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Trityl radicals in perfluorocarbon emulsions as stable, sensitive, and biocompatible oximetry probes

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Abstract

EPR oximetry with the use of trityl radicals can enable sensitive O₂ measurement in biological cells and tissues. However, *in vitro* cellular and *in vivo* biological applications are limited by rapid trityl probe degradation or biological clearance and the need to enhance probe O₂ sensitivity. We synthesized novel perfluorocarbon (PFC) emulsions, ~200 nm droplet size, containing esterified perchlorinated triphenyl methyl (PTM) radicals dispersed in physiological aqueous buffers. These formulations exhibit excellent EPR signal stability, over 20-fold greater than free PTM probes, with high oxygen sensitivity ~17 mG/mmHg enabling pO₂ measurement in aqueous solutions or cell suspensions with sensitivity > 0.5 mmHg. Thus, PFC-PTM probes hold great promise to enable combined O₂ delivery and sensing as needed to restore or enhance tissue oxygenation in disease.

Graphical Abstract

Keywords

EPR oximetry; Oxygen sensitivity; Perchlorotriphenyl methyl radicals; Perfluorocarbon emulsions; Cellular respiration

Electron Paramagnetic Resonance (EPR) oximetry with the use of thioacetal-based, water-soluble and stable trityl probes has enabled sensitive and quantitative measurement and

Supplementary Material

Supplementary data associated with this work, includes synthetic procedures, NMR, HRMS, Dynamic Light Scattering analysis, and EPR spectra.

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imaging of O₂ in large aqueous samples, and in biological tissues.^{1–4} Further *in vivo* translation of these probes has been limited by the need for efficient synthesis, biocompatibility, slow metabolism and clearance. There is also a need to enhance their functionality, most importantly their sensitivity to O₂.

The original Oxo63 trityl (Fig. 1) remains difficult to acquire commercially, or synthesize in large amounts.⁵ Oxo63 exhibits a narrow EPR linewidth sensitive to oxygen. It has been used in oximetry applications in small animals via EPR imaging or Overhauser MRI imaging.^{6–9} In spite of its strengths, Oxo63 exhibits rapid clearance *in vivo* due to its high aqueous solubility. CT03, also known as Finland trityl radical (Fig. 1)¹⁰, is a somewhat more lipophilic version of Oxo63.¹¹ The presence of its carboxylate groups enables water solubility up to 2 mM. Our group developed a highly efficient synthetic protocol allowing CT03 synthesis in gram scale.¹² CT03, especially in its deuterated form, exhibits a very narrow EPR single line spectrum, responsive to oxygen induced broadening.^{13–15} CT03 can be derivatized and is used in many applications, including *in vivo* oximetry, and in cells using dendritic, esterified, or amide-functionalized intracellular probes.^{16–19} Due to the availability of efficient synthesis, CT03 has served as core compound for innovative bioanalytical applications, including superoxide detection, and dual-function pO₂ and pH sensitive probes.^{20–24} However, CT03 is limited by its interaction *in vivo* with proteins such as albumin and lipid surfaces, which results in signal quenching in *in vivo* experiments.²⁵

Another class of trityl radicals with narrow singlet EPR spectrum suitable for oximetry applications is perchlorinated triphenyl methyl (PTM) radicals.²⁶ Carboxylated PTM radicals are water soluble and exhibit sensitivity to O₂ in aqueous solutions comparable to CT03 or Oxo63, and demonstrate limited stability towards redox compounds *in vivo*.^{27,28} A distinct, attractive property of esterified PTM radicals is their high solubility in hexafluorobenzene (HFB). Due to very high solubility of O₂ in HFB, O₂-induced broadening of PTM radicals dissolved in HFB is an order of magnitude higher, ~17.71 mG/mmHg, compared to tricarboxylated trityls in aqueous solutions.²⁹ These observations led us to formulate PFC emulsions optimized for oximetry measurements with PTM radicals. The design of perfluorocarbon emulsions is illustrated in Fig. 2. There are several advantages to this approach. Perfluorocarbons (PFC) have exceptional chemical and thermal stability (fig. 2) and PFC emulsions can function as excellent oxygen carriers, and have proven largely nontoxic in human trials as blood substitutes.³⁰ As designed, the PFC core disperses PTM radicals in aqueous phase, thus enabling their use in biological studies. In this study, we kept the design complexity to a minimum. The main focus was to produce stable emulsions with particle size ~200 nm, which can dissolve and retain high loads of PTM radicals within the PFC core. The idea of using emulsions to encapsulate trityl radicals has been described in the literature.^{31–33} However, only low submillimolar concentrations were achieved, not sufficient for high quality *in vivo* EPR oximetry measurements or EPR imaging. The synthesis of PTM radicals was performed according to literature procedures with minor modifications (Scheme 1). Experimental details are included in supporting data. Compound 6 was synthesized reliably according to the procedure by Ballester et al.³⁴ Esters 7–9 were synthesized upon deprotonation with equivalent amounts of butyl lithium, and subsequent esterification.²⁷ Methyl ester 7 was produced consistently in high yields, and could be used without purification in the next step. Ethyl ester 8, and tert-butyl ester 9 required meticulous

chromatographic purification to remove di- and mono-substituted esters. PTM 3–5 were generated in high yield as red crystals by reacting with p-chloranil, and were stored in the dark.³⁵

