcoming of EPRI is the fact that it does not provide any anatomical information, but efforts are underway to improve methods for co-registration of EPRI with MRI.[58,106] Co-registration of EPRI will allow determination of regional pO_2 by EPR and brain anatomical sites by MRI.

Additionally, while the use of EPR oximetry has been thoroughly used in laboratory animal models, advancements and investigations are ongoing for the translation of EPR oximetry to some clinical applications which may prove to be useful and effective.[57,72,107] One example is the use of EPR oximetry with india ink, the only currently approved material for human subjects, to measure the tissue pO_2 level in the tumor; thus, providing a critical guidance for radiation therapy in cancer patients as tumor pO_2 level plays a major role in the clinical outcome of radiation therapy. New probes are being developed, both superior in terms of sensitivity and biocompatibility, including safety enhancements for clinical studies, but realization for EPR oximetry in a wide-range of clinical studies, particularly the brain, is also challenged by EPR instrumentation and regulatory constraints.

Conclusions

Determination of O_2 in biology is of great interest and several techniques have been developed for the *in vivo* measurement of tissue pO_2. EPR oximetry is a promising method for monitoring this parameter conveniently and readily with significant advantages over other available techniques, including the capability of making repeated measurements from the same site in tissues, high sensitivity to changes to O_2, and ability of measuring absolute tissue pO_2 with minimal invasiveness. Hence, using EPR oximetry we are able to measure cerebral pO_2 at a single site or globally. A number of research groups are contributing to the development of *in vivo* EPR oximetry probes, EPR instrumentation, and methods for data collection and processing. With continued advancements in *in vivo* EPR,

it should become possible to carry out a large number of studies bearing on biological problems in which a significant role of O_2 has been postulated, helping to elucidate the pathogenesis of oxidative damage and to develop means to modify potentially damaging effects. We feel that there is a bright future for the use and development of *in vivo* EPR oximetry techniques. Specifically, novel EPRI with excellent *in vivo* O_2 sensors may be useful to map and quantify O_2 levels throughout the brain and other deep tissues, yielding important information regarding the pathophysiology of many diseases, which would not otherwise be elucidated in such a rigorous fashion.

Author contributions

JMW and KJL participated in the overall design of the review and wrote the manuscript. KJL edited, improved, participated in the overall design of the review and obtained funding. All authors have read and approved the final review.

Conflicts of interest

All figures were reproduced with permission from the original authors. The authors declare that they have no competing interests or conflicts of interests.

Plagiarism check

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