Published in final edited form as:

Free Radic Biol Med. 2009 September 1; 47(5): 654–658. doi:10.1016/j.freeradbiomed.2009.06.007.

Trityl-based EPR probe with enhanced sensitivity to oxygen

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Abstract

An asymmetric derivative of triarylmethyl radical, TAM-H, containing one aldehyde and two carboxyl groups was synthesized. The electron paramagnetic resonance, EPR, spectrum of TAM-H is characterized by a doublet of narrow lines with linewidth of 105 mG in anoxic conditions and hyperfine interaction constant 245 mG. The partial overlap of the components of the doublet results in enhanced sensitivity of the spectral amplitudes ratio to oxygen compared with oxygen-induced linewidth broadening of a single line. Application of the TAM-H probe allows for EPR measurements in an extended range of oxygen pressures from atmospheric to 1 mmHg whereas the EPR spectrum linewidth of the popular TAM-based oxygen sensor, Oxo63, is practically insensitive to oxygen partial pressures below 20 mmHg. Enhanced sensitivity of TAM-H probe relative to Oxo63 was demonstrated in detection of oxygen consumption by Met-1 cancer cells. The TAM-H probe allowed prolonged measurements of oxygen depletion during the hypoxia stage and down to true anoxia (≤ 1.5 mmHg).

Keywords

trityl; TAM; oximetry; water soluble oxygen probes; EPR; electron paramagnetic resonance

Introduction

Oxygen concentration is one of the most crucial parameters in living tissues, organs and the body. Hence, monitoring and imaging of oxygen concentration distribution and variation with time is very important for diagnostic and treatment purposes in biology and medicine. A reasonable depth of penetration of magnetic field in living tissues makes magnetic resonance based techniques an appropriate approach for noninvasive oxygen concentration assessment [1,2]. Electron paramagnetic resonance (EPR) based techniques for spectroscopy and imaging of oxygen possess high intrinsic sensitivity to O_2 but require exogenous spin probes with stability in biological milieu. There are two kinds of spin probes which can be used for oxygen concentration monitoring by EPR. Paramagnetic particles (lithium phthalocyanine [3], India ink [4], gloxy [5], etc.) are very persistent in tissues, non toxic and can be easily implanted and monitored over several weeks or even months and show high sensitivity to oxygen (down to 0.1–0.01 mmHg [2]). However, application of paramagnetic particles is limited in the

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monitoring of oxygen concentration to the point of implantation. Water soluble paramagnetic oxygen probes are more suitable for imaging of tissues, organs or the full body. At present triarylmethyl (TAM) and nitroxyl radicals (NRs) are among the most promising soluble probes for EPR-based oximetry [1,2,5–9]. The disadvantage of the nitroxides is their fast reduction in biological milieu to their corresponding EPR-silent products, hydroxylamines [10]. At the same time, TAM radicals demonstrate excellent stability in the presence of various reducing and oxidizing agents [11] and in biological fluids [2]. Moreover, EPR spectra of “winged” TAM radicals [12,13] provide a significant improvement in signal-to-noise ratio due to their very narrow single line (linewidth ≈ 0.1 G) compared with the much broader lines (linewidth ≈ 1 G) of the triplet EPR spectrum of NRs. Unfortunately, synthetic chemistry of TAM radicals is not excessively developed which hampers the EPR oximetry application of these spin probes. Creative efforts have been undertaken for the synthesis of these complex molecules [14–16]. Fluorinated TAMs possessing a high affinity to fluorinated media were specially designed [17] for assessment of tumor oxygenation using biocompatible perfluorocarbon emulsions. Recently we developed a series of dual function pH and oxygen probes [18,19] and esterified probes for intracellular targeting [16,20]. In this paper we describe synthesis and properties of a new TAM-based EPR spin probe with an extended range of oxygen pressures sensitivities from atmospheric pressure to 1 mmHg.

