J|A|C|S
COMMUNICATIONS

Published on Web 05/19/2007

Trityl Radicals as Persistent Dual Function pH and Oxygen Probes for in Vivo Electron Paramagnetic Resonance Spectroscopy and Imaging: Concept and Experiment

Andrey A. Bobko, Ilirian Dhimitruka, Jay L. Zweier, and Valery V. Khramtsov*

Dorothy M. Davis Heart & Lung Research Institute, The Ohio State University, Columbus, Ohio 43210

Received March 3, 2007; E-mail: valery.khramtsov@osumc.edu

Local pH and oxygen concentration are among the most important parameters in the biochemistry of living organisms. In certain stress conditions, for example, high exercise levels, interruption of normal blood supply or biochemical shock, the oxygen supply and body's ability to regulate pH, at least locally, may be compromised. A reasonable depth of penetration of magnetic field in living tissues makes NMR and low-field electron paramagnetic resonance (EPR) based techniques the most appropriate approaches for noninvasive in vivo oxygen and pH assessment. Several NMR techniques for $[O_2]$ measurement have been described, among them ^{19}F NMR spectroscopy/imaging using perfluorocarbon emulsions[1,2] and fluorinated nitroimidazoles.[3] However, spin−lattice relaxation rates of fluorinated probes may depend on other physiological or histological parameters.[2] For in vivo pH measurements, ^{31}P NMR has proven to be the most suitable noninvasive approach. However pH assessments using ^{31}P NMR and inorganic phosphate, P_i, have their own limitations, including lack of resolution, the fact that P_i concentrations vary with metabolism and ischemia, and the chemical shifts dependence on ionic strength.[4] Because of these problems, exogenous pH probes are being designed for NMR spectroscopy to improve detection of tissue acidity.[5]

Exogenous EPR probes have certain advantages over exogenous NMR probes owing to the much higher intrinsic sensitivity of EPR for the same probe concentration. Among soluble paramagnetic materials, two types of structures are particularly interesting: nitroxyl radicals, NRs, and triarylmethyl radicals, TAMs. NRs were the first paramagnetic probes used for EPR oximetry[6−9] and still have advantages in variability of structure, solubility, and ability to be targeted. A wide set of pH-sensitive NRs have been developed and used for real time pH monitoring both in vitro and in vivo.[10−12] However, comparatively fast reduction of the NRs to EPR-silent hydroxylamines limits their applications, particularly for EPR oxygen and pH mapping.

The recent development of TAMs[13] has significantly expanded the potential for using oxygen-sensitive free radicals in EPR, EPR imaging, and related techniques such as dynamic nuclear polarization. Their extreme stability toward tissue redox processes gives TAM radicals an important advantage over NRs. In human blood, the stability of TAMs varies from a half-life of a few hours to more than 24 h depending on the particular structure of the compound.[13] These compounds were first developed for biomedical applications by Nycomed Innovation.[13,14] General synthesis of these types of products has also been recently described.[15] The EPR spectrum of these compounds displays one very narrow single line with the line width being linearly dependent on the O_2 concentration. Presently, applications of TAM radicals are limited to EPR oximetry and their recently reported sensitivity to the superoxide anion.[16]

In this paper, we describe a general concept allowing for enhanced functionality of the TAM radicals. We hypothesize that TAMs containing ionizable groups may demonstrate pH sensitive magnetic resonance parameters similar to the previously reported phenomenon for pH-sensitive NRs. At the same time TAMs have the advantages of being highly sensitive EPR oxygen probes with excellent stability in living tissues. To test this hypothesis, we analyzed the EPR spectra of the previously described TAM, Oxo63, derivative containing a carboxyl group[9] (Scheme 1).

