Bioorganic & Medicinal Chemistry Letters 26 (2016) 5685–5688

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

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

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ARTICLE INFO

Article history:
Received 6 September 2016
Revised 20 October 2016
Accepted 21 October 2016
Available online 24 October 2016

Keywords:
EPR oximetry
Oxygen sensitivity
Perchlorotriphenyl methyl radicals
Perfluorocarbon emulsions
Cellular respiration

ABSTRACT

EPR oximetry with the use of trityl radicals can enable sensitive O_2 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 O_2 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 pO_2 measurement in aqueous solutions or cell suspensions with sensitivity >0.5 mmHg. Thus, PFC-PTM probes hold great promise to enable combined O_2 delivery and sensing as needed to restore or enhance tissue oxygenation in disease.

Electron Paramagnetic Resonance (EPR) oximetry with the use of thioacetal-based, water-soluble and stable trityl probes has enabled sensitive and quantitative measurement and imaging of O_2 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 O_2.

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 some-what 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 pO_2 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 O_2 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 O_2 in HFB, O_2-induced broadening of PTM radicals dissolved in HFB is an order of magnitude higher, ~17.71 mG/mmHg, compared to tricarboxylated tritys 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 Figure 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

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http://dx.doi.org/10.1016/j.bmcl.2016.10.066
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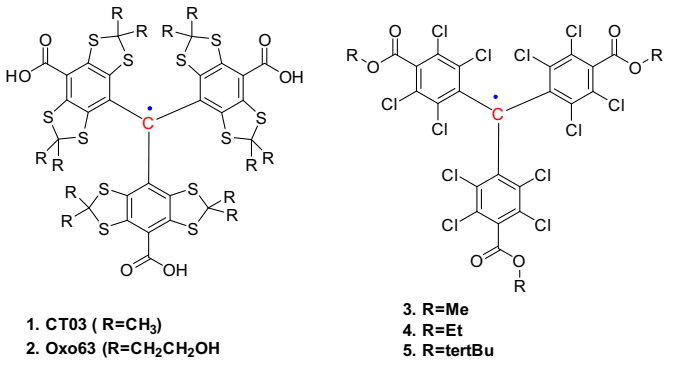

Figure 1. Structures of stable trityl radicals.

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-chloranyl, 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 sonicater. 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 % O_2 and linewidth measured in gauss (G) as ΔB 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 ΔB ~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 Figure 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 dilutioned emulsions. Very similar oximetry calibration curves were observed between dilutions (SD, Fig. 13). Figure 3B illustrates time-dependent O_2 depletion of three dilutions, placed under a stream of nitrogen at 1 atm to simulate O_2 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 O_2 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 O_2.

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 h, the average droplet size remains ~200 nm. The droplet size is well under

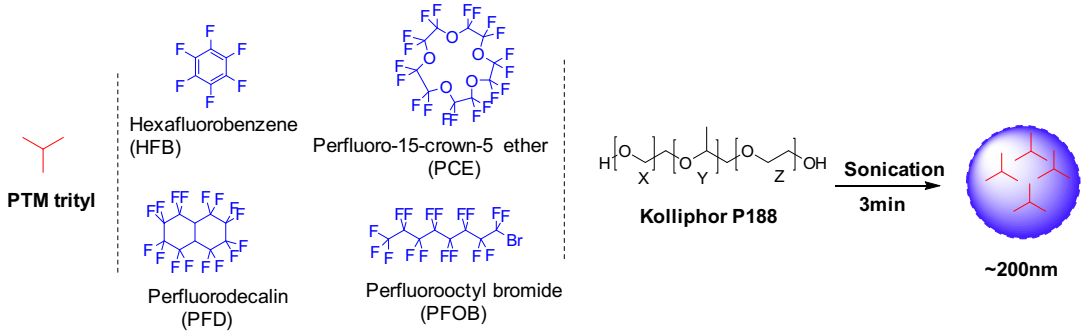

Figure 2. Design of perfluorocarbon emulsions in aqueous solutions. A 1:1 mixture (v/v) of HFB with one other perfluorocarbon constitutes the PFC dispersed phase, which contains dissolved PTM trityls. Sonication of 1:3 (v/v) PFC with 5% P188 aqueous solution yields emulsions of ~200 nm size droplets.