Published in final edited form as:

J Magn Reson. 2016 October ; 271: 68–74. doi:10.1016/j.jmr.2016.08.006.

***In vivo* EPR oximetry using an isotopically-substituted nitroxide: Potential for quantitative measurement of tissue oxygen**

John Weaver^{a,b,*}, Scott R. Burks^{c,d,e}, Ke Jian Liu^{a,b}, Joseph P.Y. Kao^{c,d,e}, and Gerald M. Rosen^{c,e,f}

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, United States

^bCenter of Biomedical Research Excellence, College of Pharmacy, University of New Mexico, Albuquerque, NM 87131, United States

^cCenter for Biomedical Engineering and Technology, University of Maryland School of Medicine, Baltimore, MD 21201, United States

^dDepartment of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201, United States

^eCenter for EPR Imaging In Vivo Physiology, University of Maryland, Baltimore, MD 21201, United States

^fDepartment of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD 21201, United States

Abstract

Variations in brain oxygen (O₂) concentration can have profound effects on brain physiology. Thus, the ability to quantitate local O₂ concentrations noninvasively *in vivo* could significantly enhance understanding of several brain pathologies. However, quantitative O₂ mapping in the brain has proven difficult. The electron paramagnetic resonance (EPR) spectra of nitroxides are sensitive to molecular O₂ and can be used to estimate O₂ concentrations in aqueous media. We recently synthesized labile-ester-containing nitroxides, such as 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **4**), which accumulate in cerebral tissue after *in situ* hydrolysis, and thus enable spatial mapping of O₂ concentrations in the mouse brain by EPR imaging. In an effort to improve O₂ quantitation, we prepared 3-acetoxymethoxycarbonyl-2,2,5,5-tetra(²H₃)methyl-1-(3,4,4-²H₃,1-¹⁵N)pyrrolidinyloxy (nitroxide **2**), which proved to be a more sensitive probe than its normo-isotopic version for quantifying O₂ in aqueous solutions of various O₂ concentrations. We now demonstrate that this isotopically substituted nitroxide is ~2-fold more sensitive *in vivo* than the normo-isotopic nitroxide **4**. Moreover, *in vitro* and *in vivo* EPR spectral-spatial imaging results with nitroxide **2** demonstrate significant improvement in resolution, reconstruction and spectral response to local O₂ concentrations in cerebral tissue. Thus, isotopic-substituted nitroxides, such as **2**, are excellent sensors for *in vivo* O₂ quantitation in tissues, such as the brain.

*Corresponding author at: University of New Mexico BRaIN Center, 1101 Yale NE, Albuquerque, NM 87131, United States. jmweaver@salud.unm.edu (J. Weaver).

Keywords

Labile-ester-containing nitroxides; ^{15}N -perdeuterionitroxide; Electron paramagnetic resonance; *In vivo* oxygen probes; Blood brain barrier; Brain oxygen

1. Introduction

Molecular oxygen (O_2) plays a central role in regulating a myriad of brain functions, including serving as the terminal electron acceptor for a broad spectrum of enzymes that catalyze the formation and metabolism of neurotransmitters, such as dopamine and serotonin. Local changes in O_2 concentrations can therefore markedly impact brain physiology. Surprisingly, in light of the significance of O_2 in brain physiology, O_2 measurement in brain with different imaging modalities remains problematic. For instance, blood-oxygen-level-dependent (BOLD) MR imaging provides only *qualitative* estimates of O_2 in tissues [1–3]. Thus, a noninvasive imaging method for real-time, *in vivo* O_2 quantitation in brain would be valuable to understand the role of O_2 in physiology and pathophysiology.

Molecular O_2 , being paramagnetic, broadens the electron paramagnetic resonance (EPR) spectral lines of other paramagnetic species, such as trityl radicals or nitroxides [2,4,5–9]. Measured changes in EPR spectral linewidth for these compounds have been used to estimate O_2 concentrations in homogenous solutions [2]. With developments in low-frequency EPR spectroscopy with imaging capability [10] and the appropriate molecular probes, it is now feasible to make reliable real-time estimations of local O_2 concentrations *in vivo* [11–20]. Despite the encouraging findings in [2], transporting O_2 -sensitive probes across the blood-brain barrier (BBB) has been problematic, when developing minimally invasive EPR imaging of O_2 in the brain.

In a series of papers, we reported a family of labile-ester-containing nitroxides [21–24] that can cross the BBB to enter brain tissue, where esterases hydrolyze the esters to the corresponding carboxylates. The anionic character of the resulting carboxynitroxides, such as **4** (Fig. 1), at physiologic pH facilitates their trapping and accumulation in brain tissue, and confers resistance to bioreduction [11]. We have previously achieved sufficient levels of entrapped nitroxides in cerebral tissue to allow quantitation of O_2 in different regions of the mouse brain following focal cerebral ischemia [4,12], while other groups have shown quantification of O_2 in discrete tissues or targeting tumors using similar spin probes [13–16,18,20].