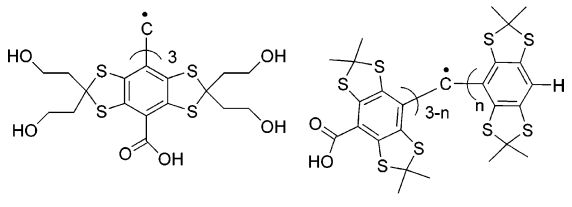
The EPR spectrum of Oxo63 is represented by a single line with a line width less than 0.1 G in the absence of oxygen. A reversible shift of the EPR line position was observed in acidic medium (see Figure 1) in agreement with reversible deprotonation of the COOH group. The value of the line shift is proportional to an EPR frequency equal to 0.26 G for 10 GHz X-band and 0.81 G for 34 GHz Q-band spectra, which are characteristic for changes in *g*-factor. The values of *g*-factors for neutral RH and deprotonated, R^-, forms of the radical were found to be equal to 2.00329 and 2.00315, correspondingly (see Supporting Information).

The low pK_a value of the Oxo63 derivative limits its application for pH measurements to a range from 2 to 4, which still could be useful, for example, for studies of stomach acidity.[11] However, another limitation of this particular pH probe is its frequency-dependent pH shift, which becomes impractically small at low-field (about 30 mG at L-band). pH-Sensitive nitroxides keep their sensitivity at low-field EPR owing to a frequency-independent pH effect on the hyperfine splitting, a_N.[12] Winged TAM radicals,[14] in part, were designed to eliminate numerous hyperfine splittings of the original triphenyl radical[18] which appear because of the spin density delocalization on the protons of three phenyl rings. Apparently, there's not a problem to leave a limited number of hyperfine splittings in pH-sensitive TAM derivatives by keeping or adding atoms with nonzero nuclear spin such as hydrogen, nitrogen, or phosphorus. To optimize the EPR peak intensity, a probe with the minimum number of EPR lines and pH sensitive distance between them would be ideal. To illustrate the concept we synthesized TAM derivatives containing carboxyl groups, $cTAM_n$ (Scheme 1), according to a modified general synthetic approach.[15,17] The special feature of the $cTAM_1$ and $cTAM_2$ is the presence of one hydrogen atom in one or two of their phenyl rings while in the symmetrical analogues, $cTAM_0$ or Oxo63, these protons are substituted by carboxyl groups. The corresponding EPR spectra of Oxo63, $cTAM_0$, $cTAM_1$, and $cTAM_2$ are shown in Figure 2, left, representing single line, doublet, and triplet patterns. Significant line width broadening of individual components of the EPR spectra (0.3−0.5 G) was observed for all the radicals after oxygenation of the solutions in agreement with previous data on oxygen sensitivity of Oxo63[9] and $cTAM_0$.[17] In acidic pH all four compounds underwent reversible EPR spectral shift, ΔH (see Figure 2, right), owing to corresponding changes of *g*-factor.

Figure 3 represents the pH effect on the hydrogen hyperfine splitting, a_H, of $cTAM_1$ and $cTAM_2$ measured by X-band EPR

10.1021/ja071515u CCC: $37.00

Scheme 1. The Chemical Structures of TAMs Containing Carboxyl Groups[a]



Oxo63 $cTAM_n$ (n = 0, 1, 2)

[a] Synthesis of the $cTAM_1$ and $cTAM_2$ were previously unreported and are given in Supporting Information.

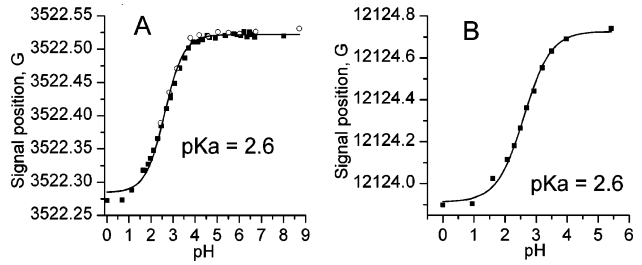


Figure 1. pH Dependence of the EPR spectral line position of 50 μM solution of Oxo63 TAM in 1.5 mM sodium citrate buffer measured by X-band (A) and Q-band (B) EPR spectroscopy. To enhance the accuracy of the measured EPR signal position, an internal standard, 1 mM TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) solution, was used (X-band) or high precision frequency measurements were performed (Q-band). The symbols ■ and ○ denote the data obtained upon titration from alkaline to acidic pH and acidic to alkaline pH, respectively.