We optimized an emulsion formulation protocol that consistently yields droplet sizes of ~200 nm diameter. Emulsions are characterized by two concentrations: (1) the concentration of PTMs in perfluorocarbon phase, and (2) concentration of the PTMs overall volume of the emulsion. We kept constant the v/v ratio of perfluorocarbon phase (dispersed phase) vs aqueous phase. A single surfactant, Kolliphor® P188, a biocompatible polymer, which was dissolved in water or phosphate-buffered saline (PBS) at 5% w/v was used as emulsifier. Mixtures of 1:3 (v/v) PFC phases vs. P188 aqueous solution in a glass beaker placed in an ice bath were sonicated for 3 min using a 22.5 kHz horn-tip sonicator. Upon resting, within 5 min a clear red solution was formed, which was stored at 4°C in the dark until used. Clear and stable emulsions were consistently produced. PTMs are insoluble in water; therefore, any amount of probe not encapsulated within the perfluorocarbon core precipitates as red crystals. In addition to visual inspection, EPR spectroscopy was used to verify emulsion signal stability and intensity. Dynamic light scattering (DLS) analysis of droplet size was performed using a Zetasizer NanoS, Malvern Instruments, immediately upon synthesis of emulsions, and periodically prior to EPR spectroscopy measurements. EPR spectra were recorded on a Bruker EMX X-band spectrometer, equipped with Temperature and Gas Controller BIOIII EMX. Probe oxygen response (calibration) in emulsions was measured by EPR spectroscopy after equilibration with known oxygen/nitrogen mixtures in gas permeable Teflon tubes. Borosilicate glass capillaries were used for cell oxygen consumption measurements. Peak to peak EPR spectral linewidths were plotted against % oxygen. Overall pressure was kept at one atmosphere. All oximetry measurements show a linear relation between % O₂ and linewidth measured in gauss (G) as B distance peak to peak. All measurements are an average of three repeats.

Emulsions of PTM 3 and PTM 5 exhibit very similar EPR spectral profile, signal intensity, and oxygen sensitivity. We tested two other common perfluorocarbons used *in vivo*- PCE, and PFD. Detailed EPR spectra and calibration curves are included in supplementary data for various PTMs concentrations in perfluorocarbon phase, and corresponding emulsions. Oximetry calibration curves are all linear in formulation prepared in PBS and water. Changes from anoxic to air conditions resulted in B ~ 2.5G in EPR spectral linewidth for all formulations. Fresh emulsions prepared with 1:1 mixture of PFOB and HFB consistently yield the smallest ~200 nm droplet size, therefore such formulation were used further in cellular studies. High concentrations of PTMs within the perfluorocarbon droplets of the emulsion, stability of emulsions upon dilution, and lack of cellular toxicity are necessary for oximetry applications *in vivo*. PTM 5 was dissolved at highest concentrations of 6.8 mM in the PFC phase, versus 3 mM for PTM 3. In Fig. 3, oximetry measurements are performed on 1.7 mM PTM 5 in PBS stock emulsion (6.8 mM in PFC phase), 5-fold, and 25-fold diluted emulsions. Very similar oximetry calibration curves were observed between dilutions (SD, Fig. 13). Figure 3B illustrates time-dependent O₂ depletion of three dilutions, placed under a stream of nitrogen at 1 atm to simulate O₂ consumption or hypoxic conditions *in vivo*. The 1.7 mM PTM 5 stock emulsion contains 40% w/v dispersed perfluorocarbon in phosphate buffered saline (PBS) and retains more O₂ concentrated in the

PFC droplets Therefore, it equilibrates to hypoxia at a slower rate compared to diluted emulsions. Diluted emulsions equilibrate close to hypoxic conditions in less than 10 min. This behavior would be useful for *in vivo* applications to provide simultaneous delivery and measurement of O₂.

For cellular respiration studies we opted to work with emulsions containing 2 mM PTM 5 in PFC phase, suspended in PBS (0.5 mM final PTM 5 concentration). The stability of the emulsion over time was measured by DLS analysis (Fig. 4B). Immediately upon formulation (SD, Fig. 16), and within 24 hours, the average droplet size remains ~200 nm. The droplet size is well under 300 nm over the next 72 hours (Fig. 4B). PFC emulsions experience a gradual increase in particle size according to the Ostwald Ripening effect.^{36,37} However, the droplet size remains well under 500 nm for over two weeks without affecting oximetry measurements. Considering the simplicity of the preparation process, use of fresh formulations is recommended.

Oximetry calibration curves were constructed for 0.5 mM PTM 5 stock solution in PBS, 5-fold dilutions to 100 μ M, and 25-fold dilutions to 20 μ M. EPR measurements yielded virtually identical oximetry calibration curves (Figs. 4D, 4E). With an EPR measurement resolution of 5 mG, the slope of 16.8 mG/mmHg provides a high O₂ measurement sensitivity of 0.3 mmHg. This remains unchanged between 20 μ M–100 μ M, which is a likely concentration range to be used for *in vivo* delivery. The slope of 0.5 mM stock solution is 16.1 mG/mmHg (SD, Fig. 15). Time dependent O₂ depletion was followed via EPR spectroscopy (Fig. 4F). Similar to measurements in Fig. 3B, diluted solutions equilibrate to hypoxic conditions within minutes, thus EPR measurements can be used effectively to follow O₂ consumption.