We envisioned improving the quality of O_2 images by using isotopically-substituted nitroxides [15,17]. EPR imaging instrumentation measures the peak-to-peak height of one line in an EPR spectrum, thus wasting two-thirds of the EPR signal in the 3-line spectrum of an ^{14}N - nitroxide. Substitution of ^{14}N with ^{15}N increases the signal-to-noise ratio (SNR) by splitting the nitroxide EPR signal into only two spectral lines that are each 50% taller. Perdeuteration further improves SNR by reducing spectral line width and consequently increasing peak height. Narrower line widths also improve the fundamental spatial resolution of nitroxides by reducing the distance the spectral signal occupies after spatial

encoding by the magnetic field gradient [25–27]. Previously, we reported the synthesis of a ^{15}N -perdeuterio-nitroxide, **1**, and its acetoxymethyl (AM) ester, nitroxide **2** (Fig. 1) [27]. EPR linewidth measurements in aqueous solution showed that nitroxide **1** is ~3-fold more sensitive to O_2 than its normo-isotopic counterpart, nitroxide **3**. In the present study, we report that nitroxide **2** is a superior *in vivo* O_2 indicator, and we determine the extent to which isotopically-substituted nitroxide **1** may improve O_2 measurement in the brain of a mouse.

2. Material and methods

2.1. Nitroxides

3-(^2H)carboxy-2,2,5,5-tetra($^2\text{H}_3$)methyl-1-(3,4,4- $^2\text{H}_3$,1- ^{15}N)pyrrolidinyloxy (nitroxide **1**), 3-acetoxymethoxycarbonyl-2,2,5,5-tetra($^2\text{H}_3$)methyl-1-(3,4,4- $^2\text{H}_3$,1- ^{15}N)pyrrolidinyloxy (nitroxide **2**), 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **3**), and 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (nitroxide **4**) were synthesized as previously described [11,21,27].

2.2. Animals

Animal housing, care, and experimental protocols were approved and in accordance with the guidelines of Laboratory Animal Care and Use Committee of the UNM HSC. Male C57BL/6 mice, 16–20 g, were obtained from Charles River Laboratory (Wilmington, MA, USA). Mice were maintained in a climate-controlled vivarium with a 12 h light–dark cycle and free access to food and water.

2.3. In vitro 1 GHz (L-band) EPR spectroscopy

A solution of nitroxide **1** or **3** (100 μM in PBS) was placed in a gas-permeable Teflon tube (inside diameter: 0.813 mm; wall thickness: 0.038 ± 0.014 mm; Zeus Industries, Raritan, NJ, USA). The Teflon tube was folded into a “W” shape and fit into a quartz tube that was open at both ends; this assembly was inserted into an E540 surface probe resonator. The quartz tube was continually perfused with a gas mixture containing N_2 and different concentrations of O_2 (0%, 10%, 21%, and 95%). The O_2 concentration in the samples was constantly monitored with a PA-1B oxygen analyzer (Sable Systems, Las Vegas, NV). EPR spectra were recorded with a Bruker E540L L-band EPR spectrometer (Bruker BioSpin, Billerica, MA) with the following parameters: microwave frequency, 1.07 GHz; microwave power, 5.7 mW; center field, 387 G (for full spectrum) and 361 G, or 356 G; modulation frequency, 50 kHz; receiver gain, 70 dB; modulation amplitude, 0.1 G and 0.3 G for nitroxides **1** and **3**, respectively. For Fig. 2, linewidth and peak-to-peak amplitude were measured on the center peak in the 3-peak spectrum of nitroxide **3** and on the first peak of the 2-peak spectrum of nitroxide **1**.

2.4. In vivo 1 GHz (L-band) EPR spectroscopy

Male C57BL/6 mice (16–20 g) were from Charles River Laboratories, Inc. (Wilmington, MA, USA). Anesthesia was induced with 4% isoflurane inhalant in $\text{N}_2\text{O}/\text{O}_2$ (7:3) and maintained with 1% isoflurane in $\text{N}_2\text{O}/\text{O}_2$ (7:3). Animal core temperature was maintained at