Materials and Methods

Chemicals

DMEM (Dulbecco's Modified Eagle Media) media was purchased from Gibco Invitrogen, Carlsbad, CA. FBS was obtained from Atlanta Biologicals, Lawrenceville, GA. rhEGF was bought from R&D Systems, Inc. Recombinant Human Insulin (Novolin R) was obtained from Novo Nordisk Inc, Princeton, NJ. TAM Oxo63 probe was a gift from Nicomed Innovations (Malmö, Sweden).

Synthesis of TAM derivative, TAM-H

The TAM-H was synthesized according to the Scheme 1. Compound **1** was synthesized as previously described [19]. Compound **2** was synthesized efficiently, with 90 % yield by treatment with multiple equivalents of a 0.3 M Dess-Martin periodine solution in dichloromethane [21,22]. Hydrolysis of compound **2** with a solution of potassium hydroxide, followed by treatment with neat trifluoroacetic acid afforded the asymmetric TAM-H radical (see Scheme 1). Unless otherwise noted all syntheses were carried out under an argon atmosphere (see Appendix A for the details).

Calibration of EPR spectral sensitivity of TAM-H and Oxo63 probes to oxygen

TAM-H (50 μ M) and Oxo63 (100 μ M) in DMEM buffer solution were placed in teflon tubes with a diameter of 1.14 mm and wall thickness of 60 μ m (Zeus Inc, USA) and EPR spectra were recorded under nitrogen-oxygen atmosphere at 37 °C. Gas composition and temperature were controlled by Temperature and Gas Controller (Noxygen, Germany) attached to the EMX EPR spectrometer (Bruker, Germany) which allows for gas mixing accuracy ± 1 mm Hg in the range of oxygen partial tension from 0 to 25 mmHg. Peak-to-peak width values of Oxo63 single line spectra and peak intensity values of TAM-H doublet spectra were calculated after EPR spectra smoothing using adjacent-averaging method by Origin 7.5 program. Values of the linewidth and ratio of line intensities (I_{in}/I_{out} , see Figure 1) were calculated as average mean of three measurements for each oxygen concentration.

Studies of oxygen consumption kinetics by Met-1 cells

Suspensions of Met-1 cells ($4.5 \cdot 10^6$ cells/ml) in DMEM buffer in the presence of Oxo63 (100 μ M) or TAM-H (50 μ M) were placed in 50 μ L glass capillaries sealed afterwards to avoid oxygen penetration from the atmosphere. Glass capillary was placed into an EPR spectrometer and probe spectra were recorded at 37 °C. Temperature was maintained using Temperature and Gas Controller (Noxygen, Germany). The values of oxygen partial pressure were calculated using calibration curves.

Met-1 cell culture

Met-1 tumor cells (a cell line derived from tumor-bearing MMTV-PyMT mice) were cultured in DMEM media containing 10% heat-denatured FBS, 10 μ g/ml insulin, and 5 ng/ml rhEGF until 80% confluent. These cells were trypsinized, washed, and resuspended in DMEM media at 5×10^6 cells per 1 ml media.

Results

Scheme 2 shows chemical structures of the well-known TAM-based oxygen probes, Oxo63, and newly synthesized TAM-H radical. The EPR spectra of Oxo63 and TAM-H are represented by a single line and doublet, correspondingly (Figure 1). The hyperfine splitting (hfs) constant in the EPR spectrum of TAM-H radical (245 mG) originates from a single proton nucleus in the aldehyde group attached to the aryl ring of the asymmetric trytil molecule. In the absence of oxygen peak-to-peak linewidths of Oxo63 and TAM-H probes were found to be equal to 164 ± 1 mG and 105 ± 1 mG, correspondingly.

The partial overlap of the components of the doublet spectrum of TAM-H results in enhanced sensitivity of its spectral shape to oxygen compared with Oxo63 as clearly seen from Figure 1. In particular, the peak intensity ratio, I_{in}/I_{out} exhibits higher sensitivity to oxygen than linewidth, in part, due to the fact that the ratio of EPR signal amplitudes is proportional to the square of the ratio of EPR signal widths.