We performed measurement of cellular O₂ consumption in human non-small cell lung carcinoma cells (NSCLC) cell line H1229, which are shown in Fig. 4A. No significant cellular toxicity was seen. Greater than 97 % cell viability was observed even in the presence of high concentrations of the PFC emulsions (Fig. 4C) for 1 hour.

A low concentration of 20 μ M PTM 5 in PBS emulsion was sufficient to follow the O₂ consumption in these cells where % O₂ decreased to 0.71 % over 20 min and addition of KCN blocked O₂ consumption. To ensure that formulations report on extracellular % O₂, H1229 cells were incubated with 20 μ M PTM 5 in PBS for 30 min. The supernatant was removed upon differential centrifugation at 110 x g for 1 min, and found to contain the entire PTM 5 EPR signal, without any loss in signal intensity. The cells re-suspended in fresh PBS contained no signal, indicating that there was no cellular uptake of the PTM emulsion over this time.

The EPR signal intensity remained stable during cellular respiration studies and the concentration of the PTM radical remained constant at 20 μ M. This is an indication that PTM esters formulated in PFC emulsions are protected from the superoxide or other radicals generated during cellular respiration.

In conclusion, we have demonstrated that PTM radicals in PFC emulsions can provide highly sensitive O₂ measurements in solutions and cellular systems. The high solubility of

PTM probes in these PFC emulsions along with their high O₂ solubility enables sensitive EPR based oximetry with excellent signal to noise ratio. These formulations are well suited to measure O₂ consumption in cells during cellular respiration. Many pathologies, including ischemic vascular disease and cancer are characterized by lack of O₂ supply.³⁸ In cancer, tumor hypoxia limits the efficacy of radiation or other therapies in tumor killing.³⁹ Based on the efficacy of PFC emulsions as O₂ carriers, and the developments reported herein, PFC emulsions containing PTM probes could be applied in the future for combined O₂ delivery and O₂ sensing to restore or enhance tissue oxygenation in disease.^{40,41}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of stable trityl radicals.

Figure 2. Design of perfluorocarbon emulsions in aqueous solutions

A 1:1 mixture (v/v) of HFB with one other perfluorocarbon constitutes the PFC dispersed phase, which contains dissolved PTM trityls. Sonication of 1:3 (v/v) PFC with 5% P188 aqueous solution yields emulsions of ~200 nm size droplets.

Figure 3. Oximetry measurements via EPR spectroscopy

3A. EPR spectra of 1.7mM PTM 5 stock emulsion in PBS (6.8mM in PFC), under air (solid line) and anoxic (dashed line) conditions. **Insert:** Oximetry calibration curve. **3B.** Time dependent oxygen depletion measurements of 1.7mM PTM 5 stock emulsion in PBS, 5 fold, and 25 fold diluted PTM 5 emulsions in PBS, performed under a stream of nitrogen, at one atmosphere. Spectral parameters were as follows: microwave power=1 mW, time constant=81.92ms, conversion time=40ms, sweep time=40.96 s, modulation frequency=100 KHz, modulation amplitude=0.25 G (air), 0.05G (anoxic), 0.1G (O₂-N₂ exchange), and sweep width=20 G, number of points=1024.

Figure 4. Measurements of cellular respiration using emulsified PTM 5 trityl

4A. H1229 cells ($25 \times 10^6/\text{ml}$); (●) Cells were suspended in PBS. PTM 5 from 0.5mM stock emulsion in PBS was added to a final concentration of $20\mu\text{M}$. The cell suspension was placed in a sealed glass capillary, and oxygen depletion was followed via EPR spectroscopy. (■) Inhibition of cellular respiration was observed with addition of $333\mu\text{M}$ KCN. **4B.** Dynamic light scattering measurements was performed in diluted solutions of 0.5mM PTM 5 stock emulsion over a period of 18 days. **4C.** Cell viability was determined via Trypan blue assay. **4D.** Oximetry calibration curve of $20\mu\text{M}$ PTM 5 in PBS prepared from 0.5 mM stock emulsion in PBS. **4E.** Oximetry calibration curve of $100\mu\text{M}$ PTM 5 in PBS prepared from 0.5 mM stock emulsion in PBS. **4F.** Time dependent oxygen depletion measurements of 0.5mM PTM 5 stock emulsion in PBS, 5 fold, and 25 fold dilutions in PBS, performed under a stream of nitrogen, at one atmosphere.

Scheme 1. Reagents and conditions

i) -78°C , THF, butyl lithium 3.3 equiv., then excess of methyl chloroformate (7), ethyl chloroformate (8), or di-tert-butyl carbonate (9); ii) Bu_4NOH 1 M in THF, then p-chloranil 2 equiv.