37 °C with a heating pad or heat lamp. Nitroxides **2** and **4** were prepared as 50 mM solutions in PBS from a 0.5 M stock. Nitroxides were intravenously injected through the lateral tail vein at 0.4 mmol/kg body weight as previously described [4,28,29]. Prone positioned anesthetized mice (n = 5) were placed in a custom-made head holder for the small-animal head resonator of a Bruker E540L L-band EPR spectrometer. After placement of the mouse head in the center of the cavity, the resonator was tuned. Neither nitroxide caused obvious changes in animal behavior or any signs of acute toxicity. For each mouse, EPR spectra were recorded immediately after injection and instrument tuning, at different concentrations of respired measured O₂ (0%, 10%, 21%, and 95%). The O₂ concentration was constantly monitored with a PA-1B oxygen analyzer. Each acquired spectrum measures the total EPR signal in the mouse head, and is the sum of the signals from the injected nitroxides **2** and **4** and the hydrolytically-generated nitroxides **1** and **3**. The following EPR acquisition parameters were used: microwave frequency, 1.04 GHz; microwave power, 18 mW; center field, 361 G and 356 G for nitroxides **2** and **4**, respectively; modulation frequency, 50 kHz; receiver gain, 70 dB; modulation amplitude, 0.1 G and 0.3 G for nitroxides **2** and **4**, respectively. Because of instrumental tuning and optimization, 60–90 s typically elapsed between nitroxide injection and start of EPR spectral acquisition. EPR spectral linewidths and peak amplitudes were estimated by spectral fitting.

2.5. In vitro 1 GHz (L-band) EPR imaging

A phantom containing capillaries (1.20 ± 0.05 mm ID; 1.65 ± 0.05 mm OD) of nitroxide **2** or **4** (100 μ M in PBS) was placed in a custom holder for the small-animal head resonator of a Bruker E540L L-band EPR imaging system (Bruker BioSpin, Billerica, MA) equipped with 3D planar gradient sets. After placement of the phantom in the cavity, the resonator was tuned. Two-dimensional (2D) spectral-spatial images were acquired at room temperature with the same spectroscopic parameters as listed above and the following imaging parameters: gradient, 10 G/cm; spectrum width, 5 G; spectral resolution, 0.20833 G/p; pixel size, 0.5 mm; field of view, 12 mm; number of projections, 216; and acquisition time, 18 min. Other parameters were the same as for conventional 2D images. The 2D images were reconstructed by filtered back-projection and linewidth fitted using Bruker Xepr software. Peak-to-peak and Lorentzian derivative fitting gave the best linewidth image fit for nitroxide **2** and nitroxide **4** with a threshold of 1.699 and 0.020 determined by the back-projection, respectively.

2.6. In vivo 1 GHz (L-band) EPR imaging

Male C57BL/6 mice (16–20 g) were from Charles River Laboratories, Inc. (Wilmington, MA, USA). Anesthesia was induced with 4% isoflurane inhalant in N₂O/O₂ (7:3) and maintained with 1% isoflurane in N₂O/O₂ (7:3). Animal core temperature was maintained at 37 °C with a heating pad or heat lamp. Nitroxide **2** (1.95 mmole/kg body weight) was intraperitoneally (i.p.) administered into a mouse, 5 min prior to EPR acquisition as previously described [12]. Prone positioned anesthetized mouse was then transferred into the EPR cavity using a dedicated custom-made head holder for the small-animal-head resonator of a Bruker E540L L-band EPR imaging system equipped with 3D planar gradient sets. After positioning the mouse head in the center of the cavity, the resonator was tuned. EPR images were recorded immediately after thereafter, at different concentrations of respired

measured O₂ (21%, and 100%) during the experiments. The 2D spectral-spatial images were acquired with the same spectroscopy parameters as listed above and the following imaging parameters: gradient, 5 G/cm; spectrum width, 5 G; spectral resolution, 0.16667 G/p; pixel size, 0.5 mm; field of view, 15 mm; number of projections, 322; and acquisition time, 26.5 min. Other parameters were the same as for conventional 2D images. The 2D images were reconstructed by filtered back projection and linewidth fitted using Bruker Xepr software. Lorentzian derivative fitting (threshold of 0.214 (21%) and 0.170 (100%)) gave the best linewidth image fit for nitroxide **1**.

2.7. Calibration of EPR spectrometer response

To ensure consistency in measurements, the Bruker EPR spectrometer is calibrated daily before experiments. Because the spectrometer detects the total number of unpaired electrons within the resonant cavity, a standard sample of a stable free radical is used for daily calibrations. The standard is DPPH (2,2-diphenyl-1-picrylhydrazyl; supplied by Bruker BioSpin). Reproducible recording of spectral parameters (*e.g.* EPR signal intensity and linewidth) for the standard confirms proper spectrometer function.

2.8. Statistical analysis

OriginPro (OriginLab, Northampton, MA) was used for data analysis and presentation. Data are represented as mean value $\pm$ SD.

3. Results and discussion

We previously described the spectral characteristics of nitroxides **1** and **3** and the dependence of their EPR spectral linewidths as a function of O₂ concentration at 9.5 GHz [27]. Microwaves at this frequency penetrate tissue poorly, making *in vivo* EPR imaging impractical. Therefore, we initially characterized nitroxides **1** and **3** *in vitro* at 1 GHz, which is suitable for both *in vivo* spectroscopy and imaging in small animals. The EPR spectra of nitroxides **1** and **3** (as K⁺ salts, 100 μ M in air-equilibrated phosphate-buffered saline, PBS) are shown in Fig. 2. The peak-to-peak linewidth of nitroxide **1** was 0.54 G, compared to 1.18 G for nitroxide **3**. Moreover, the spectral peak amplitude of nitroxide **1** was ~2.5-fold greater than that of nitroxide **3**.