Figure 2 shows dependences on oxygen partial pressure of the EPR peak-to-peak linewidth of Oxo63 probe and EPR signal ratio I_{in}/I_{out} of TAM-H probe. The EPR linewidth of Oxo63 does not change significantly at oxygen partial pressures below 20 mmHg while I_{in}/I_{out} spectral ratio of TAM-H retains its sensitivity to oxygen down to 1 mmHg. At oxygen pressures above 20 mmHg linewidth of EPR spectrum of Oxo63 linearly depends on $[O_2]$ allowing for accuracy of oxygen pressure detection ± 2.5 mmHg (slope of linear fit is equal to 0.42 mG/mmHg whereas accuracy of linewidth measurement is ± 1 mG). TAM-H probe provides significantly higher sensitivity and accuracy of oxygen measurements, namely oxygen partial pressure can be measured continuously from 1 to 160 mmHg with an accuracy no less than ± 1 mmHg which is comparable with the accuracy of the gas mixture preparation (see Materials and Methods).

Figure 3 represents the kinetics of oxygen consumption measured by EPR in a suspension of Met-1 cells. Application of Oxo63 probe allowed us to follow oxygen depletion down to 20 mmHg which corresponds to normoxia while the probe became oxygen insensitive below the hypoxia threshold (about 15 mmHg [23]). At the same time, application of the TAM-H probe allowed us to follow oxygen consumption during an extra 20 minutes of increased hypoxia (about 1.5 – 15 mmHg [23]) and even to detect a threshold of true anoxia (≤ 1.5 mmHg).

It should be noted that the introduction of an aldehyde group in the structure of the TAM-H radical did not compromise its stability compared with Oxo63 or “Finland” [19] TAM derivatives. There were no notable changes in the EPR spectra of TAM-H during a 3 hour incubation in aqueous solutions, pH 7.0, in the presence of one hundred time excess of glutathione (10 mM) or arginine (10 mM) supporting the evidence of inactivity of the aldehyde

group of TAM-H toward compounds containing amino or thiol functions (data not shown). There were also no significant changes in EPR spectra intensity during at least one hour incubation in reducing medium, namely in the presence of 10 mM of ascorbic acid at pH 7 or in the presence of cells (as in the samples used to generate data in Figure 3).

Discussion

EPR oximetry is one of the most promising and rapidly developing techniques for measurement of oxygen in living tissues [24]. While oxygen in its triplet ground state demonstrates strong X-band EPR signal in the gas phase of 120 lines at low pressure [25], no EPR spectra have ever been reported for dissolved oxygen due to line broadening. Hence, biological EPR oximetry relies on Heisenberg exchange between exogenous paramagnetic probe and a stable diradical molecule of oxygen. Because of pure physical interaction between the probe and oxygen, the EPR oximetry method does not interfere with oxygen metabolism providing a basis for noninvasive oxygen measurements in biological systems, including those *in vivo*.

Nitroxides were the first soluble paramagnetic probes used for EPR oximetry [7,26–28]. However, bioreduction of the NRs into EPR-silent products and comparatively broad spectral lines complicate their applications, particularly for EPR oxygen mapping. In this respect, TAM radicals developed for biomedical applications by Nycomed Innovation [12], have the advantage of extraordinary stability *in vivo*. The oxygen-induced broadening of the TAMs in water is about 300–500 mG/mM of oxygen [13] similar to that for the NRs while the EPR linewidth of the TAMs, about 100 mG, is on average one order of magnitude lower than EPR linewidths of the nitroxides. On the other hand, the concentration broadening of the TAMs is about 10 mG/mM [13] which is one order of magnitude less than that for the NRs (about 100–200 mG/mM [29]). These properties make TAM radicals superior oximetric probes for EPR oximetry applications [13,30].