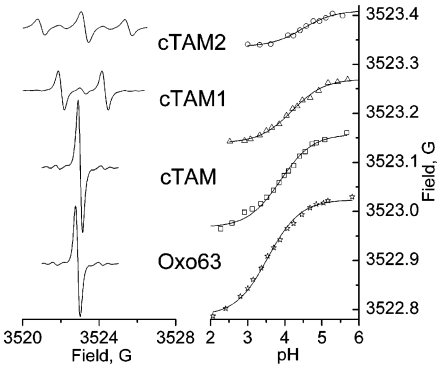


Figure 2. (Left) X-band EPR spectra of 10 μM solutions of $cTAM_n$ and Oxo63. The EPR spectra titration was performed in 25% aqueous ethanol to avoid the limited solubility of $cTAM_n$ in RH form at low pH. (Right) The pH dependences of the position of the spectrum center measured relative to the internal standard, TEMPOL nitroxide. Note that ΔH changes proportionally increase with the number of COOH groups, ranging from 84 mG for $cTAM_2$, 130 mG for $cTAM_1$, 190 mG for $cTAM_0$, and 237 mG for Oxo63. Solid curves are the best fits to titration equations, yielding observed pK_a values equal to 4.5, 4.2, 3.9, and 3.5 for $cTAM_2$, $cTAM_1$, $cTAM_0$, and Oxo63, respectively. Note that the shift of pK_a(Oxo63) from 2.6 in aqueous solution to 3.5 in 25% ethanol is a well-known polarity effect.[19]

spectroscopy. Similar to the pH-induced *g*-factor change, the pH effect on a_H is proportional to the number of carboxyl groups, being about twice as large for $cTAM_1$. While the absolute a_H changes are comparatively small, in accordance with the long distance between the COOH group and the H-atom, which are located in different phenyl rings, the data supports the possibility of using the hyperfine splitting parameter of TAM derivatives as a pH marker. Taking into account the frequency-independent character of this parameter, this makes it possible to use TAM derivatives for in vivo EPR pH measurements using low-field EPR-based techniques. The low pK_a of the TAM derivatives with carboxyl

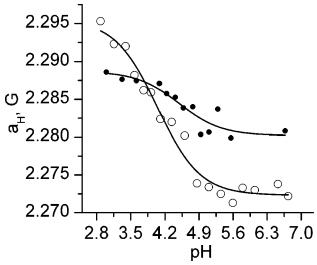


Figure 3. The dependence of a_H on pH for $cTAM_1$ (○) and $cTAM_2$ (●) radicals. Solid lines are the best fit to titration equations yielding pK_a = 4.1 and 4.5, and Δa_H = 23 mG and 8 mG for the radicals $cTAM_1$ and $cTAM_2$, respectively.

groups does not allow them to be used in a physiologically relevant pH range, around pH 7.0. Other ionizable groups, protonatable or deprotonatable, must be considered to obtain TAM derivatives with higher pK_a values. The primary TAM candidates to be tested are derivatives with −OH, $-CH_2-SH$, and CH_2-NH_2, substitutes instead of −COOH.

In summary, the obtained data support the concept of TAM radicals as dual pH and oxygen probes for in vivo EPR spectroscopy and imaging.

Acknowledgment. This work was partly supported by NIH Grants KO1 EB03519 and GM 075036 and CRDF Grant RUC1-2635-NO-05. The authors thank Drs. C. M. Hadad, F. A. Villamena, and A. Samouilov for helpful discussions and Dr. C. F. Hemann for technical assistance.