Fig. 3 shows the dependence of the EPR linewidth on O₂ concentration for nitroxides **1** and **3**. With varying O₂ concentration, the relative change in linewidth is much greater for nitroxide **1** than for nitroxide **3**. Linear least-squares fits of these data show that the line-broadening effect of O₂ is 2.88-fold greater for nitroxide **1** than for nitroxide **3**. Therefore, nitroxide **1** is more O₂-sensitive than nitroxide **3** and should be able to resolve smaller changes in O₂. As expected, at 1 GHz, the spectral characteristics of nitroxides **1** and **3**, and their relative responsiveness to O₂ are comparable to our previous findings and for other similar spin labels at 9.5 GHz [26,27,30].

We next assessed the utility of nitroxides **1** and **3** as an O₂ sensor *in vivo* in proof-of-concept experiments. Mice (n = 5) were intravenously injected with the AM ester pro-imaging agents (nitroxides **2** and **4**). As demonstrated previously [4,12,28,29], nitroxide **4** (at analogous

concentrations) can cross the BBB and be hydrolyzed to nitroxide **3**, which becomes entrapped in cerebral tissue with an *in vivo* $T_{1/2}$ of ~20–30 min dependent on injection route and 50% retention; thus, allowing sufficient amount of nitroxide to be retained in the brain tissue for quantitation of O_2 without interference from concentration-dependent linewidth self-broadening [4]. We anticipate nitroxide **2** to exhibit similar characteristics. After intravenous injection with nitroxide **2** or **4**, the head of the mouse was positioned in the cavity of an L-band EPR spectrometer, and the mouse respired N_2 mixed with various concentrations of O_2 for up to 60 min. EPR spectral data were acquired, and the dependence of the linewidth of nitroxides **1** and **3** on respired $[O_2]$ was measured *in vivo*. Peak-to-peak linewidth values obtained by spectral fitting are plotted in Fig. 4A and B. The dependence of nitroxide EPR spectral linewidth on respired $[O_2]$ was nonlinear and was fit with a single exponential (Fig. 5). It can then be estimated that the O_2 concentration required for half-saturation of the sensor response was $[O_2]_{1/2} = 15.9 \pm 5.2 \%O_2$ for nitroxide **3** and $[O_2]_{1/2} = 36.3 \pm 2.6 \%O_2$ for nitroxide **1**. This suggests that nitroxide **1** is >2-fold more sensitive than nitroxide **3** as an O_2 sensor.

While the relationship between nitroxide EPR spectral linewidth and O_2 concentration in aqueous solution is linear, the observed nonlinear relation between nitroxide linewidth and respired O_2 concentration *in vivo* is not unexpected, and can be rationalized on the basis of O_2 homeostasis. We expect that line broadening at higher respired O_2 concentrations would be less than that observed at the same O_2 concentration *in vitro*, because of limited local bioavailability of O_2 in cerebral tissue. Even as the respired O_2 concentration approaches 100%, cerebral tissue O_2 would not track respired O_2 because of the high rate of brain O_2 consumption, and because of reduced delivery of O_2 caused by hyperoxia-induced vasoconstriction [31]. Analogously, we expect *in vivo* linewidths at hypoxic concentrations to be less than those observed at the same nominal concentration *in vitro*. During isocapnic hypoxia (decrease in pO_2 without change in pCO_2), vasodilation occurs leading to increased cerebral blood flow in an attempt to minimize the effects of hypoxia on brain tissue oxygenation [31,32].

We have demonstrated that O_2 -dependent line broadening of nitroxide **1** is greater than that of nitroxide **3** *in vivo*. This suggests that nitroxide **1** is a superior probe for quantitative EPR oximetry. The EPR spectral linewidths at 1 GHz are also substantially reduced in isotopically-substituted nitroxides (0.54 G for nitroxide **1** vs 1.18 G for nitroxide **3** in air-equilibrated aqueous solutions, Fig. 2). This should enable acquisition of EPR images of greater spatial resolution than possible with non-isotopically substituted nitroxides. Magnetic resonance images are acquired in the presence of a magnetic field gradient, and the intrinsic linewidth of the probe is a limiting factor in achieving high spatial resolution. This is especially true of EPR imaging with nitroxides, whose intrinsic linewidths are relatively large (1.1 G or up to several parts per thousand of $B_0 = 356$ G). We therefore performed proof-of-concept *in vitro* phantom spectral spatial EPR images of nitroxides **4** and **2** as shown in Fig. 6. The dramatic increase in EPR spectral-spatial intensity image of nitroxide **2** compared to **4** (Fig. 6B and C), and corresponding representative linewidth image (Fig. 6D and E) demonstrate the significant improvement in spectral resolution and

reconstruction made possible by the higher SNR and ~2-fold narrower linewidth of nitroxide **2**.