The measurements of oxygen tension in living tissues are of crucial importance for monitoring the energetic metabolism from a physiological and pathological point of view. In certain stress conditions, e.g. high exercise levels, interruption of normal blood supply or biochemical shock, the oxygen homeostasis, at least locally, may be compromised. The effectiveness of cancer therapy is affected by the oxygenation status of normal and tumor tissues [31,32]. Oxygenation status of ischemic tissue in stroke and myocardial infarction is critically important for clinical application. Tissues maintain normoxic oxygen levels within a range from 100 mmHg in arterial blood to about 15 mmHg in perivenous tissues and renal medulla [33]. Strongly exercised muscle in mammal may even become essentially anoxic with $pO_2 \leq 1.5$ mmHg where mitochondrial respiration is choked off [34]. Therefore, real-time measurement of oxygen status at oxygen pressures below 15 mmHg is extremely important to identify hypoxic areas.

EPR measurements of oxygen-induced line broadening both of the nitroxyl and TAM radicals provide reasonable sensitivity at oxygen pressure above 15 mmHg. However, the peak-to-peak linewidths of these probes become rather oxygen-insensitive in the hypoxia range below 15 mmHg (see Figure 1A and 2A for Oxo63). The similar “threshold” in sensitivity of the peak-to-peak linewidth of TAM radical to oxygen concentration was previously shown (see ref. [13], fig. 10). The origin of this “threshold” is due to the existence of oxygen-independent unresolved hyperfine structure described by Gaussian lineshape contribution. Therefore at low oxygen concentrations, oxygen-dependent variations in the peak-to-peak linewidth became significantly lower than the Gaussian linewidth and are masked within unresolved hyperfine pattern. The observed “threshold” at 20 mmHg of oxygen corresponds to about 8 mG oxygen-induced broadening contribution which is about 10 % of the Gaussian linewidth (about 100 mG [19]).

Application of perdeuterated nitroxides [35–38] or, alternatively, measurement of “the depth of resolution” of the superhyperfine structure of the NRs [26,27,39,40] provides enhanced sensitivity to oxygen. However, among the drawbacks of the both approaches is overlapping line broadening effects induced by respective variations of oxygen and nitroxide concentrations. Typically, the line-broadening effect of the NR concentration on its linewidth is about 100–200 mG/mM, and may interfere with accurate oxygen measurements at high NR concentrations [29]. Therefore, application of these NRs in *in vivo* systems using low-field EPR can be complicated by the need to limit probe concentration (ideally < 100 μ M) and modulation amplitude, which compromise spectral intensity.

Halpern et al. [41] proposed the application of a selectively deuterated NR with only one hydrogen superhyperfine splitting in order to discriminate the contributions of variations in oxygen and NR concentrations on EPR line-shape. Variations in both oxygen and radical concentrations affect the “the depth of resolution” of the doublet spectrum characterized by the ratiometric parameter similar to the ratio I_{in}/I_{out} shown in Figure 2B for the doublet spectrum of TAM-H probe. Additionally, increases in spin label concentration but not oxygen resulted in narrowing of hydrogen hyperfine splitting allowing for discrimination between these two factors and accurate quantification of the oxygen concentration in tumor tissue in living mice with the accuracy ± 8 mmHg [7].

In this paper we described synthesis of new trityl radical, TAM-H, with partially overlapped doublet EPR spectrum and its superior properties for oxygen measurements, particularly at low oxygen tension. TAM-H demonstrates an advantage over single line TAM probes in sensitivity to oxygen (see Figure 1 and Figure 2) similar to its nitroxide ancestor [7]. The ratiometric parameter I_{in}/I_{out} (Figure 1B) is a more oxygen sensitive parameter than linewidth, in part, due to the fact that the ratio of EPR signal amplitudes is proportional to square of ratio of EPR signal widths. TAM-H probe provides significantly higher sensitivity and accuracy of oxygen measurements than another popular TAM-based oxygen sensor, Oxo63, allowing for oxygen partial pressure measurements down to 1 mmHg with an accuracy no less than ± 1 mmHg. This superior oxygen sensitivity of TAM-H probe is well demonstrated by monitoring oxygen consumption in cell suspensions (Figure 3). Application of TAM-H probe allowed to follow oxygen depletion during hypoxia (≤ 15 mmHg [23]) and even to detect a threshold of true anoxia (≤ 1.5 mmHg) where the Oxo63 probe was oxygen insensitive below 20 mmHg.