Supporting Information Available: Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Clark, L. C., Jr.; Ackerman, J. L.; Thomas, S. R.; Millard, R. W.; Hoffman, R. E.; Pratt, R. G.; Ragle-Cole, H.; Kinsey, R. A.; Janakiraman, R. *Adv. Exp. Med. Biol.* **1984**, *180*, 835−45.
(2) Mason, R. P.; Rodbumrung, W.; Antich, P. P. *NMR Biomed.* **1996**, *9* (3), 125−34.
(3) Aboagye, E. O.; Kelson, A. B.; Tracy, M.; Workman, P. *Anticancer Drug Des.* **1998**, *13* (6), 703−30.
(4) Gillies, R. J.; Alger, J. R.; den Hollander, J. A.; Shulman, R. G. In *Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions*; Nuccitelli, R., Deamer, D. W., Eds.; Alan R. Liss: New York, 1982; pp 79−104.
(5) Pietri, S.; Martel, S.; Culcasi, M.; Delmas-Beauvieux, M. C.; Canioni, P.; Gallis, J. L. *J. Biol. Chem.* **2001**, *276* (3), 1750−8.
(6) Backer, J. M.; Budker, V. G.; Eremenko, S. I.; Molin, Y. N. *Biochim. Biophys. Acta* **1977**, *460* (1), 152−6.
(7) Glockner, J. F.; Norby, S. W.; Swartz, H. M. *Magn. Reson. Med.* **1993**, *29* (1), 12−8.
(8) Halpern, H. J.; Yu, C.; Peric, M.; Barth, E.; Grdina, D. J.; Teicher, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91* (26), 13047−51.
(9) Krishna, M. C.; English, S.; Yamada, K.; Yoo, J.; Murugesan, R.; Devasahayam, N.; Cook, J. A.; Golman, K.; Ardenkjaer-Larsen, J. H.; Subramanian, S.; Mitchell, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (4), 2216−21.
(10) Khramtsov, V. V.; Grigor'ev, I. A.; Foster, M. A.; Lurie, D. J.; Nicholson, I. *Cell. Mol. Biol.* **2000**, *46* (8), 1361−74.
(11) Potapenko, D. I.; Foster, M. A.; Lurie, D. J.; Kirilyuk, I. A.; Hutchison, J. M.; Grigor'ev, I. A.; Bagryanskaya, E. G.; Khramtsov, V. V. *J. Magn. Reson.* **2006**, *182* (1), 1−11.
(12) Khramtsov, V. V. *Curr. Org. Chem.* **2005**, *9*, 909−923.
(13) Ardenkjaer-Larsen, J. H.; Laursen, I.; Leunbach, I.; Ehnholm, G.; Wistrand, L. G.; Petersson, J. S.; Golman, K. *J. Magn. Reson.* **1998**, *133* (1), 1−12.
(14) Anderson, S.; Golman, K.; Rise, F.; Wikström, H.; Wistrand, L.-G. U.S. Patent 5,530,140, 1996.
(15) Reddy, T. J.; Iwama, T.; Halpern, H. J.; Rawal, V. H. *J. Org. Chem.* **2002**, *67* (14), 4635−9.
(16) Kutala, V. K.; Parinandi, N. L.; Zweier, J. L.; Kuppusamy, P. *Arch. Biochem. Biophys.* **2004**, *424* (1), 81−8.
(17) Xia, S.; Villamena, F. A.; Hadad, C. M.; Kuppusamy, P.; Li, Y.; Zhu, H.; Zweier, J. L. *J. Org. Chem.* **2006**, *71* (19), 7268−79.
(18) Gomberg, M. *J. Am. Chem. Soc.* **1900**, *22*, 757−71.
(19) Khramtsov, V. V.; Marsh, D.; Weiner, L.; Reznikov, V. A. *Biochim. Biophys. Acta* **1992**, *1104* (2), 317−24.

JA071515U