It is clear that nitroxide **2** offers significant improvement in linewidth EPR imaging as fitting from projection data showed significantly reduced image artifacts and improved background, i.e. a zero background is observed and only nitroxide-containing tubes are observed in the reconstructed linewidth EPR image (Fig. 6E). Under present experimental conditions, the ~2-fold narrower linewidth of nitroxide **1** compared to nitroxide **3** should result in significant improvement in *in vivo* spatial resolution of EPR images. Thus, nitroxide **2** was injected into mice to observe *in vivo* images of nitroxide **1** (after esterase hydrolysis of nitroxide **2**) [4,12,29] in the brain of a mouse breathing different O₂ concentrations (21%, and 100%) (Fig. 7). As expected, when nitroxide **4** was used for the same experiment, the time required for acquiring EPR spectral-spatial images of adequate quality increased significantly (data not shown), which is less ideal for *in vivo* imaging. These results establish that *in vivo* EPR imaging with ¹⁵N-perdeuterionitroxide provides images with sufficiently short acquisition time (~27 min) and sufficiently high spatial resolution to permit accurate mapping and quantitation of pO₂ in the brain. Of importance was the observation that when the percentage of O₂ in the inspired gas was increased from 21% to 100%, a corresponding increase can be observed in the *in vivo* EPR images indicating that pO₂ levels significantly increased in the brain.

4. Conclusions

Nitroxide **1** has spectral characteristics that make it better suited for *in vivo* EPR applications. Currently, EPR spectrometers measure signal intensity as the peak-to-peak amplitude of a single spectral line. Because full isotopic substitution (¹⁵N and deuterium) of the present nitroxides has the net effect of increasing peak amplitude by ~2.5-fold, a corresponding improvement in SNR is expected relative to non-isotopically-substituted nitroxides. At 1 GHz, the *in vitro* peak amplitude of nitroxide **1** was 2.5-fold larger than that of nitroxide **3** (Fig. 2). Correspondingly, the *in vivo* peak amplitude of nitroxide **1** was ~2-fold higher than that of nitroxide **3**. This ~2-fold increase of *in vivo* peak amplitude significantly improves the sensitivity and resolution of O₂ mapping in the brain. This proof-of-principle demonstration that isotopically-substituted nitroxides, such as nitroxide **1**, are superior for *in vivo* O₂ quantification in the brain motivates their use for superior and higher-resolution O₂ mapping in pathological states such as ischemia [12,33], cognitive vascular impairment [34] and methamphetamine abuse [35–37], where O₂ levels are compromised. While these studies present evidence of tissue pO₂ as a potential important initiation event that culminates in oxidative stress, leading to subsequent injuries [33–37], inserted O₂ electrodes or single-site EPR oximetry, used in these studies, does not account for other regions of the brain that may likewise experience similar or more severe changes in O₂. Novel EPR imaging with excellent *in vivo* O₂ sensors may be useful to map and quantify O₂ levels throughout the brain; thus, yielding important information regarding the pathophysiology of many diseases, which would not otherwise be elucidated in such a rigorous fashion. These efforts are under way in our laboratory.

Acknowledgments

This research was supported in part by National Institutes of Health grants P30-RR-031156 (JW and KJL), R21DA023473 (JW, KJL and GMR), P41-EB-2034 (GMR) and GM-56481 (JPYK). We thank Dr. Gary Fiskum for a discussion of the *in vivo* effect of O₂.