The chemical structure of TAM-H probe contains benzaldehyde group, which may affect its stability in the presence of strong reducing or oxidizing agents. However, TAM-H probe was found to be stable in aqueous solutions, in the presence of mild oxidant (oxygen) and biologically relevant reducing agents (glutathione and ascorbate) and in the presence of cell culture.

Limited aqueous solubility of “Finland” type TAM radicals makes difficult their *in vivo* applications. Recently we reported that “Finland” TAM has an aggregation tendency at pH below 4.0 initiated by protonation of carboxyl group [15]. An increase of carboxyl group pK_a in compartments with low dielectric constants [18], e.g. biomembranes, will further aggravate aggregation problems and related toxicity of the TAM. Therefore, the development of TAM-H derivatives based on more hydrophilic structures, such as Oxo63, may be important for elaboration of nontoxic probes with enhanced oxygen sensitivity.

In summary, the synthesized TAM-H probe possesses the highest oxygen sensitivity among reported soluble paramagnetic probes, and provides a unique noninvasive tool for the oxygen quantification at low oxygen tension, including that during ischemia and anoxia in biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was partly supported by grants from NIH (KO1 EB03519, EB0490, EB00890, R21 CA132068, R21 HL091423 and R21 EB009433).

Appendix

Appendix A

Supplementary data.

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Abbreviations

TAM, triarylmethyl radical; EPR, electron paramagnetic resonance; NR, nitroxyl radical.

Figure 1.

EPR spectra of aqueous solutions of 100 μM Oxo63 (A) and 50 μM TAM-H (B) radicals measured at 37 $^{\circ}\text{C}$ and various oxygen concentrations. The spectra are scaled to the same maximum lineheight in the plots. Spectral parameters were as following: microwave power, 0.63 mW; time constant, 20.48 ms (A) and 40.96 ms (B); conversion time, 20 ms (A) and 40 ms (B); sweep time, 20.48 s (A) and 40.96 s (B); modulation amplitude, 0.1 G; sweep width, 0.9 G; number of points, 1024.

Figure 2.

A. The dependence of peak-to-peak EPR spectrum linewidth of Oxo63 radical on oxygen partial pressure. Solid line represents linear regression of the data in the range from 20 to 160 mmHg yielding line slope 0.42 mG/mmHg. Insert: expanded scale for low oxygen tensions in the range from 0 to 20 mmHg. **B.** The dependence of ratio, I_{in}/I_{out} , calculated from the EPR spectra of TAM-H radical (see Fig. 1B) on oxygen partial pressure. Insert: expanded scale for low oxygen tensions in the range from 0 to 20 mmHg. Solid line represents linear regression of the data in the range from 0 to 20 mmHg with the slope equal to $3.2 \cdot 10^{-3}$ 1/mmHg. X axis error bars denote accuracy of gas mixture preparation (see Materials and Methods).

Figure 3.

The kinetics of oxygen consumption by Met-1 cells ($4.5 \cdot 10^6$ cells/ml) measured by Oxo63 (■, 100 μ M) and TAM-H (○, 50 μ M) in the presence of DMEM buffer solution. (A) The dependences of EPR signals peak-to-peak linewidth and I_{in}/I_{out} ratio values on time for TAM Oxo63 and TAM-H probes, respectively. (B) The dependences of oxygen partial pressure measured by TAM Oxo63 and TAM-H probes over time. Note that the Oxo63 probe becomes insensitive to changes in oxygen partial pressure when it falls below 20 mmHg.

Scheme 1.

(a) 0.3 M in DCM Dess-Martin periodane, 90 %; (b) KOH, water/1,4-dioxane; (c) TFA, 66 % over two steps.

Scheme 2.

Chemical structures of trityl-based radicals Oxo63 and TAM-H.