References

1. Baudelet B, Gallez B. How does blood oxygen level-dependent (BOLD) contrast correlate with oxygen partial pressure (pO₂) insider tumors. *Magn. Reson. Med.* 2002; 48:980–986. <http://dx.doi.org/10.1002/mm.1038>. [PubMed: 12465107]
2. Elas M, Williams BB, Parasca A, Mailer C, Pelizzari CA, Lewis MA, River JN, Karczmar GS, Barth ED, Halpern HJ. Quantitative tumor oxymetric images from 4D electron paramagnetic resonance imaging (EPRI): methodology and comparison with blood oxygen level-dependent (BOLD) MRI. *Magn. Reson. Med.* 2003; 49:682–691. <http://dx.doi.org/10.1002/mm.10408>. [PubMed: 12652539]
3. Matsumoto K, Bernardo M, Subramanian S, Choyke P, Mitchell JB, Krishna MC, Lizak MJ. MR assessment of changes of tumor in response to hyperbaric oxygen treatment. *Magn. Reson. Med.* 2006; 56:240–246. <http://dx.doi.org/10.1002/mm.20961>. [PubMed: 16795082]
4. Shen J, Liu S, Miyake M, Liu W, Pritchard A, Kao JPY, Rosen GM, Tong Y, Liu KJ. Use of 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy as an EPR oximetry probe: potential for *in vivo* measurement of tissue oxygenation in mouse brain. *Magn. Reson. Med.* 2006; 55:1433–1440. <http://dx.doi.org/10.1002/mm.20894>. [PubMed: 16680679]
5. Kutala VK, Parinandi NL, Pandian RP, Kuppusamy P. Simultaneous measurement of oxygenation in intracellular and extracellular compartments of lung microvascular endothelial cells. *Antioxid. Redox Signal.* 2004; 6:597–603. <http://dx.doi.org/10.1089/152308604773934350>. [PubMed: 15130286]
6. Shen J, Khan N, Lewis LD, Armand R, Grinberg O, Demidenko E, Swartz H. Oxygen consumption rates and oxygen concentration in molt-4 cells and their mtDNA depleted (rho0) mutants. *Biophys. J.* 2003; 84:1291–1298. [http://dx.doi.org/10.1016/S0006-3495\(03\)74944-3](http://dx.doi.org/10.1016/S0006-3495(03)74944-3). [PubMed: 12547809]
7. Khan N, Shen J, Chang TY, Chang CC, Fung PC, Grinberg O, Demidenko E, Swartz H. Plasma membrane cholesterol: a possible barrier to intracellular oxygen in normal and mutant CHO cells defective in cholesterol metabolism. *Biochemistry.* 2003; 42:23–29. <http://dx.doi.org/10.1021/bi026039t>. [PubMed: 12515536]
8. Liu S, Timmins GS, Shi H, Gasparovic CM, Liu KJ. Application of *in vivo* EPR in brain research: monitoring tissue oxygenation, blood flow, and oxidative stress. *NMR Biomed.* 2004; 17:327–334. <http://dx.doi.org/10.1002/nbm.899>. [PubMed: 15366032]
9. Gallez B, Bacic G, Goda F, Jiang J, O'Hara JA, Dunn JF, Swartz HM. Use of nitroxides for assessing perfusion, oxygenation, and viability of tissues: *in vivo* EPR and MRI Studies. *Magn. Res. Med.* 1996; 35:97–106.
10. Halpern HJ, Spencer DP, van Polen J, Bowman MK, Massoth RJ, Nelson AC, Dowey EM, Teicher BA. An imaging radiofrequency electron spin resonance spectrometer with high resolution and sensitivity for *in vivo* measurements. *Rev. Sci. Instrum.* 1989; 60:1040–1050. <http://dx.doi.org/10.1063/1.1140314>.
11. Pou S, Davis PL, Wolf GL, Rosen GM. Use of nitroxides as NMR contrast enhancing agents for joints. *Free Rad. Res.* 1995; 23:353–364. <http://dx.doi.org/10.3109/10715769509065256>.
12. Shen J, Sood R, Weaver J, Timmins GS, Schnell A, Miyake AM, Kao JPY, Rosen GM, Liu KJ. Direct visualization of mouse brain oxygen distribution by electron paramagnetic resonance imaging: application to focal cerebral ischemia. *J. Cereb. Blood Flow Metab.* 2009; 29:1695–1703. <http://dx.doi.org/10.1038/jcbfm.2009.89>. [PubMed: 19675560]
13. For a review on this topic, see: Swartz HM, Sentjurs M, Kocherginsky N. Nitroxide Spin Labels: Reactions in Biology and Chemistry. 1995 Boca Raton CRC Press:175–198.
14. Redler G, Barth EG, Bauer KS Jr, Kao JPY, Rosen GM, Halpern HJ. *In vivo* EPR imaging of differential tumor targeting using cis-3,4-di(acetoxymethoxycarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy. *Magn. Res. Med.* 2014; 71:1650–1656. <http://dx.doi.org/10.1002/mrm.24813>.

15. Bacic G, Walczak T, Demsar F, Swartz HM. Electron spin resonance imaging of tissues with lipid areas. *Magn. Res. Med.* 1988; 8:209–219.
16. Kuppusamy P, Shankar R, Zweier JL, et al. In vivo measurement of arterial and venous oxygenation in the rat using 3D spectral-spatial electron paramagnetic resonance imaging. *Phys. Med. Biol.* 1998; 43:1837–1844. [PubMed: 9703045]
17. Hyodo F, Matsumoto S, Devasahayam N, Dharmaraj C, Subramanian S, Mitchell JB, Krishna MC. Pulsed EPR imaging of nitroxides in mice. *J. Magn. Reson.* 2009; 197:181–185. <http://dx.doi.org/10.1016/j.jmr.2008.12.018>. [PubMed: 19157932]
18. Halpern HJ, Peril M, Nguyen T-D, Spencer DP, Teicher BA, Lin YJ, Bowman MK, et al. Selective isotopic labeling of a nitroxide spin label to enhance sensitivity for T₂ oxymetry. *J. Magn. Res.* 1969; 1990(90):40–51. [http://dx.doi.org/10.1016/0022-2364\(90\)90364-F](http://dx.doi.org/10.1016/0022-2364(90)90364-F).
19. Halpern HJ, Peric M, Yu C, Bales BL. Rapid quantitation of parameters from inhomogeneously broadened EPR spectra. *J. Magn. Res. Ser. A.* 1990; 103:13–22. <http://dx.doi.org/10.1006/jmra.1993.1125>.
20. Halpern HJ, Yu C, Peric M, Barth E, Grdina DJ, Teicher BA, et al. Oxymetry deep in tissues with low-frequency electron paramagnetic resonance. *Proc. Natl. Acad. Sci. USA.* 1994; 91:13047–13051. [PubMed: 7809170]
21. Kao JPK, Rosen GM. Esterase-assisted accumulation of 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl into lymphocytes. *Org. Biomol. Chem.* 2004; 2:99–102. <http://dx.doi.org/10.1039/B310467B>. [PubMed: 14737666]
22. Rosen GM, Burks SR, Kohr MJ, Kao JPY. Synthesis and biological testing of aminoxyls designed for long-term retention by living cells. *Org. Biomol. Chem.* 2005; 3:645–648. <http://dx.doi.org/10.1039/B415586F>. [PubMed: 15703801]
23. Burks SR, Ni J, Muralidharan S, Coop A, Kao JPY, Rosen GM. Optimization of labile esters for esterase-assisted accumulation of nitroxides into cells: a model for in vivo EPR imaging. *Bioconjug. Chem.* 2008; 19:2068–2071. <http://dx.doi.org/10.1021/bc800222e>. [PubMed: 18783260]
24. Burks SR, Makowsky MA, Yaffe ZA, Hogg C, Tsai P, Muralidharan S, Bowman MK, Kao JPY, Rosen GM. The effect of structure on nitroxide EPR spectral linewidth. *J. Org. Chem.* 2010; 75:4737–4741. <http://dx.doi.org/10.1021/jo1005747>. [PubMed: 20540511]
25. Liu Y, Villamena FA, Song Y, Sun J, Rockenbauer A, Zwier JL. Synthesis of ¹⁴N and ¹⁵N-labeled trityl nitroxide biradicals with strong spin-spin interaction and improved sensitivity to redox status and oxygen. *J. Org. Chem.* 2010; 75:7796–7802. <http://dx.doi.org/10.1021/jo1016844>. [PubMed: 21028905]
26. Khan N, Blinco JP, Bottle SE, Hosokawa K, Swartz HM, Micallef AS. The evaluation of new and isotopically labeled isoindoline nitroxides and an azaphalene nitroxide for EPR oximetry. *J. Magn. Reson.* 2011; 211:170–177. <http://dx.doi.org/10.1016/j.jmr.2011.05.007>. [PubMed: 21665499]
27. Burks SR, Bakhashai J, Makowsky MA, Muralidharan S, Tsai P, Rosen GM, Kao JPY. ²H, ¹⁵N-Substituted nitroxides as sensitive probes for electron paramagnetic resonance imaging. *J. Org. Chem.* 2010; 75:6463–6467. <http://dx.doi.org/10.1021/jo1011619>. [PubMed: 20828113]
28. Miyake M, Shen J, Liu S, Shi H, Liu W, Yuan Z, Pritchard A, Kao JPY, Liu KJ, Rosen GM. Acetoxymethoxycarbonyl nitroxides as EPR pro-imaging agents to measure O₂ levels in mouse brain: a pharmacokinetic and pharmacodynamic study. *J. Pharmacol. Exp. Therap.* 2006; 318:1187–1193. <http://dx.doi.org/10.1124/jpet.106.106245>. [PubMed: 16757536]
29. Miyake M, Burks SR, Weaver J, Tsai P, Liu W, Bigio D, Bauer KS, Liu KJ, Rosen GM, Kao JPY. Comparison of two nitroxides labile esters for delivering electron paramagnetic resonance probes into mouse brain. *J. Pharm. Sci.* 2010; 99:3594–3600. <http://dx.doi.org/10.1002/jps.22102>. [PubMed: 20201101]
30. Beth AH, Venkataramu SD, Balasubramanian K, Dalton LR, Robinson BH, Pearson DE, Park CR, Park JH. ¹⁵N- and ²H-substituted maleimide spin labels: improved sensitivity and resolution for biological EPR studies. *Proc. Natl. Acad. Sci. USA.* 1981; 78:967–971. [PubMed: 6262786]
31. Hlatky R, Valadka AB, Gopinath SP, Robertson CS. Brain tissue oxygen tension response to induced hyperoxia reduced in hypoperfused brain. *J. Neurosurg.* 2008; 108:53–58. <http://dx.doi.org/10.3171/JNS.2008.108.01.0053>. [PubMed: 18173310]

32. Severinghaus, JW. Cerebral circulation at altitude. In: Schoene, THR., editor. High Altitude: An Exploration of the Human Adaptation. Vol. 161. New York: Marcel Dekker; 2001. p. 343-375.
33. Liu S, Shi H, Liu W, Furuichi T, Timmins GS, Liu KJ. Interstitial pO₂ in ischemic penumbra and core are differentially affected following transient focal cerebral ischemia in rats. *J. Cereb. Blood Flow Metab.* 2004; 24:343–349. <http://dx.doi.org/10.1097/01.WCB.0000110047.43905.01>. [PubMed: 15091115]
34. Weaver J, Jalal FY, Yang Y, Thompson J, Rosenberg GA, Liu KJ. Tissue oxygen is reduced in white matter of spontaneously hypertensive-stroke prone rats: a longitudinal study with electron paramagnetic resonance. *J. Cereb. Blood Flow Metab.* 2014; 34:890–896. <http://dx.doi.org/10.1038/jcbfm.2014.35>. [PubMed: 24549186]
35. Kousik SM, Graves SM, Napier TC, Zhao C, Carvey PM. Methamphetamine-induced vascular changes lead to striatal hypoxia and dopamine reduction. *NeuroReport.* 2011; 22:923–928. <http://dx.doi.org/10.1097/WNR.0b013e32834d0bc8>. [PubMed: 21979424]
36. Shiba T, Yamato M, Kudo W, Watanabe T, Utsumi H, Yamada K. *In vivo* imaging of mitochondrial function in methamphetamine-treated rats. *Neuroimage.* 2011; 57:866–872. <http://dx.doi.org/10.1016/j.neuroimage.2011.05.041>. [PubMed: 21624473]
37. Weaver J, Yang Y, Purvis R, Weatherwax T, Rosen GM, Liu KJ. *In vivo* evidence of methamphetamine induced attenuation of brain tissue oxygenation as measured by EPR oximetry. *Toxicol. Appl. Pharmacol.* 2014; 275:73–78. <http://dx.doi.org/10.1016/j.taap.2013.12.023>. [PubMed: 24412707]

Fig. 1.
Chemical structures of nitroxides used in this study.

Fig. 2. L-band EPR spectra of nitroxide **1** (solid line) and **3** (dashed line) in air-equilibrated phosphate-buffered saline. Peak-to-peak linewidths and hyperfine splittings are indicated.

Fig. 3.

Relative changes in linewidth of nitroxides **1** and **3** in aqueous solution equilibrated with different concentrations of O_2 (0, 10, 21 or 95%) at 1 GHz. Solid lines are least-squares fits of the data; the slope of each line is indicated on the graph. Where not shown, error bars are smaller than the symbols ($n = 5$).

Fig. 4.

Changes in peak-to-peak EPR linewidths of nitroxides **1** and **3** in the mouse brain. (A) Nitroxide **2** was injected into mice (i.v., 0.4 mmol/kg body weight; n = 5). (B) Nitroxide **4** was injected into mice (i.v., 0.4 mmol/kg body weight; n = 5). EPR spectra were acquired as mice inhaled different concentrations of O₂ (0, 10, 21 and 95% balance is N₂).

Fig. 5. Relative changes in EPR linewidth of nitroxides **1** and **3** in the mouse brain. Nitroxide **2** or **4** was injected into mice (i.v., 0.4 mmol/kg body weight; $n = 5$ for each nitroxide). EPR spectra were acquired as mice inhaled different concentrations of O_2 (0, 10, 21 and 95% balance is N_2). Solid lines are least-squares fit of the function, $y = (ax + b)/(x + b)$, to the data. The O_2 concentration required for 50% saturation of the response, $[O_2]_{1/2}$, is indicated for each curve.

Fig. 6.

In vitro 2D spectral-spatial L-band EPR imaging of nitroxides **2** and **4**. (A) A photograph of the phantom comprising capillary tubes, some of which contain air-equilibrated nitroxide solution. (B) 2D spectral-spatial intensity image of capillary phantom containing nitroxide **4**. (C) 2D spectral-spatial intensity image of capillary phantom containing nitroxide **2**. Note that (B) and (C) have the same intensity scale. (D) 2D spectral-spatial linewidth image of phantom containing nitroxide **4**. (E) 2D spectral-spatial linewidth image of phantom containing nitroxide **2**. Note that (D) and (E) have the same linewidth scale. Scale bar = 1 mm.

Fig. 7.

In vivo 2D spectral-spatial L-band EPR images of pO₂ distribution in the mouse brain. (A) The 2D spectral-spatial linewidth image of nitroxide **1** in the brain of a mouse breathing a gas mixture containing 21% O₂. (B) The 2D spectral-spatial linewidth image of nitroxide **1** in the brain of a mouse breathing 100% O₂. The prone-mouse was placed head first into the custom-made head holder for the EPR small-animal head resonator and L and R labels on the image refers to left and right hemispheres, respectively. Scale bar = 1 mm.